Involvement of a Mate Chaperone (TorD) in the Maturation Pathway of Molybdoenzyme TorA

Marianne Ilbert†, Vincent Méjean, Marie-Thérèse Giudici-Orticini, Jean-Pierre Samama‡, and Chantal Iobbi-Nivol

From the Laboratoire de Chimie Bactérienne and §Laboratoire de Bioénergétique et Ingénierie des Protéines, Institut de Biologie Structurale et Microbiologie, CNRS, 31, chemin Joseph Aiguier, 13402 Marseille Cedex 20, France and *Département de Biologie et de Génétique Structurales, Institut de Genétique et de Biologie Moléculaire et Cellulaire, BP 10142, 1, rue Laurent Fries, 67404 Illkirch, France

As many prokaryotic molybdoenzymes, the trimethylamine oxide reductase (TorA) of Escherichia coli requires the insertion of a bis(molybdopterin guanine dinucleotide)molybdenum cofactor in its catalytic site to be active and translocated to the periplasm. We show in vitro that the purified apo form of TorA was activated weakly when an appropriate bis(molybdopterin guanine dinucleotide)molybdenum source was provided, whereas addition of the TorD chaperone increased apo-TorA activation up to 4-fold, allowing maturation of most of the apoprotein. We demonstrate that TorD alone is sufficient for the efficient activation of apo-TorA by performing a minimal in vitro assay containing only the components for the cofactor synthesis, apo-TorA and TorD. Interestingly, incubation of apo-TorA with TorD before cofactor addition led to a significant increase of apo-TorA activation, suggesting that TorD acts on apo-TorA before cofactor insertion. This result is consistent with the fact that TorD binds to apoTorA and probably modifies its conformation in the absence of cofactor. Therefore, we propose that TorD is involved in the first step of TorA maturation to make it competent to receive the cofactor.

The molybdenum cofactor is a ubiquitous molecule associated with a wide range of redox enzymes and is found in most organisms from bacteria to humans (1, 2). Except for nitrogenase, the metal in molybdoenzymes is coordinated to a pterin derivative called molybdopterin (MPT)1 to form the MPT-Mo cofactor (3). In bacteria, the basic form of the molybdenum cofactor is generally modified by the attachment of a nucleotide moiety to the phosphate group of MPT-Mo. In many cases, the final step of the cofactor synthesis is the linkage of GMP to MPT-Mo, giving rise to molybdopterin guanine dinucleotide (MGD-Mo) (3). In Escherichia coli, it is now clearly established that the conversion of MPT-Mo to MGD-Mo is catalyzed by the metalloenzyme TorA, belonging to the Me₂SO reductase family described above, and TorC, a membrane-anchored pentahemicytochrome. A recent study showed that the interaction of the two proteins allows the electron transfer from TorC to the terminal reductase TorA (17). The genes encoding TorC and TorA are organized in the torCAD operon. The last gene of this operon codes for TorD, a 22-kDa cytoplasmic protein (18). The related operons, torECAD and dorCDA (also called dmsCBA), were characterized in Shewanella and Rhodobacter species, respectively, and they encode E. coli TorC, TorA, and TorD homologous proteins (19–21).

We showed previously that the TorD proteins from E. coli and Shewanella massilitta bind specifically to their cognate TorA enzymes (22, 23). Moreover, the absence of TorD in E. coli leads to a significant decrease of the amount of TorA (22). Strikingly, in Rhodobacter capsulatus the absence of DorD results in a complete loss of DorA (24). Finally, in an E. coli MGD-deficient strain, an excess of TorD limits the proteolytic degradation of the TorA cytoplasmic apo form (22). All these results suggest that the proteins of the TorD/DorD family play

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‡ To whom correspondence should be addressed. Tel.: 33-4-91-16-44-27; Fax: 33-4-91-71-89-14; E-mail iobbi@ibsm.cnrs-mrs.fr.

1 The abbreviations used are: MPT, molybdopterin; MGD, molybdopterin guanine dinucleotide; bis(MGD)Mo, bis(molybdopterin guanine dinucleotide)molybdenum; TMAO, trimethylamine oxide; BMH, 1,6-bismaleimidohexane; BSA, bovine serum albumin.

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the role of chaperones dedicated to molybdoenzymes of the $\text{Me}_2\text{SO}$ reductase family. However, they can act either passively by protecting the reductases against cytotoxic proteins or more actively during the folding of the enzymes or even during the insertion of the bis(MGD)Mo cofactor. The X-ray structure of TorD of $\text{S. massilia}$ was recently solved (25). It reveals that the protein presents an all helical architecture organized in a globular domain showing no similarity with any known protein structures. Based on sequence homology, proteins of the TorD/DorD family could share a similar all helical three-dimensional fold. This new protein fold lets us imagine that this protein family might play an original role during molybdoenzyme biosynthesis. Moreover, DmaD, an $E. coli$ TorD homologue, was proposed to act as an escort protein targeting periplasmic molybdoenzymes to the twin arginine translocation machinery through its binding to their leader sequence (26). This chaperone family could, therefore, possess a dual function in the maturation process of the enzyme and the targeting to the export machinery.

In this study, using an in vitro approach we showed that TorD is required for the efficient maturation of the apo form of TorA and that it probably acts just before the bis(MGD)Mo cofactor insertion. We thus propose that TorD binds the apo form of TorA to favor a conformation of the apoenzyme that is competent for accepting the cofactor.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, Media, and Growth Conditions**—Bacterial strains used in this study are MC4100 (araD139 $\Delta$ (lacIPOZA-argF) U169 rpsL thi), LCB436 (MC4100 $\Delta$torSTRCDA KM) (27), LCB504 (MC4100 torC2::Spc’ $\Delta$mds’ Km) (28), RK5208 (araD139 $\Delta$ (lacIPOZA-argF) U169 rpsL gvr $\Delta$mobA207::Muc) (29), and BL21(DE3) (F’ompT $\Delta$hsdR’ $\Delta$myc $\Delta$dm $\Delta$(DE3)) (Novagen). The strains were grown in Luria broth medium at 37 °C, except strain RK5208, which was grown at 30 °C. To maintain plasmid selection, antibiotics were added at the following concentrations: ampicillin, 50 µg/ml; kanamycin, 10 µg/ml. When necessary isopropyl $\beta$-thiogalactopyranoside (1 mM) was added to induce gene expression.

**Constructions of Plasmids**—To construct plasmid pTorA, allowing the synthesis of His$_6$-tagged holo- and apoTorA, the complete torA-coding sequence was amplified by PCR, performed with MC4100 chromosomal DNA as template, and primer TorAN, which corresponds to an M; TorD, 28 Kbp; TorD, 21 Kbp. TorD was PCR product was cloned into the corresponding sites of expression vector pJF191EH (30). The recombinant plasmid was then introduced into strains LCB436 and RK5208.

To construct plasmid pTorD, allowing the synthesis of His$_6$-tagged TorD, the same cloning strategy was used except that one primer (TorDNS310) corresponds to an XbaI site followed by a Shine-Dalgarno consensus motif and the torD 5’-coding sequence, and primer TorAC, which corresponds to a HindIII site followed by a sequence encoding a C-terminal His$_6$ tag and the complementary sequence of the 3’ end of torD. The purified PCR product was digested by the corresponding restriction enzymes and cloned into the corresponding sites of expression vector pJF191EH (30). The recombinant plasmid was then introduced into strains LCB436 and RK5208.

**Purification of Recombinant Proteins**—Strains harboring plasmid pTorA or in addition of TorD (2.1 µg/ml) were grown at 30 °C under nitrogen atmosphere. Release of MPT-Mo from mola Soluble Fraction—Cells were grown aerobically at 37 °C by shaking at 200 rpm and centrifuged at 10,000 x g.

**Native Gel Experiments**—ApoTorA (1.68 µg) and TorD (21 µg) were incubated in phosphate buffer (20 mM, pH 7), final volume of the sample, 20 µl were incubated together or separately for 90 min at 37 °C. Before loading the samples onto a 10% polyacrylamide gel, both samples were treated with 10 µl of either native sample buffer (200 mM Tris-HCl, pH 8.8, 5 mM EDTA, 1% sucrose, 0.1% bromphenol blue) or denaturing-reducing sample buffer (66% native sample buffer, 6% SDS, 0.1% dithiothreitol, and 1.7% β-mercaptoethanol). When indicated, the samples were heated at 70 °C for 5 min before loading. The migration patterns were revealed by Western blots using TorA or TorD antisera.

**Chemical Cross-linking Studies**—These experiments were carried out by using 1,6-hexamethylenediamine (HMA) as a cross-linker. Proteins (apoTorA, 1.6 µg; TorD, 28 µg) were incubated for 30 min at room temperature in phosphate-buffered saline with HMA (1 mM). The interactions were analyzed by SDS-PAGE. Western blot detection was performed with TorA antibodies.

**Activation of Purified ApoTorA**—In the assays apoTorA (0.55 µg) was added to strain LCB504 (40 µg/ml) and TorD (21 µg/ml) were added in the following conditions: 20 μM TMAO (34). TMAO reductase activity was measured spectrophotometrically at 37 °C by following the oxidation of reduced benzyl viologen by 600 nm coupled to the reduction of TMAO (35).

**Interaction Study by Biosensor Experiments**—The surface plasmon resonance (BIACore apparatus, Biensor) was used to analyze the binding between TorD (M, 22,427) and apoTorA (M, 94,263). All experiments were carried out at 25 °C. TorD in 10 mM sodium acetate buffer, pH 5, was immobilized on a Chip CM5 of the CM5 chip. Western blotting, proteins were transferred to a Hybond ECL nitrocellulose membrane, and the ECL + Plus Western blotting system was used as recommended by the supplier (Amersham Biosciences). Protein concentrations were determined by the method of Lowry et al. (34). TMAO reductase activity was measured spectrophotometrically at 37 °C by following the oxidation of reduced benzyl viologen by 600 nm coupled to the reduction of TMAO (35).

**Activation of Purified ApoTorA**—In the assays apoTorA (0.55 µg) was added to strain LCB504 (40 µg/ml) and various concentration of TorD (from 0 to 2.1 µg), and phosphate buffer (20 mM, pH 7) was added to bring the total volume to 150 µl. All solutions were oxygen-depleted. The mixture was incubated from 0 to 240 min at 37 °C under a nitrogen atmosphere. To monitor the extent of activation, the TMAO reductase activity recovered in the samples was measured as a function of time and was expressed as the percentage of the activity measured with holoTorA (0.55 µg). As a control, the same assays were performed using bovine serum albumin (BSA) (20 µg/ml) instead of TorD or in addition of TorD (2.1 µg/ml). The experimental data that correspond to at least three assays for each point were fitted to exponential curves. As a control, the same assays were performed in the absence of apoTorA and apoTorA$, \text{SN504}$, and no activity of this activity was observed.

**ApoTorA and TorD Preincubation Experiments**—Three assays were performed. ApoTorA (3.3 µg, 25 µl) and TorD (12.5 µg, 2.5 µl) were mixed together and incubated for 60 min at 37 °C before the addition of SN504 (100 µl, 40 mg/ml) and phosphate buffer (20 mM, pH 7) to bring...
the final volume to 150 μl. As a control, the same assay was performed except that no TorD was added. In a last assay, apoTorA (3.3 μM, 25 μl) and TorD (1.25 μM, 25 μl) were incubated separately and pooled just before the addition of SN504. Activation was monitored as a function of time, as described above.

**Defined In Vitro Assay.—** The assays (150 μl) performed in 20 mM phosphate buffer, pH 7, contained apoTorA (0.55 μM), TorD (0 or 2.1 μM), and the bis(MGD)Mo source made up of MobA (0.06 μM), GTP (1 mM), and MgCl₂ (1 mM) and 96 μl of a solution containing MPT-Mo released from heat denatured supernatant of mobA cells. All these compounds were added at the same time and kept under a nitrogen atmosphere. The samples were then incubated at 37 °C, and the extent of activation was measured as described above. As a control, assays containing all the compounds except either apoTorA, MobA, or MPT-Mo source were incubated in the same conditions as described above and led to no TMAO reductase activity.

**RESULTS**

**Purification of TorD and of the Holo and Apo Forms of TorA.—** To investigate the putative role of TorD during the maturation process of TorA, we produced and purified each protein as C-terminal His₆-tagged recombinant proteins. The different forms of His₆-tagged TMAO reductase were obtained from expression plasmid harboring the coding sequence of tagged TorA. The plasmid was introduced either into a torA strain or into a mobA tagged TorA. The plasmid was introduced either into a torA strain or into a mobA strain and was expressed in E. coli JM109 (top15). The expression of TorA and TorD was confirmed by Western blotting with TorA antibodies. The mobility of this band is consistent with the molecular mass of the His₆-tagged TorA, respectively. Tagged-holoTorA was purified from the periplasmic fraction of the torA mutant. The specific activity (65 μmol of TMAO reduced/min/mg) was close to that observed with untagged TorA, indicating that the addition of the His₆ tag modifies neither the activity of the enzyme nor its maturation. apoTorA, the inactive tagged protein devoid of His₆ tag modifies neither the activity of the enzyme nor its maturation.

**TorD Interacts with ApoTorA.—** To characterize a possible binding between apoTorA and TorD, we studied the formation of an apoTorA-TorD complex using various complementary techniques. We first analyzed a potential interaction between the two proteins using a surface plasmon resonance (BIAcore) technique, and for this purpose, the purified TorD protein was coupled to the dextran matrix of a sensor chip, and apoTorA was injected over it. The sensorgram obtained reflected the association and dissociation of two proteins, indicating that apoTorA directly interacts with TorD (Fig. 2A). The apoTorA-TorD interaction was also revealed by cross-linking experiments using BMH or 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide as chemical ligands (Fig. 2B and data not shown). TorD and apoTorA alone or together were incubated with BMH, and the mixtures were loaded onto an SDS-polyacrylamide gel. Western blotting was then carried out with TorA antibodies.

Another approach to confirm the interaction was to analyze the behavior of these proteins by loading an apoTorA-TorD mixture previously incubated at 37 °C onto a 10% polyacrylamide gel. After electrophoresis under native conditions, the migration patterns were revealed by Western blot using antibodies specifically raised against each of the proteins (Fig. 3, A and B). When TorA-specific antibodies were used, a new band below that of apoTorA alone, appeared in the lane corresponding to the mixture of both proteins (Fig. 3A). In contrast, no additional band other than that corresponding to the migration pattern of TorD alone was revealed using TorD antibodies (Fig. 3B). This result suggests that the additional band corresponds either to a specific proteolysis of apoTorA mediated by TorD or to a new migrating form of apoTorA induced by TorD. To draw a distinction between the two possibilities, the samples were treated with a non-denaturating or a reducing-denaturating loading buffer and heated at 70 °C before being loaded on native polyacrylamide gel (Fig. 3, C and D). The fact that the band was still present after heating the sample in non-reducing buffer but disappeared in reducing conditions indicates that this band corresponds to a new fold of apoTorA stabilized by at least one disulfide bridge and discards the possibility of apoTorA proteolysis mediated by TorD. We suspect that the conformational change in apoTorA induced by TorD brings in vicinity two cysteine residues, allowing the formation of a disulfide bridge in some cases. Altogether, these results suggest that TorD not only interacts with apoTorA but also modifies the conformation of apoTorA.

**Fig. 3.** Comparison of the migration patterns of apoTorA, TorD, and apoTorA-TorD mixtures separated on native polyacrylamide gel. Lane 1 corresponds to apoTorA, lane 2 corresponds to TorD, lane 3 corresponds to apoTorA-TorD mixture, lane 4 corresponds to apoTorA-TorD mixture loaded on reducing-denaturating loading buffer, lane 5 corresponds to apoTorA-TorD mixture loaded on native polyacrylamide gel.
TorD Increases the Activation of ApoTorA in Vitro—The fact that TorD interacts with apoTorA and apparently induces apoTorA conformational change prompts us to study whether TorD contributes to the maturation of this enzyme and possibly to the bis(MGD)Mo insertion. Because apoTorA has no TMAO reductase activity but can be activated upon bis(MGD)Mo insertion, we measured apoTorA activation in the presence and in the absence of TorD. The bis(MGD)Mo source used in this experiment was the supernatant fraction of strain LB504 (referred as SN504). This strain, mutated on both tor and dms operons is devoid of TMAO reductase activity and does not synthesize TorD. On the other hand, this strain produces a complete bis(MGD)Mo and can, thus, be used as a convenient cofactor source for the assay. ApoTorA (0.55 μM) was mixed with SN504 (100 μl, 40 mg/ml of proteins) under anaerobic conditions and incubated at 37 °C. The extent of apoTorA activation was determined by measuring the TMAO reductase activity generated in the sample as a function of the incubation time (Fig. 4A). In this assay, the amplitude value of the recovered activity reached about 20% of the activity of the same amount of holoTorA. Because the activity measured corresponds to the quantity of active enzyme obtained in the sample, we conclude that only one-fifth of apoTorA can be activated even after a long time of incubation. To check whether the amount of SN504 was or was not limiting in the assay, increasing it did not lead to a change in the recovered activity, whereas decreasing it led to a decrease in the level of apoTorA activation (data not shown).

To assess a possible role of TorD in the TorA maturation process, increasing amounts of TorD were added to the assay defined above. As shown in Fig. 4A, the recovered TMAO reductase activity increased from 20 to 80% that of holoTorA when TorD concentration was raised from 0 to 2.1 μM. TorD concentration higher than 2.1 μM did not further increase the apoTorA activation (data not shown). In conclusion, in the absence of TorD, only a fraction of apoTorA could be activated in the assay, whereas the presence of TorD allowed the maturation of most of the apoprotein. The fact that TorD seems to facilitate the bis(MGD)Mo insertion into apoTorA strongly suggests that it plays an active role in the maturation process of the apoenzyme. To verify that TorD has a specific action toward apoTorA, it was substituted by the BSA. As expected, the apoTorA activation was not increased in the presence of a high concentration of BSA compared with that obtained in absence of TorD (Fig. 4A). Moreover, when both TorD and BSA were added together in the same assay, no decrease of apoTorA activation was observed compared with that measured with TorD alone (data not shown). These experiments show that the increase of apoTorA activation is specifically due to the presence of TorD in the assay.

FIG. 4. Time course for the in vitro activation of apoTorA. TMAO reductase activities, given as a percentage of the activity of holoTorA (0.55 μM), were measured as a function of incubation time. The curves are exponentials fitted to experimental data. A, activities from aliquots of mixtures containing apoTorA (0.55 μM), SN504 (100 μl, 40 mg/ml), TorD (1.2 μM, 0.4 μM, 0.21 μM), BSA (C, 20 μM), B, apoTorA (0.55 μM, concentration in the final assay) was mixed with TorD (0.21 μM, concentration in the final assay) and incubated for 60 min at 37 °C before the addition of SN504 (100 μl, 40 mg/ml). C, apoTorA (0.55 μM, concentration in the final assay) and TorD (0.21 μM, concentration in the final assay) were incubated separately for 60 min at 37 °C and mixed together when SN504 was added. D, as a control, apoTorA was incubated for 60 min at 37 °C before SN504 addition. The time corresponds to the time of incubation with SN504.
Maturation of the TorA Molybdoenzyme

DISCUSSION

The E. coli TorA protein is a TMAO reductase containing a bis(MGD)Mo cofactor in its catalytic site, and we showed in this study that TorD is the mate chaperone of TorA, allowing its efficient maturation. Indeed, the TMAO reductase activity that is recovered in vitro from immature apoTorA mixed with a source of molybdenum cofactor, increased as a function of TorD concentration. It reached an amplitude value corresponding to the activation of 63–80% of the apoTorA present in the assays, whereas only 13–20% of apoTorA was matured in the absence of TorD (Fig. 4A and 5).

Because preincubation of apoTorA with TorD before the cofactor addition significantly increased the quantity of recovered TMAO reductase activity (Fig. 4B), we suggest that TorD acts in a first step onto apoTorA to favor insertion of the bis(MGD)-Mo cofactor (Fig. 6). This hypothesis is also supported by the fact that TorD binds to apoTorA in the absence of any other compound and probably modifies to some extent apoTorA conformation (Figs. 2 and 3). It would be interesting to define the TorD and apoTorA binding regions and to determine whether the conformational change induced by TorD is restricted or not to the region of the TorA catalytic site. To explain that only a small fraction of apoTorA was matured in the absence of TorD even after a long incubation time, we propose that a large fraction of apoTorA evolves to a stable non-activatable conformation, whereas the remainder turns spontaneously into a competent form able to receive the molybdenum cofactor. In this line of thought, the presence of TorD could shift the equilibrium toward the competent state of apoTorA and, thus, allows the maturation of the majority of the apoenzyme (Fig. 6). However, our results do not exclude an additional role for TorD during or even after the cofactor insertion.

Using a defined in vitro system, we observed that the efficient activation of apoTorA is obtained in the presence of only two functional proteins, TorD and MobA (Fig. 5). Although this latter is required for the conversion of MPT-Mo into bis(MGD)-Mo (5), it remains possible that MobA plays some role during TorA maturation, for example by escorting the cofactor during its insertion into apoTorA. To clarify this point, it would be necessary to perform defined in vitro assays without MobA but unfortunately, pure bis(MGD)Mo is unstable, and MobA is always required for this kind of in vitro experiment (3).

It should be noted that the presence of E. coli-soluble proteins in the cofactor-containing extract does not significantly change the pattern of activation of apoTorA compared with the experiment carried out with the defined in vitro assays (Fig. 4A and 5). This observation indicates that, at least in vitro, no soluble protein can efficiently substitute for TorD during the maturation process of TorA. However, it was demonstrated that GroEL is required for the final insertion of the iron-molybdenum cofactor into the nitrogenase of Azotobacter vinelandii (36). Therefore, the possibility that a general chaperone may also be involved in the maturation process of apoTorA should be considered.

The TorD family is a large family of proteins (25), but except for TorD of E. coli, the putative role of these proteins in the maturation process of molybdoenzymes remains to be defined. Interestingly, disruption of the dorD gene, which encodes a TorD homologue in R. capsulatus, leads to the disappearance of
the molybdoenzyme DorA (24). This result points out a relationship between DorD and DorA and suggests that immature DorA is rapidly degraded unless DorD binds to it and allows maturation. It was recently shown that 73% of apoDorA can be activated in vitro after 7 h of incubation (5). This experiment was carried out in the absence of DorD, and it would, thus, be interesting to test whether the presence of DorD will accelerate and/or increase the maturation of DorA.

There is little evidence for the implication of chaperones in the maturation process of molybdoenzymes, and in addition to TorD, only two proteins, NarJ and XDHC, have been proposed to play such a role toward their cognate molybdoprotein. Although both proteins do not belong to the TorD family, NarJ was shown to act as a specific chaperone of the bis(MGD)Mo-containing subunit of the membranous nitrate reductase of *E. coli*, and XDHC was suspected to be involved in the maturation of MPT-Mo containing xanthine dehydrogenase (XDH) of *R. capsulatus* (37–39). Because several homologues of these proteins are found in different organisms, we postulate that many molybdoenzymes need a mate chaperone during their maturation process to improve cofactor insertion.

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