Molybdenum Cofactor Biosynthesis in *Escherichia coli mod* and *mog* Mutants

MAHESH S. JOSHI,[†] JEAN L. JOHNSON, AND K. V. RAJAGOPALAN*

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

Received 16 February 1996/Accepted 3 May 1996

The molybdopterin content of *Escherichia coli mod* and *mog* mutants was estimated by conversion to the form A derivative. The results are in accord with complete phenotypic repair of *mod*, and incomplete repair of *mog*, by culture in high concentrations of molybdate. A possible role for Mog as a molybdochelataase is discussed.

Molybdoenzymes in *Escherichia coli* require a molybdenum cofactor which is present as a complex of the metal with a molybdopterin guanine dinucleotide (see Fig. 1). Like other *mol* mutants of *E. coli*, *mod* and *mog* mutants show pleiotropic loss of several molybdoenzyme activities, suggesting that they are unable to synthesize the molybdenum cofactor essential for these enzymes (17, 20). Both mutants have been reported to respond to elevated levels of molybdate in the culture medium, implicating these loci in the uptake or processing of molybdate; however, the two mutants differ in the extent of phenotypic repair. Molybdoenzymes in the *mod* mutant are restored to wild-type levels by the inclusion of 0.1 mM molybdate in the medium (3), while much lower activity levels are achieved when the *mog* mutant is supplemented with molybdate. Even at nearly toxic levels of molybdate (10 mM), only modest increases in nitrate reductase (22) and biotin sulfoxide reductase (2) levels are observed in the *mog* mutant.

Transcription of the *mod* operon is controlled by levels of molybdate in the culture medium (12). Four genes have been identified at the *mod* locus, and the corresponding proteins, ModABCD, have been defined as components of a membrane transport system specific for molybdate (5, 18, 19). Less information about the biochemical role of Mog is available. The amino acid sequence of the protein suggests that it is not a membrane protein (4) but gives no direct clues as to its function. Multifunctional proteins with peptide segments showing strong sequence similarities to Mog have been identified in higher organisms (10, 16, 21), but again, the biochemical roles of these proteins are undefined.

One approach to understanding the role of Mod and Mog in *E. coli* has been to quantitate the levels of molybdenum cofactor in the *mod* and *mog* mutants using as an assay the ability of extracts of the mutant cells to reconstitute the nitrate reductase apoenzyme in extracts of the *Neurospora crassa nit-1* mutant (1, 11). The results obtained with *mod* mutants were in accord with the molybdoenzyme response to altered growth conditions (and the now-defined role of ModABCD in molybdate transport). That is, *mod* mutants were shown to have 15 to 20% of wild-type levels of molybdenum cofactor when cultured on 1 μ M molybdate and wild-type levels when grown on 1 mM molybdate. However, *mog* mutants were also reported to produce wild-type levels of molybdenum cofactor on 1 mM molybdate, results which appear to be inconsistent with the relatively low extent of molybdoenzyme repair.

The *nit-1* assay provides a very sensitive and convenient estimate of molybdenum cofactor levels (11); however, it is subject to artifacts caused by the presence of a molybdopterin precursor (precursor Z) (8, 9) and is limited in that it measures only transferable molybdenum cofactor. Cofactor that is tightly bound to proteins, be they molybdoenzymes or carrier proteins, is not quantitated unless specific steps are taken to release the cofactor by denaturation of the proteins by acid or heat. These procedures in turn produce questionable quantitative results because of the instability of the released cofactor. In this laboratory we have developed an analytical procedure that quantitates molybdenum cofactor as the fluorescent form A derivative after acid iodine oxidation (Fig. 1) (7). This method provides an accurate assessment of total molybdopterin present in the cell extract, since treatment with acid, heat, and iodine converts loosely and tightly bound forms of molybdenum cofactor, molybdopterin, and molybdopterin guanine dinucleotide to the stable form A. We have carried out these assays on *mod* and *mog* mutants cultured on low and high levels of molybdate.

E. coli RK4353 (wild type at the *mol* loci) and Mu insertion strains RK5202 (*mod*) and RK5206 (*mog*) were kindly provided by Valley Stewart of Cornell University. Cells were grown in Luria-Bertani medium for 17 h at 30°C with shaking. Where indicated, 1 mM sodium molybdate was included. The cell pellets were washed once with 0.1 M potassium phosphate (pH 7.4), suspended in 5 ml of deionized H₂O per g (wet weight) of cells, and twice passed through a French pressure cell at 20,000 lb/in². The extract was centrifuged at 10,000 $\times$ g for 15 min, and the supernatant was collected. The cell extract (25 ml) was diluted to 50 ml with H₂O and acidified to pH 2.5 with HCl. A solution of iodine-KI (1 g of iodine and 2 g of KI in 100 ml) was added in an amount sufficient to maintain the yellow I₂ color, and the samples were boiled for 20 min with excess I₂ present. The pH was adjusted to 8.5 with NH₄OH, and the sample was centrifuged at 12,000 $\times$ g for 20 min. The supernatant was passed through an Amicon PM-10 membrane by using an Amicon stirred cell unit and incubated with alkaline phosphatase overnight at room temperature in the presence of MgCl₂ (7). The dephosphorylated form A produced by this treatment was adsorbed onto a column of QAE-Sephadex (10-ml bed volume). The column was washed with 25 ml of H₂O, and dephosphorylated form A was eluted with 70 ml of 10 mM acetic acid. Fluorescent fractions were pooled, neutralized with NH₄OH, and rotoevaporated to dryness. The form A was dissolved in 10 mM NaOH and was purified by high-pressure liquid chromatography (HPLC) on a C₁₈ column with 10% methanol as the mobile phase. The amount of form A was quantitated by integration of the fluorescent peak and com-

* Corresponding author. Phone: (919) 681-8845. Fax: (919) 684-8919. Electronic mail address: raj@biochem.duke.edu.

[†] Present address: Department of Physiology, Louisiana State University Medical Center, New Orleans, LA 70112.

FIG. 1. Structures of the molybdopterin guanine dinucleotide-molybdenum complex identified in *E. coli* molybdoenzymes (A) and of the dephosphorylated form A derivative (B). In panel A, the reversible attack of the C-3' hydroxyl on C-7 of the pterin ring, forming a pyran ring, is indicated.

parison with the integrated intensity of the standard compound. HPLC instrumentation was as previously described (6).

The levels of molybdopterin in wild-type *E. coli* and in the *mod* and *mog* mutants determined by quantitating the fluorescent form A derivative are shown in Fig. 2. The amount of form A obtained from wild-type cells was independent of the concentration of molybdate in the culture medium; even with the trace amount of metal present in the normal medium, the cells produced levels of form A nearly equal to those obtained when cells were cultured on 1 mM molybdate. The levels of form A obtained from *mod* cells, on the other hand, were extremely sensitive to the availability of molybdate in the growth medium. In the absence of added metal, *mod* cells contained 11% of wild-type levels of molybdopterin; cells grown in the presence of 1 mM molybdate contained nearly wild-type levels. In contrast, *mog* cells yielded form A only at 15% of wild-type levels, whether or not the medium was supplemented with 1 mM molybdate. These experiments show that molybdenum cofactor levels are significantly decreased in both *mod* and *mog* mutants of *E. coli*. The mutation responsible for these lowered cofactor levels in the *mod* strain, but not that in the *mog* strain, is overcome by growth on 1 mM molybdate, in accord with the observed levels of phenotypic repair.

In both *mod* and *mog*, molybdopterin may be synthesized normally but subject to degradation if further assembly into molybdenum cofactor and incorporation into molybdoenzymes

is interrupted, leaving only low levels of residual pterin in the cells. The failure of the *mod* mutant to incorporate molybdenum and assemble molybdenum cofactor can be explained by failure in one or more of the four components of the molybdate uptake system. The mutant cells respond to elevated levels of the metal in the culture medium because molybdate uptake can be accomplished by mass action or through transport by cellular phosphate or sulfate uptake systems. In contrast, the failure of the *mog* mutant to incorporate molybdenum and assemble molybdenum cofactor is not reversed by molybdate supplementation. This result might be expected if Mog functions as a molybdochelatase, an enzyme responsible for the assembly of molybdenum cofactor from molybdopterin in the presence of low concentrations of molybdate. Molybdenum incorporation is apparently accomplished in vitro to some extent nonenzymatically, as documented by the formation of molybdenum cofactor from precursor Z and molybdopterin synthase in the presence of high (10 to 20 mM) concentrations of molybdate in the reconstitution assay (13–15). However, since such high cellular concentrations of molybdenum are unlikely to be attained, a chelatase with high affinity for molybdenum may be essential for in vivo molybdenum cofactor assembly, playing a role analogous to that of ferrochelatase in heme assembly.

The presence of a *mog*-encoded molybdochelatase would certainly facilitate the observed phenotypic repair of *mod* mutants by extracellular molybdate. Even very low levels of intra-

FIG. 2. Form A levels in wild-type *E. coli* and in *mod* and *mog* mutants cultured in the absence (■) and presence (▨) of 1 mM added molybdate. The molybdenum cofactor was oxidized to form A and quantitated as described in the text.

cellular molybdenum, as could be achieved by moderately elevated concentrations in the culture medium, could be efficiently scavenged by a specific intracellular molybdenum-binding protein and processed for molybdenum cofactor assembly. The *mog* mutant, lacking functional molybdochelatase, would require exceptionally high intracellular levels of molybdate to accomplish nonenzymatic molybdenum cofactor assembly. These levels may be achieved only by including inordinately high levels of molybdate in the culture medium (10 mM or higher), levels which would repress *mod* transcription and disable the normal molybdenum uptake system. Molybdate uptake under these conditions would be at the expense of phosphate and sulfate uptake, such that molybdate levels necessary to effect complete phenotypic repair would be toxic to the cells. Characterization of *Mog* to test these proposals is currently underway.

This work was supported by grant GM00091 from the National Institutes of Health.

REFERENCES

1. Amy, N. K. 1981. Identification of the molybdenum cofactor in chlorate-resistant mutants of *Escherichia coli*. *J. Bacteriol.* **148**:274–282.
2. del Campillo-Campbell, A., and A. Campbell. 1982. Molybdenum cofactor requirement for biotin sulfoxide reduction in *Escherichia coli*. *J. Bacteriol.* **149**:469–478.
3. Glaser, J. H., and J. A. DeMoss. 1971. Phenotypic restoration by molybdate of nitrate reductase activity in *chld* mutants of *Escherichia coli*. *J. Bacteriol.* **108**:854–860.
4. Hinton, S. M., and D. Dean. 1990. Biogenesis of molybdenum cofactors. *Crit. Rev. Microbiol.* **17**:169–188.
5. Johann, S., and S. M. Hinton. 1987. Cloning and nucleotide sequence of the *chld* locus. *J. Bacteriol.* **169**:1911–1916.
6. Johnson, J. L., N. R. Bastian, and K. V. Rajagopalan. 1990. Molybdopterin guanine dinucleotide: a modified form of molybdopterin identified in the molybdenum cofactor of dimethyl sulfoxide reductase from *Rhodobacter sphaeroides* forma specialis *denitrificans*. *Proc. Natl. Acad. Sci. USA* **87**:3190–3194.
7. Johnson, J. L., B. E. Hainline, K. V. Rajagopalan, and B. H. Arison. 1984. The pterin component of the molybdenum cofactor. Structural characterization of two fluorescent derivatives. *J. Biol. Chem.* **259**:5414–5422.
8. Johnson, M. E., and K. V. Rajagopalan. 1987. In vitro system for molybdopterin biosynthesis. *J. Bacteriol.* **169**:110–116.
9. Johnson, M. E., and K. V. Rajagopalan. 1987. Involvement of *chlA*, *E*, *M*, and *N* loci in *Escherichia coli* molybdopterin biosynthesis. *J. Bacteriol.* **169**:117–125.
10. Kamdar, K. P., M. E. Shelton, and V. Finnerty. 1994. The *Drosophila* molybdenum cofactor gene *cinnamon* is homologous to three *Escherichia coli* cofactor proteins and to the rat protein gephyrin. *Genetics* **137**:791–801.
11. Miller, J. B., and N. K. Amy. 1983. Molybdenum cofactor in chlorate-resistant and nitrate reductase-deficient insertion mutants of *Escherichia coli*. *J. Bacteriol.* **155**:793–801.
12. Miller, J. B., D. J. Scott, and N. K. Amy. 1987. Molybdenum-sensitive transcriptional regulation of the *chld* locus of *Escherichia coli*. *J. Bacteriol.* **169**:1853–1860.
13. Pitterle, D. M., J. L. Johnson, and K. V. Rajagopalan. 1993. In vitro synthesis of molybdopterin from precursor Z using purified converting factor. Role of protein-bound sulfur in formation of the dithiolene. *J. Biol. Chem.* **268**:13506–13509.
14. Pitterle, D. M., and K. V. Rajagopalan. 1989. Two proteins encoded at the *chlA* locus constitute the converting factor of *Escherichia coli chlA1*. *J. Bacteriol.* **171**:3373–3378.
15. Pitterle, D. M., and K. V. Rajagopalan. 1993. The biosynthesis of molybdopterin in *Escherichia coli*. Purification and characterization of the converting factor. *J. Biol. Chem.* **268**:13499–13505.
16. Prior, P., B. Schmitt, G. Grenningloh, I. Pribilla, G. Multhaup, K. Beyreuther, Y. Maulet, P. Werner, D. Langosch, J. Kirsch, and H. Betz. 1992. Primary structure and alternative splice variants of gephyrin, a putative glycine receptor-tubulin linker protein. *Neuron* **8**:1161–1170.
17. Rajagopalan, K. V., and J. L. Johnson. 1992. The pterin molybdenum cofactors. *J. Biol. Chem.* **267**:10199–10202.
18. Rech, S., U. Deppenmeier, and R. P. Gunsalus. 1995. Regulation of the molybdate transport operon, *modABCD*, of *Escherichia coli* in response to molybdate availability. *J. Bacteriol.* **177**:1023–1029.
19. Rech, S., C. Wolin, and R. P. Gunsalus. 1996. Properties of the periplasmic ModA molybdate-binding protein of *Escherichia coli*. *J. Biol. Chem.* **271**:2557–2562.
20. Shanmugam, K. T., V. Stewart, R. P. Gunsalus, D. H. Boxer, J. A. Cole, M. Chippaux, J. A. DeMoss, G. Giordano, E. C. C. Lin, and K. V. Rajagopalan. 1992. Proposed nomenclature for the genes involved in molybdenum metabolism in *Escherichia coli* and *Salmonella typhimurium*. *Mol. Microbiol.* **6**:3452–3454.
21. Stallmeyer, B., A. Nerlich, J. Schiemann, H. Brinkmann, and R. R. Mendel. 1995. Molybdenum cofactor biosynthesis: the *A. thaliana* cDNA *cnx1* encodes a multifunctional two-domain protein homologous to a mammalian neuroprotein, the insect protein Cinnamon and three *E. coli* proteins. *Plant J.* **8**:751–762.
22. Stewart, V., and C. H. MacGregor. 1982. Nitrate reductase in *Escherichia coli* K-12: involvement of *chlC*, *chlE*, and *chlG* loci. *J. Bacteriol.* **151**:788–799.