NarJ Chaperone Binds on Two Distinct Sites of the Aponitrate Reductase of *Escherichia coli* to Coordinate Molybdenum Cofactor Insertion and Assembly*  

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Understanding when and how metal cofactor insertion occurs into a multisubunit metalloenzyme is of fundamental importance. Molybdenum cofactor insertion is a tightly controlled process that involves specific interactions between the proteins that promote cofactor delivery, enzyme-specific chaperones, and the apoenzyme. In the assembly pathway of the multisubunit molybdenum, membrane-bound nitrate reductase A from *Escherichia coli*, a NarJ-assisted molybdenum cofactor (Moco) insertion step, must precede membrane anchoring of the apoenzyme. Here, we have shown that the NarJ chaperone interacts at two distinct binding sites of the apoenzyme, one interfering with its membrane anchoring and another one being involved in molybdenum cofactor insertion. The presence of the two NarJ-binding sites within NarG is required to ensure productive formation of active nitrate reductase. Our findings supported the view that enzyme-specific chaperones play a central role in the biogenesis of multisubunit molybdenum enzymes by coordinating subunits assembly and molybdenum cofactor insertion.

Molybdenum enzymes are involved in numerous metabolic reactions in the carbon, nitrogen, and sulfur cycles and crucial for all forms of life (1). With the exception of nitrogenase, the active site of molybdenum enzymes contains a molybdenum cofactor (Moco) that has an ubiquitous basic structure composed of a molybdenum atom coordinated to one or two molecules of a tricyclic pyranopterin (2, 3). The past few years have seen spectacular advances in our understanding of the molecular mechanisms of Moco biosynthesis, a highly conserved biosynthetic pathway (4–8). In contrast, information concerning biogenesis of molybdenum enzymes is scarce. Molybdenum enzyme biogenesis, the process that ensures productive formation of active molybdenum enzymes, generally involves both metal cofactor insertion and multisubunit assembly. In prokaryotes, the Moco insertion process is a cytoplasmic post-translational event (9) often assisted by enzyme-specific chaperones (10–13).

Dissimilatory nitrate reductase A from *Escherichia coli* (NarGHI) is one of the best studied multisubunit molybdenum enzymes (14) and can be considered as a model system for studying the biogenesis process in prokaryotic enzymes. NarGHI is a heterotrimeric enzyme comprising a Moco and an iron-sulfur-containing catalytic subunit (NarG, 139 kDa), an iron-sulfur-containing subunit (NarH, 58 kDa) and a quinol-oxidizing membrane-bound heme b subunit (Narl, 26 kDa) (14, 15). NarGHI is located in the cytoplasm, anchored to the cytoplasmic membrane by NarI. When liberated from the membrane, the NarGHI complex retains its activity using artificial electron donors such as benzyl viologen. Finally, the enzyme-specific chaperone NarJ plays an essential role for nitrate reductase A activity, facilitating Moco insertion into NarG (11).

As observed for other known molybdenum enzymes (16–19), the crystal structure of the NarGHI complex (20, 21) reveals that Moco is an extended molecule deeply buried into the enzyme complex at the NarG-H subunit interface. This observation suggests that the subunit assembly and the Moco incorporation must be tightly coordinated. Such coordination has been shown using the membrane-bound nitrate reductase from *Thermus thermophilus*. Indeed, the NarJ-assisted Moco insertion step can only occur once the apoenzyme complex is attached to the cytoplasmic membrane (22).

In this work, we have provided new insights about molybdenum enzyme biogenesis in *E. coli*. We have revealed a novel function for the enzyme-specific chaperone NarJ that goes beyond its reported implication in the Moco incorporation process. Our results demonstrated that NarJ acts at two distinct binding sites on the NarG subunit within the apoenzyme complex, one interfering with membrane anchoring and another being involved in Moco insertion. Overall, the enzyme-specific NarJ protein coordinates assembly and molybdenum cofactor acquisition of the heterotrimeric enzyme during the biogenesis process.

**MATERIALS AND METHODS**

**Bacterial Strains and Growth Conditions**—The *E. coli* strains and plasmids used in this work are described in Table 1. BL21(DE3) was employed for protein overexpression using pET22b-type expression plasmids (Novagen). BTH101 was used as recipient strain for two-hybrid assays. In-frame deletion of *narJ* was performed according to the Hamilton procedure (23). The *mob*/*narJ* double mutant strain was obtained by P1 transduction as described before (15). Bacterial cultures were grown aerobically in L-broth medium at 37°C. For biochemical studies, strains were grown anaerobically in L-broth medium supplemented with glucose (0.3%) and nitrate (0.2%). When required, the appropriate antibiotics and 0.2 mM isopropyl-1-thio-β-D-galactopyranoside were added.

**Plasmids**—Deletions in *narG* gene were performed by PCR using pT18-*narG*, pT25-*narG*, or pNarGHJI plasmids as templates. Plasmids marked with the (Δ1–40) designation produce a truncated NarG protein for the first 40 amino acids. Conversely, pT18-*narG*-Δ(1–40) plasmid expresses the first 40 amino acids of NarG fused to the N terminus of the T18 domain of adenylate cyclase.
for 10 min at 20,000 × g to remove any unbroken cells. The crude extract obtained (CE) was diluted 5-fold with buffer A and centrifuged at 250,000 × g for 90 min. The cell supernatant and the membrane fraction were again centrifuged for 90 min at 250,000 × g, yielding the soluble (S) and membrane (M) fractions.

**Immunological Quantitation**—The amount of nitrate reductase antigen present in cell fractions was quantified by performing rocket immunoelectrophoresis analysis using a polyclonal antiserum specific for nitrate reductase A as described previously (26).

**Activation of Nitrate Reductase in Crude Cell Extracts**—The MobA-dependent activation of nitrate reductase was based on that described previously (10). The specified quantities of purified MobA-His6 protein were added to 100 µl of crude extract; the volume of the reaction medium was adjusted to 150 µl with buffer A. The NarJ-dependent activation experiment was performed using the same procedure by adding specified quantities of purified NarJ-His6 (11). In this work, *in vitro* activation of nitrate reductase precursors present in crude extracts was only considered to avoid a time-dependent inactivation of the sample occurring during cell fractionation. The assay mixtures were incubated under strictly anaerobic conditions in a glove box (95% N2, 5% H2) at 37 °C for 90 min and placed on ice to stop the reaction. Aliquots of the reaction mixture were assayed for nitrate reductase activity.

**Enzyme Assays**—Nitrate reductase activity in cell fractions was measured at 37 °C by following the oxidation of reduced benzyl viologen spectrophotometrically at 600 nm coupled to the reduction of potassium nitrate (27). Nitrate reductase specific activity is expressed in µmol of nitrite produced min⁻¹ mg⁻¹ of nitrate reductase as detected by rocket immunoelectrophoresis.

**Overexpression and Purification of His₆-tagged Proteins**—MobA-His₆ was purified as described (28). Overexpression of NarJ-His₆ was performed as described previously (11). Soluble fractions were prepared as described above using 20 mM Tris-HCl, pH 7.6, 0.5 mM NaCl, 5 mM imidazole buffer (binding buffer) and applied to a 4-ml Ni²⁺ affinity column (Qiagen) equilibrated with the binding buffer. After washing the column, the NarJ-His₆ protein was eluted using binding buffer supplemented with 200 mM imidazole. The pooled fractions were immedi-
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ately dialyzed against 20 mM Tris-HCl, pH 7.6, 0.1 M NaCl buffer, frozen in liquid nitrogen, and stored at −80 °C until used. The whole procedure was carried out at 4 °C.

Interaction Study by Biosensor Experiments—The surface plasmon resonance (BIAcore apparatus) was used to analyze the interaction between NarJ and NarG variants. All experiments were carried out at 25 °C. NarJ-His6 was purified as indicated above and immobilized on a sensor chip CM5 (BIAcore) through amine coupling as described previously (11). Crude extracts (70 ml at 50 mg of total protein/ml containing NarG variants were prepared in HBS buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20) and injected over the test and control (no protein immobilized) surfaces at a flow rate of 10 ml/min. The sensor surface was regenerated with a 1-min injection of 1 mM NaOH.

RESULTS

NarJ Maintains the Aponitrate Reductase in a Soluble State before Moco Insertion—Enzyme-specific chaperones interact with the apoenzyme early during the maturation process. The apoenzyme acquires thereby a conformation suitable for subsequent incorporation of the metal cofactor (29). In the same way, NarJ attachment to the aponitrate reductase is a prerequisite for Moco incorporation (11, 30). In the case of the nitrate reductase from T. thermophilus, it has been demonstrated that NarJ-assisted Moco incorporation occurs within the membrane-bound apoenzyme complex. Further, NarJ is required for membrane attachment of the thermophilic apoenzyme complex (22). Thus, we investigated whether the cellular distribution of the apoenzyme from E. coli is influenced by its interaction with NarJ (Fig. 1). Initially, the absence of NarJ did not affect the cellular distribution of the apoenzyme; the apoenzyme produced by a narJ strain was mainly associated with the membrane fraction as in the wild-type parent (Fig. 1). However, in a mob mutant in which Moco biosynthesis is arrested, chromosomal or plasmidic expression of NarJ increased significantly the amount of soluble enzyme. Although only 15% of soluble apoenzyme was found in the mob/narJ strain, nearly 40 and 80% were present in the mob and in mob/pNarJ strains, respectively. Conversely, in a Moco-sufficient strain (WT), overexpression of NarJ did not affect the localization of the holonitrate reductase. Thus, before Moco insertion, the NarJ chaperone maintains the apoenzyme in a soluble state.

NarJ-assisted Moco Insertion Step Precedes Membrane Anchoring of the Apenzyme—We next asked whether the NarJ-driven modification of apoenzyme distribution influences the NarJ-assisted Moco incorporation process. To address this point, we followed Moco insertion using in vitro activation assays on crude extracts from strains displaying either mostly soluble or membrane-bound apoenitrate reductases (Fig. 2). Initially, in vitro MobA-dependent activation assays were performed with mob or mob/pNarJ strains expressing 40 and 80% of soluble apoenzyme, respectively. Interestingly, the highest activity value was obtained with the strain mob/pNarJ that expressed a mainly soluble apoenzyme (Fig. 2A). In a different set of experiments, in vitro NarJ-dependent activation assays were performed using a nar strain transformed with a plasmid expressing either the soluble NarGHI complex or the membrane-associated NarGHI complex (Fig. 2B). Here again, the highest level of activation was observed with the nar/pNarGHI extract displaying 80% of soluble apoenzyme in contrast to the nar/pNarGHI displaying 20% of soluble enzyme. In both approaches, the higher level of soluble apoenzyme in the crude extracts resulted in a higher level of activation. Further, as seen in Fig. 2, the highest activity values were obtained with extracts in which the apoenzyme had interacted in vivo with NarJ (mob strain) as compared with the cases in which NarJ has been added in vitro (nar derivatives). This is in full agreement with the reported chaperone function of NarJ i.e. apoenzyme stabilization (31).

Since maintaining the apoenzyme in a soluble state improved the yield of activation, we next asked whether, once solubilized, the membrane-bound apoenzyme can be activated in vitro. Solubilization by Triton X-100 did not improve the in vitro activation level of the mob crude extract in which 60% of the apoenzyme is membrane-bound (data not shown). As a control, Triton X-100 addition to a mob/pNarJ extract in which the apoenzyme is mainly soluble did not inhibit the level of activation. These results indicated that, despite solubilization, the membrane-bound apoenzyme can no longer incorporate Moco.

Taken together, these results clearly indicated that (i) the apoenzyme maintained in a soluble state by NarJ in a mob strain is in a competent conformation for Moco insertion and (ii) once bound to the NarJ subunit, the apoNarGHI complex has acquired a definitive conformation that is no longer compatible with a NarJ-assisted Moco insertion proc-
Moco incorporation is apparently a cytoplasmic event that must take place at a particular stage of nitrate reductase biogenesis before membrane attachment of the apoenzyme.

Evidence for Two NarJ-binding Sites on the NarG Precursor—We showed that NarJ maintains the apoNarGH complex in a soluble state. One explanation could be that NarJ interferes with membrane anchoring of the apoenzyme. Previous studies from this laboratory indicated that NarJ specifically interacts with the catalytic subunit NarG within the apoenzyme complex (11). The x-ray structure of the NarGHI complex reveals that both NarG and NarH subunits associate with NarI, the membrane-anchor subunit (Fig. 3) (20). A major interaction with NarI centers on the N-terminal tail of NarG. Such an extended domain of NarG would constitute a good candidate for NarJ binding in terms of membrane-anchoring interference. To verify this hypothesis, the region extending between Met-1 and Gln-40 (Fig. 3) was fused to the N terminus of the T18 domain of the adenylate cyclase and tested for NarJ interaction in the two-hybrid system. Remarkably, the fusion strongly interacts with NarJ (Fig. 4A). As reported before, the NarJ-NarG protein interaction can also be followed using BIAcore (11). NarJ protein was thus immobilized and tested for its interaction with the NarG-(1–40)-T18 fusion protein. As compared with the sensorgram obtained with a T18 derivative plasmids were employed: pT18-Zip, pT18-NarG, and pT18-NarG-(1–40). In C, T25 derivative plasmids were employed: pT25-Zip, pT25-NarG, and pT25-NarG-(11–40).
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oxidoreductase activity (Table 2), indicating the presence of Moco in the enzyme complex and maintenance of its structural integrity (32). The finding that the enzyme is active with an artificial electron donor (benzyl viologen), but not with the physiological electron donor (quinate), suggested a disturbed membrane anchoring to NarI resulting in the loss of electron transfer throughout the NarGHI complex. Further, localization analysis showed that membrane anchoring is affected (55% as compared with 97% for the wild-type active enzyme) (Table 2). Such an observation is not surprising considering that the N-terminal tail of NarG is involved in NarI subunit interaction together with NarH (20). Overall, these results demonstrated that the NarI-binding site located within the truncated protein is essential for Moco incorporation.

**NarJ Controls the Quality of the Enzyme Addressed to the Membrane via Binding to the N-terminal Tail of NarG**—According to our hypothesis, NarJ binding to the N-terminal tail of NarG interferes with membrane anchoring of the apoenzyme complex. Deletion of this fragment should thus enhance anchoring of the apoenzyme prior to the Moco insertion step. This should be visualized by a low specific enzymatic activity of the N-terminally truncated NarG variant present in the membrane fractions. Indeed, the benzyl viologen:nitrate oxidoreductase specific activity (expressed in μmol of nitrite produced, min⁻¹ mg⁻¹ nitrate reductase) is an intrinsic parameter of the enzyme that assesses the presence of Moco and enzyme integrity. In a wild-type context (nar/pNarGHJI strain), no significant differences in terms of specific activity were observed between the enzyme present in the soluble or the membrane fractions (70–100 units) (Table 2). Interestingly, the specific activity of the truncated enzyme located into the membrane fraction was much lower (5-fold less) than the one measured for the soluble counterpart. This indicated a low proportion of Moco-containing and active enzymes in the membrane fractions. These data suggested that an uncontrolled membrane anchoring of both the apoenzyme and holoenzyme complexes occurs in the absence of the N-terminal tail of NarG.

In a different but complementary set of experiments, we determined whether the N-terminal tail of NarG provided in trans in a wild-type strain could titrate in vivo NarJ from the nitrate reductase biogenesis process (Table 2). A plasmid construction allowing expression of a fusion protein between the first 40 amino acids of NarG and the T18 domain of the adenylate cyclase was employed to transform the wild-type strain. Interestingly, the specific enzymatic activity measured in the crude extract from the WT/pT18-NarG-(1–40) strain is much lower than the one prepared from the wild-type strain transformed with the vector control (20 versus 94 units) (Table 2). Thus, the N-terminal tail of NarG provided in trans negatively influences the level of maturation of the nitrate reductase. Cell fractionation was performed using both strains and showed that the enzyme distribution was not affected by the presence of the NarG fragment. Further, in the presence of the N-terminal tail provided in trans, the specific activity of the membrane-bound enzyme was reduced as compared with the one measured for the soluble counterpart (22 versus 75 units) (Table 2). Overall, these data indicated that deletion of the N-terminal fragment of NarG or providing it in trans results in a drastic reduction of the quality of the nitrate reductase complex attached to the cytoplasmic membrane as evaluated by the enzyme specific activity.

**DISCUSSION**

Molybdenum cofactor acquisition has become an important area of study given the wide range of functions carried out by molybdoenzymes in all domains of life. Moco is systematically found deeply buried into the enzyme complexes, suggesting that the subunit assembly and the cofactor incorporation must be coordinated. The Moco insertion process involves several accessory proteins (30), among which the enzyme-specific chaper-
ones facilitate the process by the initial formation of a complex with the apoenzyme (10, 11, 13). The work presented here has answered two fundamental questions on molybdoenzyme biogenesis. (i) Considering the multisubunit character of numerous molybdoenzymes, at which particular stage of assembly does the Moco insertion event occur? (ii) How could such an event be controlled? Here, using the heterotrimeric nitrate reductase A from E. coli as a model, we demonstrated that the enzyme-specific chaperone NarJ binds two distinct sites on the apoenzyme to coordinate both molybdaden cofactor insertion and multisubunit assembly.

Our results support a model depicted in Fig. 5 for how sequential interaction between NarJ and the apoenzyme promotes assembly and Moco incorporation within a multisubunit molybdoenzyme. At first, both the NarG and the NarH subunits associate, forming the apoNarGH complex. At this stage, we showed that the complex may interact with the membrane-anchor NarI subunit, leading to the formation of an inactive NarGHI complex that can no longer incorporate the cofactor. Such an assertion is supported by examination of the crystal structure of the membrane-bound apoenzyme, produced in a mob strain, which shows a closed conformation identical to the native enzyme (20, 33). Alternatively, the apoNarGH complex may enter the maturation pathway by the initial interaction with the NarJ protein. At this stage, NarJ determines the fate of the apoenzyme complex by shifting the equilibrium toward the Moco insertion pathway. In addition to its protective role on the apoenzyme, we showed (i) that NarJ binding to the N-terminal tail of NarG prevents premature membrane anchoring of the apoNarGH complex and (ii) that NarJ binding to another site of NarG allows the interaction with the Moco delivery machinery constituted by several proteins involved in the final stages of Moco biosynthesis (30). Accordingly, we showed that NarJ titration using the N-terminal tail of NarG not only reduces the level of Moco-containing enzyme but also redirects some of the apoNarGH complex toward membrane anchoring prior to Moco insertion. Once NarG has folded consecutively to cofactor insertion, the NarJ chaperone and the Moco biosynthetic proteins are dislodged by an unknown mechanism, and the active holoNarGH complex is then free to interact with NarI via the N-terminal tail of NarG and NarH. To summarize, the fate of the nitrate reductase apoprotein complex in the cytoplasm can be determined by the equilibrium of three factors: the folding kinetic of the apoenzyme, the rate of membrane anchoring, and the efficiency of the Moco incorporation process. Our results were consistent with NarJ controlling each of these factors, an action that goes beyond its restricted chaperone function.

Likely, another NarJ-binding site exists in the N-terminally truncated version of NarG, in which NarJ facilitates Moco insertion. The exact location of this second binding site remains unclear. Considering that the association and dissociation criteria for NarJ binding onto either site of NarG are the same, i.e., binding on the apoNarGH precursor and release upon Moco incorporation, one can envision that both NarJ-binding sites are structurally connected. Such coupling may enhance the co-association and dissociation steps and minimize the possibility that NarJ remains at the N-terminal tail of NarG, preventing membrane anchoring of the holoNarGH complex. Alternatively, NarJ can be released from the N-terminal tail of NarG with an intrinsically constant time constant, providing sufficient time for Moco incorporation to be completed before membrane anchoring.

Recently, studies on the membrane-bound nitrate reductase (NarCGHI) from T. thermophilus revealed that multisubunit assembly and Moco incorporation are coupled (22). However, a different biogenesis pathway has been delineated as compared with the E. coli enzyme. Indeed, the NarJ-assisted Moco incorporation step strictly requires prior membrane anchoring of the apoNarGH complex to the NarCl anchor subunits (22). Further, sequence analysis of the NarG subunit from T. thermophilus revealed the absence of the N-terminal tail targeted by NarJ in the E. coli NarG protein. Such an observation could explain the different biogenesis scheme depicted for the thermophilic enzyme complex.

The underlying concept of this work is that coordination of multisubunit assembly and metal cofactor insertion is fundamental for productive synthesis of a multisubunit metalloprotein. Our results bring interesting comparisons with other systems such as the [NiFe] hydrogenases (34). The fully mature and active hydrogenase is a heterodimer made up of a large subunit that carries the [NiFe] center and an electron-transfer small subunit (35). A key intermediate during maturation is a complex between the precursor form of the large subunit and a chaperone protein (36, 37). After maturation of the large subunit, the final step of hydrogenase maturation is the formation of the heterodimer (38). In the case of periplasmic hydrogenases, it clearly appears that the existence of the heterodimer is a prerequisite for translocation as only the small subunit harbors the twin arginine-containing signal peptide that directs the complex to the Tat translocon (39, 40).

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Addendum—During the course of our work, Jack et al. (41) showed, for complex cofactor-containing Tat substrates such as [NiFe] hydrogenases and a monomeric molybdoenzyme, the trimethylamine N-oxide reductase, that Tat signal peptides via the binding of dedicated chaperones play a key role in coordinating metal cofactor assembly and translocation of a fully assembled metalloprotein. These observations are in complete agreement with our results in which NarJ coordinates Moco insertion and membrane anchoring of a fully assembled nitrate reductase. Consequently, such a quality control activity mediated by enzyme-specific chaperones is not restricted to exported metalloproteins nor to the presence of a Tat signal peptide, but it is rather a consequence of an evolutionary pressure for an efficient and successful synthesis of complex metalloenzymes.

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