Dedicated Metallochaperone Connects Apoenzyme and Molybdenum Cofactor Biosynthesis Components*

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The biogenesis of molybdenum-containing enzymes is a sophisticated process involving the insertion of a complex molybdenum cofactor into competent apoproteins. As for many molybdoenzymes, the maturation of trimethylamine-oxide reductase TorA requires a private chaperone. This chaperone (TorD) interacts with the signal peptide and the core of apopTorA. Using random mutagenesis, we established that α-helix 5 of TorD plays a key role in the core binding and that this binding drives the maturation of TorA. In addition, we showed for the first time that TorD interacts with molybdenum cofactor biosynthesis components, including MobA, the last enzyme of cofactor synthesis, and Mo-molybdopterin, the precursor form of the cofactor. Finally, we demonstrated that TorD also binds the mature molybdopterin-guanine dinucleotide form of the cofactor. We thus propose that TorD acts as a platform connecting the last step of the synthesis of the molybdenum cofactor just before its insertion into the catalytic site of TorA.

The molybdenum cofactor (Moco)3 is a ubiquitous and ancient prosthetic group that is essential for fundamental biological processes (1). Moco biosynthesis is an evolutionarily conserved pathway comprising several reactions starting from GTP via several intermediates to the formation of the molybdopterin (MPT) moiety, a tricyclic pyranopterin with a unique dithiolene group that provides the platform for the coordination of the molybdenum atom thus forming Mo-MPT (2–4). In prokaryotes and eukaryotes, the Mo-MPT form can become more complex by the addition of a nucleotide, generally GMP, to result in the molybdopterin-guanine dinucleotide (MGD) form of the cofactor (5). Resolution of several structures of diverse MGD-containing enzymes revealed that in the catalytic site of those proteins a bis-MGD form is present in which two MGD molecules are coordinated at the molybdenum atom (6–10). The mechanism converting MGD into bis-MGD is so far not known. Genetic deficiencies of enzymes involved in Moco biosynthesis give rise to the pleiotropic loss of molybdoenzymes. In both eukaryotes and prokaryotes, consequences are dramatic because molybdenum enzymes are essential for diverse metabolic processes. In humans, Moco deficiency leads to premature death of affected newborns, and in bacteria, Moco deficiency leads to dramatically reduced metabolic adaptability and diversity (11, 12).

After completion of biosynthesis, a mature cofactor has to be inserted into molybdenum apoenzymes. Researchers are beginning to understand the mechanisms of transfer, storage, and insertion of the different forms of Moco into the respective apoenzyme. The recent discovery of specific proteins necessary for the maturation of molybdoprotein containing either the sulfurated Mo-MPT or the bis-MGD forms of the cofactor suggests there are common broad lines of cofactor insertion (13–16). Two specific proteins involved in the maturation of Moco-containing enzymes were shown to bind the Moco and to transfer it to the apoprotein. Indeed, in Rhodobacter capsulatus, the XdhC protein coordinates Mo-MPT binding, modification to the sulfurated Mo-MPT form, and insertion into the xanthine dehydrogenase enzyme (16). Moreover, the Moco carrier protein of Chlamydomonas reinhardtii also identified in other eukaryotes was shown to bind Mo-MPT but not MPT and in vitro can transfer Moco to aminotransferase or asparaginase oxidase (17).

Molybdoenzyme TorA and its dedicated chaperone TorD from Escherichia coli represent the archetypal model couple; TorD function is the most studied and best understood in the field (14, 18–22). TorA is a bis-MGD-containing reductase involved in periplasmatic bacterial trimethylamine oxide (TMAO) respiration, and TorD is the dedicated chaperone allowing optimal production of active TorA in the cell (13, 20). So far, two roles have been experimentally attributed to TorD. First, TorD binds and protects the N-terminal twin arginine signal peptide of apoTorA (19, 22); second, TorD binds the core of apoTorA and enhances the bis-MGD insertion by forming the apoprotein in a competent shape (13, 14). It was also proposed that TorD proofreads in a GTP-dependent way the TorA preprotein for cofactor insertion and targets it to the specific twin arginine translocation system (21, 22). However, these aspects have not been definitively demonstrated, and they could be side effects of TorD binding and protection of TorA signal peptide.
Molybdoenzyme Biogenesis by Chaperone

E. coli TorD protein is part of a large family of metalloprotein-associated chaperones presenting a low homology level in their primary amino acid sequences but sharing an all-α-helical structure organized in two domains connected by a short hinge region (23–25). Site-directed mutagenesis showed that this interdomain region with two conserved residues was critical for the signal sequence recognition and the proofreading activity of TorD (21). Nevertheless the affinity of the two TorD variants for the TorA signal peptide was not dramatically modified (22).

Here we chose a strategy of random mutagenesis of the torD gene to find the region involved in TorD-TorA interactions. A poorly conserved patch in TorD protein was shown to interact with the core of apoTorA but not with the signal peptide. By using several in vitro and in vivo approaches, we revealed that the maturation of the TorA apoprotein is tightly dependent on this interaction. Finally it appears that TorD has a third function. It is a Moco-binding protein also capable of specific interaction with MobA, the enzyme involved in MGD biosynthesis. TorD thus allows cofactor insertion by connecting all the components necessary for TorA maturation. Our work thus reveals a new role for members of the TorD family.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions—The E. coli strains used in this work are MC4100 (araD139 Δ(lacl-PZYA-argF) U169 rpsL thi), LC5B15 (MC4100 torD::Ω SpeΔdmsD KmR, LC4B40 (MC4100 Δ(storSTRCAD) Δ(dmsABC) KmR) (18), BTH101 (F- cya-99 araD139 galE15 galK16 rpsL1 (Str8) hsdR2 mcrA1 mcrB1) (26), and BL21(DE3) (F- ompT hsdS2 (rP– mP–) dcm gal (DE3)) (Novagen). The strains were grown in Luria broth medium, and when necessary, ampicillin (50 μg/ml) was added to maintain plasmid selection. Plasmids pBD, pBDC79R, and pBDL83P are pBad24 derivatives allowing the synthesis of His6-tagged TorD, His6-tagged TorDC79R, and His6-tagged TorDL83P, respectively (Ref. 18 and this work). Plasmid pBDW40H allows the synthesis of His6-tagged TorDW40H. The site-directed mutagenesis used to construct the torDW40H allele was the overlap extension method of Ho et al. (27) based on two rounds of PCR amplification with two divergent complementary primers, each bearing the expected mutation, and with two convergent primers. The torD allele was introduced in pBad24 as described previously (18). Plasmid pTORA allowing His6-tagged apoTorA production was described previously (14). To construct plasmid pMobA, which allows the synthesis of His6-tagged MobA, the same cloning strategy as that described previously (14) was used except that the mobA coding sequence was cloned into the BamHI-HindIII cloning sites of pet21(+) (Novagen). Plasmid pMN56 allowing R. capsulatus MobA production was described previously (28). Transformations were carried out according to the method of Chung and Miller (29).

Isolation of Random torD Mutants—For random mutagenesis, the torD coding sequence was amplified from MC4100 chromosomal DNA by PCR using the same primers used for the construction of pTorD. To allow the introduction of mutations in the torD sequence, three successive rounds of PCR amplification (30 cycles each) were performed. After enzymatic hydrolysis, the PCR products were cloned into the corresponding EcoRI and HindIII sites of pBad24 vector. Ligation products were then transformed in strain LC5B15, and the resulting colonies were screened on MacConkey plates supplemented with 0.15% maltose and 0.4% TMAO at 43 °C. Strains producing wild type TorD proteins yielded white colonies, whereas strains producing TorD-negative mutant proteins yielded red colonies.

Purification of Recombinant Proteins—ApoTorA protein was purified as described previously (14). In these production conditions, the first 35 amino acids of the N-terminal extremity of apoTorA are missing (19). Recombinant TorD and TorD variants were purified from the soluble extract of strain LC5B15 containing either pBD, pBDC79R, pBDL83P, or pBDW40H grown aerobically at 37 °C in the presence of arabinose (0.2%). Recombinant MobA protein was purified from the soluble extract of strain BL21(DE3)/pMobA grown aerobically at 37 °C in the presence of isopropyl-β-D-galactopyranoside (1 mM). Protein purifications were performed by HisTrap chelating HP chromatography (Amersham Biosciences) as described previously (14). After purification, apoTorA and the various forms of TorD were dialyzed against 20 mM phosphate buffer, pH 7.

Preparation of Extracts—For the soluble fraction analyses, strains were grown anaerobically overnight with 0.2% TMAO at 37 and 43 °C. The cells were resuspended in 40 mM Tris-HCl, pH 7.6, washed twice, and disrupted by a French press. The extracts were centrifuged at 15,000 rpm, and the recovered supernatants were centrifuged at 45,000 rpm to obtain the soluble fractions.

Analytical Procedures—TMAO reductase activity was measured spectrophotometrically at 37 °C by following the oxidation of reduced benzyl viologen at 600 nm coupled to the reduction of TMAO (13). TMAO reductase activity units correspond to μmol of TMAO reduced/min/mg of proteins. Proteins were revealed after SDS-PAGE either by Coomassie Blue staining or Western blotting using TorA antibodies.

In Vitro Activation of apoTorA—The cofactor source (SN440) corresponds to the soluble fraction (40 mg·ml⁻¹, in 20 mM phosphate buffer, pH 7) of strain LC5B40 grown anaerobically at 37 °C as described previously (14). ApoTorA (0.5 μM) was mixed with 100 μl of SN440 in the presence of either TorD or TorD variants (2.3 μM). The mixture was incubated for 120 min at 37 °C under nitrogen atmosphere. Units correspond to μmol of TMAO reduced/min/mg of apoTorA.

Circular Dichroism Spectra—Far-UV circular dichroism spectra of purified proteins (3.6 μM) were monitored from 195 to 260 nm. The spectra were recorded on a Jasco J-715 instrument equipped with a Peltier-type temperature control system (model PTC-348WI) with a 2-mm path length and a scan rate of 20 nm·min⁻¹ at 20 °C. Base lines were corrected by subtracting the buffer spectrum.

Surface Plasmon Resonance Experiments—Binding experiments were performed with the surface plasmon resonance-based instrument Biacore™ 2000 and sensor chips (CM5) using the control software 2.1 and evaluation software 3.0 (Biacore AB, Upssala, Sweden). TorD was immobilized via amine coupling with 300, 1000, and 2100 resonance units/flow cell. As a control bovine serum albumin was immobilized via amine coupling with 900 resonance units. The running buffer was 10
**Chemical Cross-linking Studies**—Interactions of TorD and TorD variants (23 μM) with truncated apoTorA (1.6 μM) or interactions of TorD and TorD variants (40 μM) with MobA (40 μM) were tested using 5 mM bismaleimidoalane or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, respectively, as cross-linkers. Proteins and cross-linkers were incubated for 30 min at room temperature. The interactions were analyzed by SDS-PAGE and revealed either by Western blotting using TorA antibodies or Coomassie Blue staining.

**Isothermal Titration Calorimetry**—Isothermal titration calorimetry experiments were performed at 20 °C using a MicroCal MCS instrument. TorD and TorD variants were dialyzed against 50 mM Tris-HCl, 200 mM NaCl buffer, pH 7.2. The protein concentration was 10 μM in the calorimetric cell. Each experiment consisted of 10-μl injections of 100 μM peptide prepared in the same buffer (30 injections in total). For every injection, the binding enthalpy was calculated by integration of the peak area using the ORIGIN software. The association constant $K_a$ and additional binding parameters (enthalpy and entropy) were obtained through curve fitting with ORIGIN; $ΔG$ values were $-7.6\times10^5$ and $-7.5\times10^3\text{kJ}\text{mol}^{-1}$ for TorD and TorDL83P, respectively. Peptide $\text{St}_{\text{TorA10-50}}$ was chemically synthesized by Genosphere Biotechnologies (Paris, France).

**Form A Detection**—Mo-MPT was quantified by conversion of Form A by the addition of MgCl$_2$, nucleotide pyrophosphatase, and alkaline phosphatase at pH 8.0. After the addition of 10 μl of acetic acid, Form A from Mo-MPT and MGD was identified and quantified by HPLC analysis with a C$_{18}$ reversed phase HPLC column (4.6 × 250-mm ODS Hypersil; particle size, 5 μm) with 5 mM ammonium acetate, 15% methanol at an isotropic flow rate of 1 ml/min. In-line fluorescence was monitored by an Agilent 1100 series detector with excitation at 383 nm and emission at 450 nm. The quantification of Form A was determined as described earlier (16, 32).

**Binding of Mo-MPT**—4.6 μM TorD (in 100 mM Tris, pH 7.2) was incubated with free Mo-MPT (0–16 μM) for 15 min at room temperature. The samples were transferred to Microcon concentrators (molecular weight cutoff, 10,000; Millipore) and centrifuged at 14,000 × g for 5 min. As a control, free Mo-MPT was used in the absence of TorD. The flow-through containing unbound Mo-MPT was converted to Form A and quantified. Fitting was based on the law of mass action for a Mo-MPT to TorD ratio of $n \times m$ (16). Bovine serum albumin, TorT (33), TorD, and TorD variants (4.6 μM each) in 100 mM Tris, pH 7.2, were mixed with free Moco (10 μM) for 15 min. To remove unbound Mo-MPT, samples were loaded on Nickel columns (gel filtration) (GE Healthcare), and fractions containing protein with bound Moco were collected. Mo-MPT was converted to Form A, quantified, and expressed as a percentage of Mo-MPT bound to TorD.

**Binding of MGD**—MobA was expressed from plasmid pMN56 and purified as described earlier (28). The His tag was removed by usage of the Thrombin CleanCleave kit (Sigma).

**For MGD synthesis**, either 2.5 or 25 μM MobA and 25 μM TorD were incubated in 100 mM Tris, pH 7.2, containing 1 mM MgCl$_2$, 1 mM GTP, and 50 μM Mo-MPT in a total volume of 400 μl. The mixtures were loaded onto a 200-μl nickel-nitrilotriacetic acid column equilibrated with 50 mM sodium phosphate, 50 mM NaCl, 10 mM imidazole, pH 8.0. The column was washed with 1 ml of buffer containing 20 mM imidazole, and TorD was eluted in 400 μl of buffer containing 250 mM imidazole. Samples were analyzed for MGD content as described above.

**RESULTS**

**Screening Method to Isolate Inactive TorD Mutants**—TMAO reductase activity is mainly due to the activity of TorA, the periplasmic reductase of the Tor system (34). At 43 °C, it was shown previously that TorD is essential for TorA stability (18). Thus TorD$^-$ strain (LCB515) cannot use TMAO as a substrate when grown at 43 °C unless it bears a plasmid carrying and expressing torD gene (18). To discriminate TorD$^+$ and TorD$^-$ strains, cells were plated on MacConkey/maltose medium containing TMAO and incubated overnight at 43 °C. Hence cells from strain LCB515 unable to use TMAO as exogenous substrate grew as dark red colonies, whereas cells from the recombinant strain carrying pTorD plasmid were white due to the alkalization of the medium by the production of trimethylamine (35).

**Generation of Random torD Mutations**—Random mutagenesis of the torD gene was performed. 20,000 colonies of recombinant strains carrying potential TorD mutants were screened. Among 1380 red candidates, 516 contained the torD gene in the
of the TorD family that is genetically related to the type III molybdoenzymes group including TorA orthologs (23). Furthermore this genetic approach revealed none of the strictly conserved residues organized in the three distinct motifs of the TorD amino acid sequence (23).

**Analysis of the Chaperone Activity of TorD Mutants**—The intriguing result obtained by the random mutagenesis approach compelled us to study further the function impaired in both TorD mutants. To investigate the action of TorD variants on TorA activity, we introduced each multicopy plasmid expressing one of the mutated torD alleles back into strain LCB515, which is defective for TorD production. Complementation analysis at 37 or 43 °C of the torD-deficient strain LCB515 producing TorDC79R and TorDL83P mutants revealed that Cys-79 and Leu-83 residues are essential for the activity of TorA enzyme (Fig. 2, A and B). To study the bis-MGD insertion activity of TorD mutants into apoTorA, we next used the dedicated in vitro maturation assay (14). Purified apoTorA was mixed with the molybdenum cofactor source, and the TMAO reductase activity generated was measured after 120 min of incubation at 37 °C in the presence of either TorDC79R or TorDL83P mutants. In both cases the TorD mutants were found to be completely inactive because they were unable to enhance TMAO reductase activity and thus TorA maturation in the in vitro system (Fig. 2C). In conclusion, TorDC79R and TorDL83P mutants have no chaperone activity in vivo and in vitro.

**TorD Variants Interact with TorA Signal Sequence in Vitro**—A previous study demonstrated the crucial role of TorD in the protection of the apoTorA protein in vivo (19). Indeed in the absence of the chaperone, apoTorA is submitted to a two-step proteolysis by a still unknown system. Whereas the first attack leads to the loss of most of the signal peptide, the second attack results in the loss of the protein core. Binding of the TorA signal peptide by TorD variants was investigated by using isothermal titration calorimetry. Part of the TorA signal peptide (SPTorA10–50) comprising the twin arginine motif, the h-region, and the sec avoidance motif (36), was chemically synthesized. Titration of the TorD amino acid sequence (23). Furthermore this genetic approach revealed none of the strictly conserved residues organized in the three distinct motifs of the TorD amino acid sequence (23).

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TorD Variants Do Not Interact with the Core of TorA—To further study the capacity of the TorD variants to bind the core of TorA, bismaleimidohexane chemical cross-linking was performed between either TorDC79R or TorDL83P and apoTorA truncated of its signal peptide. Western immunoblotting established that neither TorD mutant could interact with the apoTorA form devoid of its signal peptide contrary to wild type TorD (Fig. 4A). To gain corroborative evidence, the bacterial two-hybrid system based on functional reconstitution of adenylate cyclase activity was used. torD alleles and 5’-truncated torA encoding the TorA protein devoid of signal sequence were cloned into the bait and prey plasmid pT18 and pT25, respectively, and complementation experiments were performed. The two-hybrid system approach showed that, contrary to wild type TorD, TorDL83P and TorDC79R variants were unable to interact with the core of TorA in vivo (Fig. 4B). In summary, although the interaction of TorD variants is conserved with the signal peptide, neither variant had the capacity to interact with the core of the protein. These results establish that the part of helix 5 containing both residues involved in the recognition and the binding to the core of TorA. Moreover the concomitant loss of both the interaction with the core of the enzyme and the activity of the TorD variants in the in vitro maturation system (Fig. 2C) demonstrated that the interaction with the core of TorA is required for molybdenum cofactor insertion. In the same line of thought, our results confirmed that the function of TorD in the cofactor inser-
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TorD Binds Various Moco Forms—Having established that TorD interaction with the core of TorA is an obligate step for TorA maturation, the question arose whether TorD is able to bind the molybdenum cofactor. Unfortunately attempts to purify the bis-MGD form of the cofactor from either TorA of E. coli or DMSO reductase of Rhodobacter species failed to give enough material to work with even when large amounts of bis-MGD-containing enzyme were used. This failure is due to the instability of purified bis-MGD, and thus it cannot be used in an isolated state for direct binding studies. Instead we used Mo-MPT extracted from purified sulfite oxidase for binding studies. TorD and Mo-MPT were incubated. Unbound Mo-MPT was then separated by ultrafiltration and quantified (Fig. 5A). Fitting revealed a function according to the law of mass action for a Mo-MPT to TorD ratio of 1:1 with a Mo-MPT saturation of 1.19. A $K_D$ value of $1.39 \pm 0.1 \mu M$ was obtained for Mo-MPT binding to TorD, whereas reactions with TorD and TorDC79R variants had an apparent $K_D$ value of $1.2 \pm 0.1 \mu M$ and $1.3 \pm 0.1 \mu M$, respectively. The signal peptide, neither variant had the capacity to interact with the core of the protein. These results establish that the part of helix 5 containing both residues involved in the recognition and the binding to the core of TorA. Moreover the concomitant loss of both the interaction with the core of the enzyme and the activity of the TorD variants in the in vitro maturation system (Fig. 2C) demonstrated that the interaction with the core of TorA is required for molybdenum cofactor insertion. In the same line of thought, our results confirmed that the function of TorD in the cofactor inser-
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incubated with Mo-MPT (10 μM Mo-MPT) in vitro binding (Fig. 5) that residues Cys-79 and Leu-83 are not involved in Mo-MPT was verified by the same technique, and these data established important, the capacity of TorD variants to still bind Mo-MPT checked using proteins known to be Moco-free (Fig. 5). More experiments clearly showed that the mutated region of TorD is not involved in the interaction with MobA (Fig. 7). The interaction of both proteins was also seen by surface plasmon resonance experiments. Results obtained by real time detection of the specific interaction provided a $K_D$ value of 0.34 ± 0.05 μM between MobA and TorD. This value corroborates and extends the involvement of TorD in TorA maturation. Testing TorDL83P and TorDC79R for MobA interaction by cross-link experiments clearly showed that the mutated region of TorD is not involved in the interaction with MobA (Fig. 7).

**DISCUSSION**

The *E. coli* TorD protein is involved in both the protection and the maturation of the molybdenum cofactor. In a previous work, we showed that the protective role of TorD toward TorA is mediated by the binding of TorD to TorA signal peptide (19). In this report, we revealed that the role of TorD on TorA maturation is dependent on TorD interaction with the core of TorA, and we established that the TorD α-helix 5 is crucial for this interaction. The two mutations isolated after random mutagenesis affected poorly conserved amino acids. Indeed Cys-79 and Leu-83 are present only in the subfamily genetically.
related to TorA orthologs and are replaced by Asn or Gln and Ile, Val, or Ala, respectively, in the other subfamily (23). These data probably explain the high specificity between the two partners and the fact that *E. coli* DmsD and YcdY, two TorD homologs from other subfamilies, were unable to complement in *vitro* and in *vivo* the lack of TorD (23). It thus appears that the determinants of TorD specificity are very likely borne by regions of low conservation. To decipher whether the partner specificity is located in the same area in TorD homologs, random mutagenesis or site-directed mutagenesis on α-helix 5 will be required. None of the highly conserved residues organized in three motifs in all the TorD family members were found as a mutagenesis target. Moreover the two conserved residues, Ser-95 and Glu-142, located between α-helices 5–6 and 7–8, respectively, and proposed by others as being involved in TorA maturation, were not revealed by the genetic approach (21). In fact, none of the highly conserved residues appear to play a key role in TorA maturation. In other respects, although the TorD-TorA core interaction was lost in L83P and C79R TorD variants, the in *vitro* binding with the TorA signal peptide was maintained (Figs. 3 and 4). This highlights that two distinct regions of TorD are involved in the recognition of the two TorA binding sites. As no mutant defective in signal peptide binding was isolated in this study, the loss of this interaction probably presents less deleterious effects on TorA production in the cell. Remarkably binding of TorD to apoTorA leads to a strong interaction between the two proteins because they can be easily co-purified (19). According to the level of TorD interaction at each apoTorA site, it appears that the binding strength to apo-TorA is probably greater than the sum of each apo-TorA site binding (Fig. 3 and Ref. 19). Thus, our hypothesis is that the two apo-TorA sites are bound cooperatively by TorD.

The relationship between the TorD-TorA core interaction and TorA maturation is now experimentally established. It was shown previously that TorD binding induces a competent state in which apoTorA is able to receive the molybdenum cofactor (13). To investigate further the role of TorD in TorA maturation, a search for TorD novel partners was undertaken, and Mo-MPT and MobA were found to interact with TorD (Figs. 5 and 7). The present discovery adds a new dimension to the involvement of private chaperones in the molybdenoenzyme maturation process. Indeed we demonstrated that TorD possesses two partners, apo-TorA and MobA, and Mo-MPT as ligand. Other researchers have shown that a second ligand, GTP, also binds TorD (22). It appears thus that, like a platform for TorA maturation, the chaperone binds and connects all the components required for the ultimate step of MGD biosynthesis and completion of MGD synthesis by MobA on the TorD platform, MGD binds TorD before its insertion into apoTorA. The difficulties we encountered in producing bis-MGD in *vitro* add weight to the argument that the MGD must be synthesized close to its insertion site. Consequently the TorD binding site in the core of the apoprotein could be near the catalytic site of TorA.

The establishment that TorD acts as a platform in the proximity of the insertion site of MGD and thus connects various partners increases the scope of this type of chaperone. The affinity of TorD for Mo-MPT and MobA is reminiscent of that of XdhC, the private chaperone for Moco-containing xanthine dehydrogenase in *R. capsulatus*. XdhC, which belongs to a family different from TorD, binds sulfurred Mo-MPT and also interacts with MobA enzyme, although Mo-MPT rather than MGD is found in the catalytic site of xanthine dehydrogenase (16, 28). The binding of Mo-MPT to XdhC is required for the protection of sulfurred Mo-MPT from oxidation, whereas the binding to MobA inhibits MGD biosynthesis. In bis-MGD enzyme maturation, the mechanism is similar, but the purpose is different. It is now conceivable that TorD binding to Mo-MPT facilitates its conversion into MGD by bringing closer all the protagonists. This process could be similar in other families of molybdenoenzyme chaperones. For instance, NarJ, the auxiliary protein dedicated to nitrate reductase NarGHI maturation, was shown to interact with apoNarG at two distinct sites (37). Even if the molybdooenzyme subunit NarG was shown to interact with the molybdenum cofactor biosynthesis enzymes, the role of NarJ during this process is yet not clearly defined (38). It could be of interest to investigate whether Mo-MPT or MGD is also a NarJ ligand.

In conclusion, this work highlights the functional complexity of molybdenoenzyme-specific chaperones. The major function of these specific chaperones, revealed by this study, may be to coordinate the maturation of both the cofactor and the enzyme by acting as a connector between all the required components. The challenge will be to elucidate the molecular mechanisms leading to the formation and delivery of bis-MGD into prokaryotic molybdenoenzymes, which may have biotechnological applications.

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4 O. Genest and C. Iobbi-Nivol, unpublished results.
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