TorD, an Essential Chaperone for TorA Molybdoenzyme Maturation at High Temperature*

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TorD has been recognized as an accessory protein that improves maturation of TorA, the molybdenum cofactor-containing trimethylamine oxide reductase of Escherichia coli. In this study, we show that at 42 °C and in the absence of TorD TorA is poorly matured and almost completely degraded. Strikingly, TorD restores TorA maturation to the same level whatever the growth temperature. In vitro experiments in which apoTorA was incubated with or without TorD at various temperatures confirm that TorD is an essential chaperone for TorA at elevated temperatures preventing apoTorA misfolding before cofactor insertion.

The understanding of the mechanism of metalloprotein maturation is a challenge, because the insertion of a metal ion into a protein often requires complex pathways involving auxiliary proteins (for a review see Ref. 1). Maturation of molybdenum cofactor-containing proteins is a good example of this complexity, because it can involve cofactor escort proteins as well as specific chaperones (2, 3). Depending on the molybdoenzymes, the molybdenum cofactor (MoCo) can be a molybdopterin (MPT-Mo), a molybdopterin guanine dinucleotide (MGD), or a bis(MGD)Mo (4). This latest form was found in the large Escherichia coli reductase family of bacterial molybdoenzymes (5).

In Escherichia coli, TorA, a member of the 

Me2SO reductase family, is the main respiratory enzyme responsible for the TMAO reduction when the cells are grown anaerobically in the presence of TMAO (6). TorA is located in the periplasm and receives electrons from TorC, a pentahemec type cytochrome (7). TorA and TorC are encoded by the torCAD operon, which is induced in the presence of TMAO (6). TorA crosses the inner membrane by the TAT machinery in a folded state, meaning that the molybdenum cofactor is inserted into the apoprotein in the cytoplasm before translocation (8). TorA has been used as a model protein for the study of both cofactor insertion and translocation. Recently, we established that TorA maturation is improved two to three times by the presence of the cytoplasmic TorD protein that proved to be the specific chaperone of TorA (9–11). TorD interacts with apoTorA and allows it to become competent to receive the MoCo. Using in vitro assays containing purified apoTorA and a source of MoCo, we showed that TorD alone was sufficient to allow an optimal maturation of the enzyme (10).

A second role was also attributed to TorD during the translocation process of TorA by the TAT translocase to prevent export of immature TorA (12, 13). During this proofreading mechanism, TorD binds the signal peptide of TorA and exhibits a quality control activity to ensure that only matured TorA is addressed to the TAT translocase. These two functions of TorD appeared independent, because it was clearly demonstrated that the action of TorD during TorA maturation is independent of the presence of the signal peptide (11). Thus, TorD also binds to a second region of TorA in addition to the signal peptide (9, 13).

TorD is a member of a large family of homologous proteins associated to molybdoenzymes of the Me2SO reductase family (11, 14). Among them, TorD from Shewanella massilia was crystallized, and its three-dimensional structure was solved revealing an all-helical architecture showing no similarity with other known protein structures (15, 16). We and others have also shown that members of the TorD family are structurally related and contain mainly α-helices (11, 17). Although these proteins are part of the same family of chaperones, they probably possess a high specificity toward their partner. For instance, DmsD, a TorD homologue in E. coli, is necessary for the activity of molybdoenzyme DmsA (11, 18) but cannot replace TorD during TorA maturation, and, conversely, TorD cannot play the role of DmsD toward DmsA (11).

In this study we show that although TorD is an accessory protein at 37 °C, it becomes essential for TorA maturation at elevated temperatures. This study also reveals that misfolded apoTorA is degraded in vitro and that TorD not only avoids apoTorA degradation but also plays a key role in the first step of TorA maturation.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions—The E. coli strains used in this work are LCB514 (MC4100 ΔdmsD Km’ (11), LCB515 (MC4100 torD::Spc ΔdmsD Km’) (11), RK5208 (araD139 ΔlacIPOZYA-argF) U169 rpsL gyrA mobA207::Mucts) (19), LCB440 (MC4100 ΔtorSTRCAD) ΔdmsABC Km’, this study), and LC8620 (MC4100 torA8::MudII 1734 (torA-lexZ) (6). The strains were grown in Luria Broth medium, and, when necessary, ampicillin (50 μg/ml) was added to maintain plasmid selection. Plasmid pTorA allowing His6-tagged apoTorA production was previously described (10). To construct plasmid pBD, which allows the synthesis of His6-tagged TorD, the same cloning strategy as previously described for pTorD (11) was used except that the torD coding sequence was cloned into the EcoRI-HindIII cloning sites of pBAD24 (20).

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1 The abbreviations used are: MoCo, molybdenum cofactor; TMAO, trimethylamine oxide; bis(MGD)Mo, bis(molybdopterin guanine dinucleotide)/molybdenum; MPT-Mo, molybdopterin-molybdenum; ATPγS, adenosine 5’-O-(thiotriophosphate).

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the recombinant plasmid was checked by sequencing. Transformations were carried out according to the method of Chung and Miller (21).

Preparation of the Soluble Fractions—Strains LCB514, LCB515, LCB515/pBAD24, and LCB515/pBD were grown overnight anaerobically at 37 and 42 °C with TMAO (0.2%) to induce chromosomal tor operon expression. 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, with the recovered supernatants then being centrifuged at 45,000 rpm to obtain the soluble fractions. The periplasmic and cytoplasmic fractions of strains LCB514 and LCB515 were prepared according to the sucrose-lysozyme-EDTA procedure as described previously (9).

Analytical Procedure—Protein concentrations were measured by the method of Lowry. 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 (9). The amount of TorA present in the extracts was determined by rocket immunoelectrophoresis (10). After purification, apoTorA and TorD were dialyzed against phosphate buffer (pH 7) and stored at 0°C. TorD and TorA antibodies had previously been added. Both preparations were performed by HI Trap Chelating HP chromatography (Amersham Biosciences) as described previously (10). After purification, apoTorA and TorD were dialyzed against phosphate buffer pH 7 (20 mM).

In Vitro Maturation System—ApoTorA (3 mg/ml) and phosphate buffer, pH 7 (20 mM, 25 μl), or apoTorA (3 mg/ml, 25 μl) and TorD (60 mg/ml, 25 μl) mixed together were incubated for 30 min at 35, 37, 42, and 47 °C before the addition of 100 μl of the MoCo source. The MoCo source corresponds to the soluble fraction of strain LCB440 grown anaerobically at 37 °C, prepared in 20 mM phosphate buffer, pH 7, at a protein concentration of 40 mg/ml (10). When ATP (1 mM) or ATP-β-S (1 mM and 5 μM) and MgCl₂ (1 mM) were tested, they were added during the 30-min incubation of apoTorA in the presence or absence of TorD at various temperatures. The MoCo source is then added as mentioned above. All solutions were oxygen-depleted. After an incubation of 60 min at 37 °C the TMAO reductase activity was measured. Units corresponded to μmol TMAO reduced/min/μg of apoTorA.

Results and Discussion

TorD Is Required for TorA Maturation at High Temperature_in Vivo_—The TorA protein is fully produced when cells are grown in anaerobiosis in the presence of TMAO. In an earlier study, we showed that the loss of TorD does not abolish TorA maturation but decreases its level (9). Indeed, 30–40% of the TMAO reductase activity was measured in a TorD- strain compared with a TorD+ strain when both were grown anaerobically in the presence of TMAO. Previously, the growths were routinely performed at 30 or 37 °C. To investigate further the role of TorD as a specific chaperone of TorA, we grew both the wild type (LCB514) and mutant (LCB515) strains at an elevated temperature. After overnight growth at 42 °C, soluble extracts were prepared, and the TMAO reductase activities were measured. As shown in Fig. 1A, the level of TMAO reductase activity measured in the soluble extract of strain LCB514 (TorD+) was similar when cells were grown at 37 and at 42 °C (2.2 and 2.3 units, respectively), indicating that the high temperature of growth does not affect TMAO reductase activity in the presence of TorD. In contrast, when cells of strain LCB515 (TorD-) were grown at 42 °C, the TorA activity was dramatically affected, because it was 19 times lower than that measured in the TorD+ strain grown in the same condition (0.12 instead of 2.3 units). As expected for growth at 37 °C, the absence of TorD led to ~3 times less TMAO reductase activity (0.65 units with 2.2 units in the presence of TorD). To verify that the effect is only because of the absence of TorD in strain LCB515, the torD mutation was complemented by a plasmid (pBD) harboring the wild type torD gene. Strain LCB515 containing pBD and the control strain (LCB515/pBAD24) were grown in TorA-inducing conditions at 37 and 42 °C. A similar result was obtained because for growth at 42 °C, 16 times more TMAO reductase activity was measured when TorD was produced in trans, whereas at 37 °C, 3 times more activity was obtained in the presence of TorD. This point means that TorD is responsible for the increase of TorA maturation at elevated temperatures of growth. We also performed similar experiments at 25 °C. In this growth condition, the loss of TorD caused only two times less TMAO reductase activity (data not shown).

We also checked whether the absence of TorD affected the subcellular location of TorA at high temperature. We observed that in the presence or in the absence of TorD, at least 60% of the TMAO reductase activity was found in the periplasmic fraction of cells grown at 42 °C. This point indicates that at high temperature and in the absence of TorD, the weak activity is mainly provided by a MoCo-containing TorA, which has translocated into the periplasm. Therefore, the absence of TorD...
does not abolish the translocation of mature TorA even at high temperature.

These experiments clearly established that TorD is required for TorA maturation when cells are grown in drastic temperature conditions. In this case, the loss of TorD almost abolishes TorA activity. The essential role of TorD was confirmed by the inability of strain LCB515 (TorD−) to grow anaerobically at elevated temperature in minimal medium with glycerol as the non-fermentable carbon source and TMAO as the only terminal electron acceptor (data not shown). Altogether, these data indicated that TorD plays an essential role at elevated temperatures of growth and improves TorA maturation at lower temperatures. Furthermore, because it is now established that TorD is directly involved during TorA maturation (10), TorD could induce or stabilize the competent form of apoTorA at high temperature.

**Protective Role of TorD on apoTorA**—The strong decrease of TMAO reductase activity observed for TorD− cells grown at 42 °C compared with 37 °C is either due to a drastic decrease of the amount of TorA or to the accumulation of inactive TorA protein. To discriminate between the two possibilities, we checked the amount of TorA protein from cells grown at 37 and 42 °C, and we also monitored the effect of the temperature on the torA expression level.

To test whether temperature variations could affect the expression of torCAD, a chromosomal torA-lacZ fusion was used. Growths were performed in inducible conditions at 25, 37, and 42 °C. In the three conditions of temperature, the levels of β-galactosidase activity measured were similar (300–340 Miller units) meaning that the variations of temperature from 25 to 42 °C do not modify the level of torCAD expression. This result indicates that the changes in the TorA activity do not come from transcriptional effects.

The amount of TorA present in the extracts was estimated by rocket immunoelectrophoresis using antibodies specifically raised against TorA (Fig. 1B). When strains LCB514 and LCB515 were grown at 37 °C, about one-third of TorA protein was recovered in the soluble extract of the TorD− strain compared with the TorD+ strain (Fig. 1B, tracks 1 and 3). Moreover, a similar amount of TorA was found in extracts of LCB514 grown at either 37 or 42 °C (Fig. 1B, compare track 1 with 2). Strikingly, in the soluble extract of strain LCB515 (TorD−) grown at 42 °C, only a very low amount of TorA was recovered, and the arc was at least 15 times smaller than that observed in both temperature conditions for the TorD+ strain (Fig. 1B, compare track 4 with tracks 1 or 2). TorA is thus strongly degraded at 42 °C in the absence of TorD. This effect agrees with the drastic decrease of the TMAO reductase activity and indicates that TorD protects the immature form of TorA directly or indirectly. Our proposal is that the presence of TorD stabilizes apoTorA to prevent misfolded state induced by the high temperature and devoted to proteolysis.

**Effect of Elevated Temperatures on the In Vitro Maturation of TorA**—To test the effect of the temperature on apoTorA folding and the role of TorD in those conditions, we incubated apoTorA at various temperatures for 30 min in the presence or in the absence of TorD. We then added a MoCo source and incubated the mixture for 1 h at 37 °C to test the activation ability of apoTorA. The resulting TMAO reductase activity, which corresponds to the level of TorA maturation, thus reflects the amount of competent apoTorA in the sample.

In a first set of experiments, apoTorA was incubated alone at 37, 42, and 47 °C for 30 min. The MoCo source was then added to each sample for 1 h at 37 °C. Fig. 2A shows a 13-fold activity decrease when apoTorA was incubated at 47 °C and an ∼3-fold decrease when apoTorA was incubated at 42 °C compared with the level of activity measured in the sample incubated at 37 °C. These data indicate that high temperatures also affect the *in vitro* maturation of TorA and strongly suggest that apoTorA is rapidly misfolded in these conditions leading to a significant decrease of its *in vitro* activation (Fig. 2A).

In a second set of experiments, TorD was added to apoTorA during the incubation at 37, 42, and 47 °C. After 30 min of incubation, the MoCo source was added for 1 h at 37 °C, and the TMAO reductase activity was then measured. In contrast to what was obtained when apoTorA was incubated alone, in the presence of TorD the level of TMAO reductase activity remained constant at 37 and 42 °C and slightly decreased (1.7-fold) at 47 °C (Fig. 2A). As a consequence, when the temperature rises, the activity ratio measured in the presence or in the absence of TorD increased from 2 at 37 °C and 5 at 42 °C, up to 14 at 47 °C. These results indicated that TorD prevents the deleterious effects of elevated temperature on apoTorA probably by maintaining it in a competent state for maturation. Thus, our *in vivo* and *in vitro* experiments revealed that TorD possesses an essential role as a chaperone when apoTorA experiences elevated temperatures.

For their activity, molecular chaperones usually require ATP as an energy source. To test whether the action of TorD toward apoTorA could be enhanced by ATP, ATP-Mg2+ (1 mM), or its non-hydrolyzable analog ATPγS (1 or 5 mM) were added during the 30 min-incubation period of apoTorA and TorD at 42 and 47 °C. We observed that the activities measured after the *in vitro* activation test were not modified by the presence of ATP or ATPγS and thus remained in the same range of values as those described in Fig. 2A (data not shown). As a control, a similar
experiment was performed by incubating apoTorA at 42 and 47 °C for 30 min in presence of Mg²⁺-ATP (1 mM) or ATPγS (1 or 5 mM), and again no change was observed. These results strongly suggested that TorD does not require ATP to act as a chaperone toward apoTorA and to maintain it in a competent fold.

By rocket immunoelectrophoresis using TorA antibodies, we have quantified the amount of mature and immature TorA present in the various samples tested for TMAO reductase activity. When apoTorA was incubated in the presence of TorD, the amount of immunoprecipitated protein was constant whatever the incubation temperatures were (Fig. 2B, tracks 4–6). When apoTorA was incubated alone, the amount of protein decreased slightly when the temperature increased (Fig. 2B, tracks 1–3). This result clearly shows that misfolded apoTorA is poorly degraded in our in vitro assays in contrast to what was observed in vivo, indicating that the role of TorD is not restricted to apoTorA protection against proteolysis but rather is to allow its correct folding.

Maturation of TorA in an in Vitro Defined Assay—Using a defined assay, we have previously shown that TorD alone is sufficient to allow an efficient maturation of apoTorA at 37 °C (10). In this defined assay, apoTorA or apoTorA and TorD were mixed with MPT-Mo provided from a heated mobA supernatant (100 °C, 5 min), GTP, and the purified MobA enzyme, which catalyzed the conversion of MPT-Mo to bis(MGD)Mo. After 30 min of incubation of apoTorA or apoTorA and TorD at 37, 42, and 47 °C, we added the defined MoCo source for 2 h at 37 °C to allow the synthesis and the incorporation of the bis-(MGD)Mo. The results shown in Fig. 3 indicate that incubation of apoTorA alone at 42 and 47 °C leads, respectively, to a 2.5- and 23-fold decrease compared with the activity measured in the apoTorA sample incubated at 37 °C. This result confirms that high temperature apoTorA is rapidly misfolded. In contrast, when apoTorA was first incubated with TorD, the activity recovered was similar at 37 and 42 °C and decreases 3-fold at 47 °C (Fig. 3). The ratio of the activity recovered in the presence or in the absence of TorD increases from 4 °C at 37 °C and 10 °C at 42 °C up to 25 at 47 °C. These results confirm the key role of TorD at high temperature, but the levels of activation are lower with the defined assay compared with that obtained with the supernatant fraction (compared Fig. 2A with Fig. 3). This difference results probably from the low amount of bis(MGD)Mo available in the defined assay (10). In conclusion, using the defined activation assay, we demonstrated that TorD alone is sufficient to maintain apoTorA in a competent fold and to facilitate the MoCo insertion into the apoenzyme. However, as MobA was also present in the activation system, we cannot discard a putative role of MobA during the MoCo insertion in addition to its involvement in the last step of MoCo biosynthesis.

Conclusion—This study reveals the essential role of TorD during TorA maturation at high temperature and leads us to propose the model shown in Fig. 4. High temperatures accelerate misfolding of apoTorA, which could not insert the MoCo and becomes sensitive to proteolysis in vivo. TorD counterbalances this deleterious effect by stabilizing or inducing the competent fold of the apoprotein, which then acquires the MoCo. Therefore, apoTorA seems to be in an unstable state between a competent and an incompetent form, and the TorD chaperone modifies this equilibrium toward the competent state, and, as a consequence, TorD also contributes indirectly to the protection of apoTorA assuming that competent apoTorA is not degraded.

This study is the first description of the essential role of a molybdoenzyme-specific chaperone experiencing elevated temperatures, and this feature could be used as a powerful tool to study in details TorD involvement in both TorA maturation and translocation. It will be also very interesting to study whether this novel characteristic of TorD is shared by other members of this family of molybdoenzyme-specific chaperones.

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