Involvement of the Molybdenum Cofactor Biosynthetic Machinery in the Maturation of the Escherichia coli Nitrate Reductase A*

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The maturation of Escherichia coli nitrate reductase A requires the incorporation of the Mo-(bis-MGD) cofactor to the apoprotein. For this process, the NarJ chaperone is strictly required (Blasco, F., Dos Santos, J. P., Magalon, A., Friixon, C., Guigliarelli, B., Santini, C. L., and Giordano, G. (1998) Mol. Microbiol. 28, 435–447). We report the first description of protein interactions between molybdenum cofactor biosynthetic proteins (MogA, MoeA, MobA, and MobB) and the apoprotein reductase (NarG) using a bacterial two-hybrid approach. Two conditions have to be satisfied to allow the visualization of the interactions, (i) the presence of an active and mature molybdenum cofactor and (ii) the presence of the NarJ chaperone and of the NarG structural partner subunit, NarH. Formation of tungsten-substituted molybdenum cofactor prevents the interaction between NarG and the four biosynthetic proteins. Our results suggested that the final stages of molybdenum cofactor biosynthesis occur on a complex made up by MogA, MoeA, MobA, and MobB, which is also in charge with the delivery of the mature cofactor onto the apoprotein reductase A in a NarJ-assisted process.

Molybdenum plays a critical role in the biogeochemistry of nitrogen and sulfur and, as such, has been found to be essential in most mammals as well as in plants. It constitutes an essential trace element found associated with a large group of redox active enzymes in eukaryotes, eubacteria and Archaea. With the exception of nitrogenase, molybdenum is present as an ubiquitous basic structure composed of a molybdenum atom coordinated to one or two molecules of a tricyclic pyranopterin forming the molybdenum cofactor (Moco)§ (1).

In all organisms studied so far, Moco is synthesized by an ancient, ubiquitous, and highly conserved biosynthetic pathway. Moco biosynthesis has been extensively studied in Escherichia coli by using a combination of biochemical, genetic, and structural approaches. Moco biosynthesis can be divided into three steps, (i) the conversion of a guanine nucleotide to form precursor Z, (ii) the introduction of two sulfur atoms to give molybdopterin (MPT), and (iii) the chelation of molybdenum by MPT thus forming active Moco (2) (see Fig. 1). In prokaryotes, the cofactor can be further modified by the attachment of a nucleotide onto the terminal phosphate group of MPT (1). In E. coli, most of the molybdoenzymes require not only the GMP-modified form of MPT, i.e. MGD (molybdopterin guanine dinucleotide) for their activity but a Mo-(bis-MPT)-based cofactor where one Mo atom is coordinated by four dithiolenes of two MGD molecules (3–7). However, although the synthesis of MGD is well documented, formation of Mo-(bis-MGD) remains enigmatic. Noteworthy is that the two processes of molybdenum insertion catalyzed by MogA and MoeA proteins (8, 9) and of dinucleotide formation catalyzed by MobA (10) seem to be strongly linked (11).

Information about Moco incorporation within apomolybdoenzymes is scarce. So far, nothing is known about the Moco donor or the molecular mechanism of Moco transfer. In prokaryotes, several molybdoenzymes incorporate the cofactor by a process that involves enzyme-specific chaperones. This situation was first noted for maturation of the dissimilatory nitrate reductase A of E. coli where NarJ was shown to be needed (12, 13). The list of such chaperones has been expanded by TorD required for the maturation of the trimethylamine-N-oxide reductase (14, 15). The exact function of this new class of proteins is not yet well understood despite an active role in Moco incorporation facilitation (13, 16). Another participant in such a process is the Moco carrier defined as a molecule linking Moco biosynthesis to its subsequent incorporation into various apomolybdoenzymes (17). However, little is known concerning the identity of this carrier molecule. Interestingly, mutations affecting the activity of E. coli molybdoenzymes mapped only in loci involved in Mo transport (mod) or Moco biosynthesis (moea, mob, noe, and moeg) (18). Such an observation indicates that Mo proteins could ensure Moco protection until its delivery to the apoenzymes. Recent work (19) has shown that, in vitro, numerous protein–protein interactions exist among mgo gene products involved in the final stages of Moco biosynthesis and that some of them even require the binding of Moco intermediates to occur. One can envision that these steps occur on a multiprotein complex allowing a fast and protected transfer of oxygen-sensitive intermediates and subsequent delivery of active Moco to resident apomolybdoenzymes.

In this work, we provided the first molecular evidence for the presence of interactions between the Moco biosynthetic machinery and an apomolybdoenzyme, the E. coli apoprotein reductase A, being used as a model. Interestingly, two conditions have to be satisfied to permit any of the interactions to occur, the presence of a mature and active Moco and the presence of the NarJ chaperone. Taken together, our data point to an unexpected essential role for the enzyme-specific chaperones.

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§ This abbreviations used are: Moco, molybdenum cofactor; MPT, molybdopterin; MGD, molybdopterin guanine dinucleotide.
exemplified by NarJ in mediating the interaction between the Moco carrier and the apoenzyme.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Media—The bacterial strains and plasmids used in this work are described in Table I. Using BTH101 as recipient strain (20), BTH101narZnarA and BTH101narZnarJ were obtained by P1 transduction as described by Miller (21). In-frame deletion of narJ was performed according to the Hamilton procedure (23) thus forming the BTHmob strain. Bacterial cultures were grown in L-broth medium under semi-aerobic conditions. As needed, sodium molybdate or sodium tungstate were added at 1 and 10 mM final concentration, respectively.

Table I: Bacterial strains and plasmids used in this study

| Strain or plasmid | Description | Reference or source |
|-------------------|-------------|--------------------|
| DH5α              | supE44, ΔlacU169 (Δ80 lacZΔM15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1 | Laboratory collection |
| BTH101            | F′, cya-99, araD139, galE15, galK6, rpsL15(Str8), hsdR2, mcrA1, mcrB1 | |
| BTH101mob         | BTH101 mob254-Tn10, StrR, TeR | 19 |
| BTH101mod         | BTH101 modB247::Tn10, StrR, TeR | 19 |
| BTH101mobB        | BTH101 mobB::kan, StrR, KmR | 19 |
| BTH101narZnarJ    | BTH101 Δnar25 narJ narH narJ::kan, narH, SpecR, KmR, StrR | This work |
| BTH101narZnarJ    | BTH101 Δnar25 narJ narH narJ::kan, narH, SpecR, StrR | This work |

Two-hybrid vectors

| pT18-Zip | pT18-Leucine zipper fused to T18 fragment (225–399 amino acids of CyaA) | 24 |
| pT25-Zip | pT25-Leucine zipper fused to T25 fragment (1–224 amino acids of CyaA) | 24 |
| pT18-MoeA | pT18-MoeA-T18 fusion protein, AmpR | 19 |
| pT18-MobA | pT18-MobA-T18 fusion protein, AmpR | 19 |
| pT18-MobB | pT18-MobB-T18 fusion protein, AmpR | 19 |
| pT25-MoeA | pT25-MoeA fusion protein, CmR | 19 |
| pT25-MobA | pT25-MobA fusion protein, CmR | 19 |
| pT25-MobB | pT25-MobB fusion protein, CmR | 19 |
| pT25-NarG | pT25-NarG fusion protein, CmR | This work |
| pT25-NarGH | pT25-NarGH fusion protein, NarGH CmR | This work |
| pT25-NarGJ | pT25-NarGJ fusion protein, NarGJ CmR | This work |

* E. coli adenylate cyclase protein.

As a model were tested by a bacterial two-hybrid approach (24). In an initial test, interactions between NarG, the Moco-containing subunit, and each of the four Mo proteins tested (MobA, MobB, MobD, and MobB) were assayed in a wild-type strain BTH101. Interestingly, each of the four proteins interacted to varying extents with NarG, MobA, MobD, and MobB displaying the highest levels of interaction (Fig. 2). Because several specific interactions between Mo proteins have been shown to require binding of Moco intermediates (19), it has been tested whether the presence of Moco is also needed for interaction with NarG. At first, the effect of Moco absence on the interactions has been probed in a BTH101moa strain (Figs. 1 and 2). All of the interactions have been lost supporting the idea that NarG does not interact individually with each of the Mo proteins but rather with a Mo protein complex absent in such conditions. Indeed, one would have expected the maintenance of some of the interactions in the case that each of the tested Mo proteins interacts individually and separately with the apoenzyme. To verify such a hypothesis, the effect of several mutations interrupting Moco biosynthesis at different stages and having differential consequences on the interaction network among the four Mo proteins (19) has been studied.

The interactions between NarG and each of the four Mo proteins have been assayed in a BTH101mob strain synthesizing an active Moco (Mo-MPT) for MPT-dependent enzymes (Fig. 1). Here again, none of the Mo proteins interacted with NarG (Fig. 2). Such an observation might be the consequence of either the absence of Mo-(bis-MGD), the active Moco for MGD-containing enzymes such as nitrate reductase A, and/or the loss of integrity of a Mo protein complex because of the absence of both MobA and MobB. Although in such a strain, the presence of either T18- or T25-MobA fusion proteins and not of MobB equivalents leads to a full restoration of the mutant phenotype (12, 19) and as a consequence cannot be tested, the interaction between MobB and NarG has been assayed (Fig. 2). Interestingly, no interaction has been detected limiting the phenomenon to the absence of MobB as a crucial component of a Mo protein complex, the only complex able to interact with NarG and one which would not form in a mob mutant. As a control, interactions have been assayed in a mobB context. Interest-
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Synthesis of a Tungsten-substituted Cofactor Prevents Interactions between NarG and the Mo Proteins—Tungsten is a close analog of molybdenum and has been shown to be incorporated into the Moco biosynthetic pathway via a still unknown mechanism. In E. coli, the addition of tungstate to the growth medium of a mod mutant leads to the synthesis of inactive molybdoenzymes (17, 26) with the exception of the trimethylamine-N-oxide reductase (27). In this latter case, only a small fraction of the enzyme (<10%) has incorporated the W-(bis-MGD) cofactor (27). Moreover, tungsten prevents both molybdenum ligation by MPT and insertion of the organic component of the cofactor into the nitrate reductase A as recently confirmed by the x-ray structure of the apoenzyme (26, 28). To better understand the reason of the absence of W-(bis-MGD) cofactor within the E. coli nitrate reductase A, interactions have been assayed in a mod mutant supplemented by 10 mM tungstate (W) (Fig. 2). It is important to notice that the pairwise interaction network among Mo proteins is maintained under these conditions as reported previously (19). Although NarG still interacts with NarJ (900 Miller units), it does not interact any longer with each of the Mo proteins thus explaining the non-incorporation of W-(bis-MGD). Taken together, all of these results strongly suggest that interactions between NarG and the Mo proteins require mature and active Moco i.e., Mo-(bis-MGD) cofactor.

Interactions between Mo Proteins and NarG further Require NarJ and NarH—Because Moco incorporation within the aponitrate reductase A strictly requires the presence of the enzyme-specific chaperone protein, NarJ (13, 29), one can envision that this protein is needed for the interactions between NarG and the Mo proteins. To assess the effect of the NarJ absence, an in-frame deletion of narJ has been performed in a BTH101narZ mutant devoid of the NarJ-homolog, NarW, yielding the BTH101narZ,narJ strain. Interestingly, all of the pairwise interactions have been lost, supporting a critical role for NarJ (data not shown). To verify this point, a plasmidic construct allowing the co-expression of NarJ together with the T25NarG fusion protein has been employed. The additional presence of NarJ restores the interactions between NarG and the Mo proteins pointing to an unexpected key role for NarJ in the Moco incorporation process. To test whether among the nar-encoded proteins (NarG, -H, -J, and -I) (30) NarJ is the only protein required to allow NarG to interact with the Mo proteins, a nar-deficient strain (BTH101narA,narZ) has been constructed by P1 transduction. As predicted, all of the interactions have been lost due at least to the NarJ absence (Fig. 3). However, providing NarJ in trans does not allow a restoration of the interactions between NarG and the Mo proteins pointing to a role for NarH, the structural partner subunit absent in such a context. As a control, a similar plasmidic construct allowing co-expression of NarH together with T25NarG have been tested and does not restore the interactions of NarG with the Mo proteins. Finally, co-expression of both NarH and NarJ with T25NarG was required to restore the interactions (Fig. 3). Such a result suggests that proper folding of the NarG catalytic subunit for interaction with each of the Mo proteins can only be achieved by the action of its partner structural subunit, NarH, and of the NarJ chaperone.

Fig. 1. Molybdenum cofactor biosynthesis in E. coli. The proteins catalyzing each step are indicated in parentheses. Interactions have been probed in several strains interrupting the Moco biosynthetic pathway at different stages.

Fig. 2. Interactions between the Mo proteins and the nitrate reductase A strictly require mature Moco. The interactions have been probed in several strains interrupting the Moco biosynthetic pathway at different stages. Interactions have been measured between NarG and the Zip domain (used as a negative control), MogA, MoeA, MobA, or MobB. The β-galactosidase activity values are the average of at least three independent experiments and are expressed in Miller units. WT, wild type.
DISCUSSION

Microorganisms have the extraordinary ability to rapidly adapt their metabolism to environmental changes by inducing respiratory enzymes and altering their metabolic activity. Under anaerobic growth conditions, *E. coli* produces several molybdenenzymes almost all of which serve as terminal enzymes in the anaerobic respiratory pathway (31). Production of active molybdoenzymes depends on the coordination of apoprotein synthesis with synthesis and acquisition of the molybdenum cofactor. Once mature and active Moco is synthesized within the prokaryotic cell, various resident apomolybdoenzymes efficiently and rapidly incorporate Moco via a still unknown mechanism, which in some cases involves enzyme-specific chaperones. The present work has several implications for understanding the Moco incorporation process.

According to our previous studies (19), a transient complex made up by at least MogA, MoeA, and MobA would exist allowing efficient coupling between the two last steps of Moco biosynthesis i.e., molybdenum addition and nucleotide binding to yield active Moco, although the mechanistic details remain unclear. How do the interactions among the Mo proteins involved in the final stages of Moco biosynthesis contribute to its subsequent incorporation into various apomolybdoenzymes? In this report, using a bacterial two-hybrid approach we have demonstrated the physical interaction of an apomolybdoenzyme exemplified by NarG, the Moco-containing subunit of the *E. coli* nitrate reductase A and four distinct Mo proteins (MogA, MoeA, MobA, and MobB). It is important to note that one would have expected an interaction of NarG with a single Mo protein, i.e., MobA, because of its role in a late event of Moco biosynthesis rather than with several Mo proteins. Interestingly, all of the pairwise interactions with NarG are lost simultaneously in the absence of Moco biosynthesis, a situation encountered in a moa strain. Furthermore, our analyses have clearly shown that no interaction was observed until completion of the Moco biosynthesis, in marked contrast with the interactions existing among the Mo proteins (19). Overall, these observations support the contention that NarG interacts with a protein complex formed in the presence of Moco as reported previously (19) rather than with isolated proteins. Such a consideration has important consequences for the understanding of the Moco incorporation mechanism as a direct transfer of mature Moco from the Moco protein complex onto the apomolybdoenzymes. Hence, a Mo protein complex could represent the long time proposed Moco carrier allowing the protection and transfer of active and mature Moco onto resident apomolybdoenzymes as anticipated in our previous work (Fig. 3) (19).

Because of the crucial role of the NarJ chaperone for Moco incorporation within the *E. coli* apomolybdoenzyme, the latter representing the Moco delivery machinery, Does the chaperone interact with each of the Mo proteins? An answer to this question must await further experiments. To get a better understanding of the NarJ-assisted Moco incorporation process, there has been the question of whether pairwise interactions between NarG and the Mo proteins can also exist in the absence of the structural partner subunit NarH i.e. whether Moco is incorporated within NarG before its association with NarH during the course of the nitrate reductase biogenesis. Our results have clearly demonstrated that the interactions between NarG and each of the Mo proteins can only occur when NarH and NarJ are both provided. Consequently, a mature Moco would only be transferred onto an apo-NarGH complex. On the basis of the crystallographic view of the apo-NarGHI complex (28), it is difficult to conceive of how the Mo-(bis-MGD) cofactor can be incorporated within such a closed structure. First, does NarJ have access to such a complex i.e. the membrane-associated one? In fact, recent work has indicated that only the soluble apo-NarGH complex is able to interact with NarJ and as such to incorporate Moco. Hence, crystalization of the apo-NarGH complex capable to undergo the maturation process should be considered.

Overall, a hypothetical model describing Moco biosynthesis and incorporation within a prokaryotic molybdoenzyme, the *E. coli* nitrate reductase A can be delineated from our previous work (19) and the present results (Fig. 4). The proposed model implies that (i) the interaction between NarG and its dedicated

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NarJ chaperone is an early event as such interaction is maintained in any mu mutant (data not shown), (ii) the final stages of Moco biosynthesis occur on a protein complex made up of at least MogA, MoeA, and MobA (19) regardless of the apoenzyme, and (iii) the interaction between the Mo protein complex and the aporitate reductase (apo-NarGH) only occurs after the completion of Moco biosynthesis and in the presence of the NarJ chaperone.

Tungsten, a close analog to molybdenum, has been shown to be incorporated as such into the Moco biosynthesis pathway via a still unknown mechanism rendering most of the E. coli molybdoenzymes inactive. For instance, in contrast to the trimethylamine-N-oxide reductase (27), nitrate reductase A cannot incorporate a tungsten-substituted cofactor as recently confirmed by the crystallographic structure of the apo-NarGH complex (28). Such an observation raises the question whether the multiple pairwise interactions observed between NarG and each of the four Mo proteins will be maintained in the presence of W-(bis-MGD) cofactor. In fact, none of the interactions was observed giving an explanation to the absence of cofactor within the aporitate reductase A. One should notice that enhancement of some specific interactions among the Mo proteins has been reported previously under these conditions (19). Therefore, the inability of the Mo protein complex to deliver W-(bis-MGD) to apoenzymes such as the nitrate reductase will lead to its accumulation (Fig. 4). Because of the essential role of NarJ for these interactions and not of TorD as shown by the absence of phenotype of a torD mutant (14), it is tempting to speculate that such enzyme-specific chaperones ensure both the control and the specificity of the Moco incorporation process. According to such a hypothesis, false incorporation of W-(bis-MGD) within the E. coli trimethylamine-N-oxide reductase would be the direct consequence of the non-essential character of the TorD chaperone.

In conclusion, the present findings demonstrated a network of dynamic protein-protein interactions among and between the Mo proteins and an apoenzyme thus forming most likely a large and multimeric complex. However, biochemical visualization will be hampered by the rapid dissociation of the complex made between the Mo proteins and NarG, as soon as conditions required for information are identical to those that will lead to an effective Moco transfer and resolution of the whole complex. Until now attempts to identify the high molecular mass complexes by several techniques have been unsuccessful (data not shown). Such short lived or dynamic complexes may dissociate and reform in response to the metabolic status of the cell, thereby providing a rapid and powerful mechanism for regulating molybdoenzymes synthesis. It is important to note that the ability to overproduce in an active form either Mo-(bis-MGD)- or Mo-MPT-containing enzymes in E. coli has been reported for a long time (32–35). According to such observations, the Moco biosynthetic and delivery machinery is readily able to respond to a high demand in mature Moco. In the case of the nitrate reductase A, the cell apparently coordinates Moco biosynthesis with apoprotein synthesis at the level of mope operon transcription (36). Conversely, the mobe operon is transcribed constitutively at very low levels (37). Transcriptome analysis has also indicated that mag gene is constitutively expressed.3 Considering that the minimal set of Mo proteins allowing both the final stages of Moco biosynthesis and incorporation of Mo-(bis-MGD) cofactor within the aporitate reductase A consists of MogA, MoeA, and MobA, it is interesting to notice that MoeA is the only protein of which expression level varies. As soon as the proteins involved in the final stages of Moco biosynthesis are also in charge of Moco delivery in several apoenzymes, the latter process needs to be extremely rapid to allow recycling of the Mo proteins expressed at very low levels in the cell. The function of the enzyme-specific chaperones would then be, as such, to optimize the Moco incorporation process. In the case of the nitrate reductase A, the NarJ chaperone is strictly required to allow Moco transfer (12, 13) via the interaction with the Moco biosynthetic machinery (Fig. 4). On the other hand, the presence of the non-essential TorD chaperone enhances Moco incorporation within the E. coli trimethylamine-N-oxide reductase (15).

Many cellular processes rely upon the concerted action of multiple proteins. During such events, specific protein-protein interactions play an essential role. In this report, we provide several indications concerning the fact that Mo proteins work in concert during the last sequential steps of Moco biosynthesis (19) and transfer the active Moco to the aporitate reductase, which as a consequence lead to resolve the whole protein network. Moco intermediate channeling can thus easily be envisioned as a means to (i) attain high levels of Moco biosynthesis, (ii) coordinate the activities of MogA, MoeA, and MobA, (iii) sequester reactive intermediates, and (iv) regulate competition between branch pathways for common metabolites such as MPT. A second major insight of our analysis was that the NarJ chaperone is needed to allow the Mo proteins to interact with NarG.

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