Biogenesis of a Respiratory Complex Is Orchestrated by a Single Accessory Protein*

The biogenesis of respiratory complexes is a multistep process that requires finely tuned coordination of subunit assembly, metal cofactor insertion, and membrane-anchoring events. The dissipatory nitrate reductase of the bacterial anaerobic respiratory chain is a membrane-bound heterotrimeric complex nitrate reductase A (NarGHI) carrying no less than eight redox centers. Here, we identified different stable folding assembly intermediates of the nitrate reductase complex and analyzed their redox cofactor contents using electron paramagnetic resonance spectroscopy. Upon the absence of the accessory protein NarJ, a global defect in metal incorporation was revealed. In addition to the molybdenum cofactor, we show that NarJ is required for specific insertion of the proximal iron-sulfur cluster (FS0) within the soluble nitrate reductase (NarGH) catalytic dimer. Further, we establish that NarJ ensures complete maturation of the b-type cytochrome subunit NarI by a proper timing for membrane anchoring of the NarGH complex. Our findings demonstrate that NarJ has a multifunctional role by orchestrating both the maturation and the assembly steps.

All biological systems require the biogenesis of functional respiratory or photosynthetic complexes for their viability. In bacteria, bioenergetic electron transfer chains are associated to the inner membrane. Biogenesis of these complexes is an intricate process that requires several steps such as the synthesis of the different subunits, their assembly, the incorporation of various types of metal or organic cofactors, and the anchoring of the complex to the membrane. In the case of exported metalloproteins, the assembly and cofactor incorporation steps need to be accomplished prior to translocation of the inner membrane via the twin arginine translocase apparatus (1–3). Importantly, accessory proteins are often involved in biogenesis of metalloproteins (4–9). Although it is most likely that all these events occur in a coordinate fashion to yield a final functional multimetalloprotein, information about how this coordination is performed is scarce.

The well studied and characterized Escherichia coli dissipatory quinol-nitrate oxidoreductase of the anaerobic respiratory chain, referred to as the nitrate reductase A (NarGHI) (10, 11), can be considered as a suitable model for deciphering the biogenesis pathway of multimeric metalloproteins. NarGHI is a non-exported membrane-bound respiratory complex composed of three subunits that bind eight redox centers: (i) a catalytic subunit (NarG) containing a molybdenum-bis-molybdopterin guanine dinucleotide cofactor (Moco) and a proximal [4Fe-4S] cluster (FS0) (12, 13), (ii) an electron transfer subunit (NarH) carrying one [3Fe-4S] cluster (FS4) and three [4Fe-4S] clusters (FS1 to FS3) (14, 15), and (iii) a quinol-oxidizing membrane-bound subunit (NarI) containing two b-type hemes (bD and bP) (11, 16, 17). The NarJ protein encoded by the narGHI operon plays an essential role in nitrate reductase activity, enabling Moco insertion into NarG (4, 18). NarJ binds to two distinct sites of the apoprotein reductase (apoNarGH) (19). One site allows the interaction of apoNarGH with the molybdenum cofactor biosynthetic machinery and thus allows Moco insertion (18). The location of this site over apoNarGH remains unclear. The other site is made of the first 40 amino acids of the N-terminal tail of NarG, which are responsible for the interaction of NarG with NarI (19), as revealed by the x-ray structure of the NarGHI complex (13). It has been proposed that binding of NarJ to this site interferes with membrane anchoring of the apoenzyme, maintaining apoNarGH in a soluble form competent for Moco insertion (19). These observations raise the question concerning the possible role of NarJ in the coordination of subunit assembly and cofactor insertion processes.

A major challenge in understanding the formation of multimeric respiratory complexes is the spatial and temporal dissection of their assembly pathways. In this work, different stable folding maturation intermediates of the nitrate reductase complex are identified and characterized by using a systematic and thorough analysis by redox potentiometry and electron paramagnetic resonance (EPR) spectroscopy. A broad defect in metal incorporation is demonstrated in the absence of the accessory protein NarJ.

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4 The abbreviations used are: NarGHI, membrane-bound form of the native enzyme complex; NarGHIap, membrane-bound form of the apoenzyme complex produced in the absence of Moco synthesis; NarGHIapnar, membrane-bound form of the apoenzyme complex produced in the absence of NarJ; Δ1–41NarGHI, membrane-bound form of the enzyme complex truncated for the N-terminal tail of NarG; NarGH, soluble form of the native enzyme complex; NarGHIapmob, soluble form of the apoenzyme complex produced in the absence of Moco synthesis; NarGHIapnar, soluble form of the apoenzyme complex produced in the absence of NarJ; EPR, electron paramagnetic resonance; FS, iron-sulfur center; Moco, molybdenum cofactor.
whereas pNarGHHis6 and its derivatives allow production of membrane-bound NarGHI, its derivatives are used to produce membrane-bound NarGHI, in addition to Moco.

Medium (20) were resuspended in 5 volumes of buffer A (20 mM Tris at pH 7.6, 400 mM NaCl, 5 mM imidazole, 1 mM phenylmethyl-sulfonyl-fluoride) and were disrupted by a French press. The lysate was clarified by centrifugation at 20,000 × g for 15 min. Soluble fractions were prepared by ultracentrifugation at 200,000 × g for 90 min and then loaded onto a 10-ml Ni2+ ions affinity column (Qiagen), previously equilibrated in buffer A. The resin was washed with buffer A containing 10 mM imidazole, and proteins were eluted in buffer A containing 40 mM imidazole. The fractions containing the nitrate reductase enzyme were pooled and concentrated using Amicon Ultra-15 (molecular cutoff 5 kDa) (Millipore). All purification steps were carried out at 4 °C, and the proteins were stored at −80 °C.

**Experimental Procedures**

**Bacterial Strains and Plasmids**—The E. coli strains and plasmids used in this work are described in Table 1. pNarGHHis6 allowing expression of a His-tagged NarGH complex, was constructed by cloning a BamHI/XbaI fragment into the pNarGH plasmid (15). A XbaI/SphI narJ fragment was cloned into the pNarGHHis6 to yield the pNarGHHis6. Site-directed mutagenesis was performed on the pT25-NarG1–41 plasmid yielding the pT25-NarG1–41 H50S one. pNarGHJI and its derivatives are used to produce membrane-bound NarGHI, whereas pNarGHHis6 and its derivatives allow production of a soluble NarGHI complex.

**Nitrate Reductase Purification**—Membrane-bound NarGHI was partially purified from membrane fractions as described previously (20). For purification of NarGHI, cellular pellets issued from aerobically grown cells at 37 °C in Terrific Broth medium (20) were resuspended in 5 volumes of buffer A (20 mM Tris at pH 7.6, 400 mM NaCl, 5 mM imidazole, 1 mM phenylmethyl-sulfonyl-fluoride) and were disrupted by a French press. The lysate was clarified by centrifugation at 20,000 × g for 15 min. Soluble fractions were prepared by ultracentrifugation at 200,000 × g for 90 min and then loaded onto a 10-ml Ni2+ ions affinity column (Qiagen), previously equilibrated in buffer A. The resin was washed with buffer A containing 10 mM imidazole, and proteins were eluted in buffer A containing 40 mM imidazole. The fractions containing the nitrate reductase enzyme were pooled and concentrated using Amicon Ultra-15 (molecular cutoff 5 kDa) (Millipore). All purification steps were carried out at 4 °C, and the proteins were stored at −80 °C.

**Bacterial Two-hybrid Assay**—Protein interactions were detected using a bacterial two-hybrid approach as described previously (21, 22).

**Redox Titration Experiment and EPR Spectroscopy**—Redox titrations were performed at 25 °C in an airtight vessel flushed with oxygen-free argon in the same conditions as those described in Ref. 20. They were carried out under a range of redox potentials either by reduction with sodium dithionite or by oxidation with potassium ferricyanide (typically between +300 and −500 mV). Redox potentials are given in the text with respect to the standard hydrogen electrode. X-band EPR spectra were recorded using a Bruker-Biospin ElexSys E500 spectrometer equipped with a standard rectangular Bruker EPR cavity fitted to an Oxford Instruments helium flow cryostat (ESR900).

**EPR Quantitation of the NarGHI Redox Cofactors**—EPR tubes were calibrated prior to be used for EPR experiments to allow for direct comparison of spin intensities in each preparation. To get rid of the differences in the concentration and expression level of NarGHI or NarGH in the different preparations used in this work, quantitation of their redox cofactor content was achieved relatively to the nitrate reductase [3Fe-4S]c cluster (FS4) content in each preparation. For this purpose, EPR spectra of the cofactors having maximal signal intensity were recorded under non-saturating conditions in a fully oxidized state for heme bD and FS4, in a fully reduced state for FS0, and under a redox potential range for the MoV intermediate paramagnetic state. The double integral of the MoV EPR signal measured at 50 K was compared with the double integral of the FS4 signal measured at 12.5 K. In cases of FS0 and heme bD, the single integration of the low field feature of their EPR signal was compared with the double integral of the FS4 signal, in agreement with the method proposed by Aasa and Vånggard (23). Then, the ratios so obtained for FS4, MoV, FS0, and heme bD relative to the amount of FS4 in a given enzyme were normalized to the same ratio calculated for the membrane-bound native enzyme NarGHI. This enables us to estimate the proportion of each center with respect to the FS4 center, and the results are reported in Table 2.

**Results and Discussion**

NarJ is Required for the Insertion of FS0—In a previous study, we clearly demonstrated that, in the absence of NarJ, no Moco is present in the nitrate reductase complex (4). Resolution of the crystallographic structure of the NarGHI complex revealed that the catalytic subunit NarG comprises a [4Fe-4S]c cluster (FS0) in

| Table 1 | Bacterial strains and plasmids used in this study |
|---|---|
| **Strains or plasmids** | **Description** | **Reference** |
| LC30604 | nar25(narGH), narZ::Ω, Δnap, thi-1, leu-6, thy-1, rpsL175, lacY, KanR, SpcR | 40 |
| TP1000 | MC4100 (ΔmobAB), KanR | |
| BTH101 | F', cya-99, adaD139, galE15, galK16, rpsL1(StrR), hsdR2, mcrA1, mcrB1 | 22 |

**Plasmids**

| Plasmids | Description | Reference |
|---|---|---|
| pNarGHJI | pFI19EH, NarGHJI, AmpR | 15 |
| pNarGH(Δ1–41)HI | pFI19EH, NarGH(Δ1–41)HI, AmpR | 27 |
| pNarGH | pFI19EH, NarG(40–1247)HI, AmpR | 19 |
| pNarGHHis6 | pFI19EH, NarGHHis6, AmpR | This work |
| pNarGH(Δ1–41)HI | pFI19EH, NarGH(Δ1–41)HI, AmpR | This work |
| pT18-NarJ | pT18, NarJ-T18 fusion protein, AmpR | 21 |
| pT25-NarG(1–41) | pT25, T25-NarG(40–1247) fusion protein, CmR | 19 |
| pT25-NarG(Δ1–41)H50S | pT25, T25-NarG(Δ1–41)H50S fusion protein, CmR | This work |
| pT18-Mog | pT18, Mog-T18 fusion protein, AmpR | 21 |
| pT18-MoeA | pT18, MoeA-T18 fusion protein, AmpR | 21 |
| pT18-MobA | pT18, MobA-T18 fusion protein, AmpR | 21 |
| pT18-MobB | pT18, MobB-T18 fusion protein, AmpR | 21 |
addition to Moco (12, 13). Based on the close proximity of these two redox cofactors in NarG, we have investigated by EPR spectroscopy whether the insertion of the FS0 cluster is influenced by the absence of Moco either due to a synthesis defect (mob strain) or due to the absence of NarJ. This [4Fe-4S] cluster has an unusual coordination comprising one His residue and three Cys residues. As proposed later on, it was associated to an unusual EPR signature at g = 2.0 (24). As the other FS clusters (FS1 to FS3) give, in the whole potential range, a peculiar EPR signature, we conclude that the absence of NarJ leads to the specific loss of both Moco and FS0 cluster from NarGHI-depleted membrane preparations (Fig. 1B). By comparison with NarGH-depleted membrane preparations (Fig. 1F), no signals were observed around g = 5. Moreover, EPR signals given in the g = 5.60 region by NarGH and NarGHI mob membrane fractions were compared with samples poised in the whole potential range, with a special care between 100 and 250 mV. No differences were observed between these two membrane preparations for EPR spectra recorded at low temperature (9–12.5 K) and using a microwave power ranging from 1 to 100 mW, which exclude any conversion of the FS0 spin state into a lower spin state (data not shown). Thus, we conclude that the absence of NarJ leads to the specific loss of both Moco and FS0 cluster from NarG and has no effect on the four iron-sulfur clusters from NarH (FS1 to FS4).

Recently, we showed that Moco incorporation is a cytoplasmic event that must take place before membrane attachment of the apoenzyme (19). It is notable that, in the absence of NarJ, the apoenzyme interacts with NarL, leading to the formation of an inactive NarGHI mob complex that can no longer be matured. To investigate whether the absence of FS0 in the NarGHI mob complex was a direct consequence of an early membrane anchoring or due to the absence of NarJ, EPR experiments were performed on NarGHI-enriched membrane fractions from a narJ strain (NarGHI mob). In contrast to the native enzyme, neither the EPR signal of the Moco nor the EPR signal of the FS0 cluster were detected on a wide potential range, from +200 to −500 mV (Fig. 1D). By comparison with NarGH-depleted membrane preparations (Fig. 1F), no signals were observed around g = 5. Moreover, EPR signals given in the g = 2.0 region by NarGH and NarGHI mob membrane fractions were compared with samples poised in the whole potential range, with a special care between 100 and 250 mV. No differences were observed between these two membrane preparations for EPR spectra recorded at low temperature (9–12.5 K) and using a microwave power ranging from 1 to 100 mW, which exclude any conversion of the FS0 spin state into a lower spin state (data not shown). Thus, we conclude that the absence of NarJ leads to the specific loss of both Moco and FS0 cluster from NarG and has no effect on the four iron-sulfur clusters from NarH (FS1 to FS4).

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The biogenesis of the nitrate reductase A complex

soluble NarGHmob complex indicates the absence of the FS0 EPR signature (Fig. 2C). Moreover, the EPR signals related to the four iron-sulfur clusters present in NarH are unchanged in both NarGHmob and NarGHnarJ enzymes (data not shown). Taken together, these results show that Moco and FS0 insertion are cytoplasmic events occurring before membrane attachment of the enzyme complex and that NarJ is absolutely required for these events.

It is notable that the NarGHmob and NarGHnarJ complexes can be reconstituted and activated in vitro in a so-called “activation assay” (19). Such an assay consists of mixing, under anaerobic conditions, a crude extract containing the apoenzyme and the missing component, being either MobA (required for completion of Moco synthesis in a mob strain) or NarJ, and of measuring the recovered nitrate reductase activity. Based on this assay, we have observed a nearly complete activation of the NarGHmob, whereas a three times less activity was recovered with NarGHnarJ (19). In this latter case, a limiting factor or step most likely exists in the activation assay which may consist of the FS0 insertion step. On the contrary, efficient in vitro activation of the NarGHmob complex is likely due to the presence of the FS0 cluster. It should also be noted that a lower proportion of FS0 cluster was observed by EPR in the purified NarGHmob complex as compared with the NarGHmob one (Table 2). It is possible that the structure of the soluble NarGHmob adopts an open conformation amenable for Moco insertion with a less stable FS0 cluster, giving an explanation to the reduced amount of cluster.

In a previous study, we showed that the NarG H50S substitution leads to the synthesis of an inactive nitrate reductase complex characterized by the specific loss of Moco (27). In the present work, EPR titrations of NarG1450S HI were performed on a wide potential range and have indicated the absence of signal in the region around \( g = 5 \) (Fig. 1E). Further, no changes were observed in the \( g = 2.0 \) region excluding any conversion in a \( S = 1/2 \) spin state of FS0 (data not shown). As His-50 is a FS0 cluster ligand, our results demonstrate unambiguously that the \( S = 3/2 \) signature arises from the FS0 cluster. Therefore, the lack of Moco is likely correlated with the absence of FS0. To understand the origin of this relation in the maturation process, the two-hybrid methodology has been used. This approach allowed us to establish that NarJ binding onto a NarG protein truncated for the N-terminal tail is indispensable and sufficient to allow interaction of \( \Delta 1-41 \) NarG with the proteins in charge of Moco transfer (MobA, MoeA, MogA, and MobB) (18). Given that the NarG1450S HI complex does not contain FS0 and Moco, we have investigated the effect of this substitution on the interaction between the \( \Delta 1-41 \) NarG1450S protein with various interactants. At first, the interaction with NarJ is not affected by the H50S substitution (data not shown). This observation indicates that the absence of Moco is not due to the inability of the NarJ protein to interact with the substituted apoenzyme. In contrast, all of the pairwise interactions between \( \Delta 1-41 \) NarG1450S and MobA, MoeA, MogA, or MobB are lost (data not shown). It is notable that examination of the crystallographic structure of the NarG1450S HI complex shows no major structural changes associated with the absence of both FS0 and Moco. These observations suggest that the substitution H50S or the consecutive absence of FS0 induces a local structural perturbation that affects the ability of the Moco biosynthetic proteins to deliver the Moco.

Taken together, our results show that the presence of FS0 is a prerequisite for Moco insertion. Thus, in addition to the strong structural and functional relationship between FS0 and Moco, their respective insertion processes appear to be strongly coordinated and coupled in a NarJ-dependent manner.

NarJ Coordinates Cofactor Acquisition by NarI and NarG—Assembly of the NarGH complex is a multiple step process including metal cofactor insertion, subunit assembly, and membrane anchoring. The absence of NarJ or of Moco synthesis leads to a definitive membrane anchoring of the apoenzyme complex (19). To evaluate the consequence of such a premature anchoring on the maturation of the NarJ subunit, analysis of its heme content was performed by EPR spectroscopy on NarGH1–41 NarGH50S and NarGH1–41 NarGH50S HI complexes. Remarkably, the sharper typical EPR signal at \( g = 3.76 \) associated to the proximal heme \( b_p \) in the NarGH enzyme is clearly absent in the fully oxidized NarGH1–41 NarGH50S HI enzyme (Fig. 3, A and C). In contrast, the EPR peak at \( g = 3.34 \) associated to the distal heme \( b_d \) is still present and in a comparable amount to the native enzyme (Table 2). Similar studies carried on the NarGH1–41 complex indicate that the EPR signals of both hemes are detected (Fig. 3B). The two peaks observed for heme \( b_d \) presumably arise from a conformational heterogeneity related to the enzyme preparation as previously mentioned in the native enzyme (17).

Overall, this work shows that the NarJ protein is a key component of the biogenesis process of the multimeric nitrate reductase complex because none of the Moco, the FS0, and the heme \( b_d \) are inserted when it is missing. Given that the insertion processes of both FS0 and Moco appear to be two correlated cytoplasmic events occurring before

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anchoring of the NarGH complex to the membrane, insertion of heme $b_P$ seems to be a late step in the maturation process of the NarI subunit. It is notable that in the absence of both NarGH and NarJ, NarI is fully mature (16, 17). In contrast, premature anchoring of the NarGH complex to the membrane in the absence of NarJ leads to the insertion of a single heme within NarI. This is further supported by the observation that the NarGH$_{mob}$ apoenzyme having experienced NarJ has inserted both hemes. These results strongly suggest that the presence of NarJ is indirectly required for complete maturation of NarI by a proper timing for membrane anchoring of the NarGH complex.

**Uncoupling Redox Cofactor Incorporation from the Membrane-anchoring Process by Deletion of the N-terminal Tail of NarG**—NarJ binds on two distinct sites of NarG, one interfering with membrane anchoring of apopNarGH (located at the N-terminal tail of NarG) and another one being involved in NarGH maturation (19). As shown above, the action of NarJ on the first site allows complete maturation of the NarI subunit prior to membrane anchoring of the catalytic dimer. To gain corroborative evidence of finely tuned coordination of the nitrate reductase biogenesis process by NarJ, we have examined the effect of the deletion of the N-terminal tail of NarG on both FS0 insertion and NarI maturation by EPR spectroscopy.

Interestingly, both the EPR signals of FS0 cluster and the EPR signals of Moco are present in $\Delta 1–41$NarGHI (Fig. 1C, data not shown). The relative proportion of FS0 is comparable with that of the native enzyme, whereas that of Moco is significantly reduced (Table 2). These observations are consistent with the interpretation given above that the insertion of FS0 occurs before the insertion of Moco. The EPR spectrum of the heme region is shown in Fig. 3D. Although the signal of heme $b_P$ is present at $g = 3.29$, the $g = 3.76$ signal associated to the $b_P$ heme is lacking. Instead, a peak at $g = 2.92$ is clearly resolved. This peak has been well characterized in NarI-enriched membranes and attributed to the $b_P$ heme in a relaxed NarI conformation occurring upon the absence of the NarGH dimer (16, 17, 28). The quantitation of the EPR signals associated to the $b_P$ heme shows that the proportion of this center is significantly higher than those of Moco and FS0 in $\Delta 1–41$NarGHI (Table 2). Therefore, only a fraction of the $b_P$ hemes detected is associated to a $\Delta 1–41$NarGHI complex, the rest being likely present in isolated NarI subunits. These observations are consistent with the fact that deletion of the N-terminal tail of NarG results in disturbed membrane anchoring of the $\Delta 1–41$NarGHI complex and in the presence of isolated NarI subunits (19). Further, the specific absence of the $b_P$ heme is most likely responsible for the reported lack of physiological quinol:nitrate oxidoreductase activity of the $\Delta 1–41$NarGHI enzyme (19). Overall, these observations provide spectroscopic evidence that NarJ is required for concerted coordination of the cofactor incorporation and membrane-anchoring processes.

**Concluding Remarks**—A complete understanding of the biogenesis process of multimeric metalloproteins requires spatial and temporal dissection of the assembly intermediates. EPR spectroscopy has proven to be a valuable technique in characterizing several stable folding intermediates in the case of the nitrate reductase from *E. coli*. In this report, we demonstrate that the NarJ protein orchestrates the cofactor insertion, subunit assembly, and membrane-anchoring steps during the assembly of the NarGHI complex. A detailed biogenesis model of the nitrate reductase complex can be inferred from all these data (Fig. 4). An important point is the separate maturation of the NarI subunit involving sequential insertion of the two $b$-type hemes without the aid of NarJ. In parallel, the apopNarGH complex interacts with NarJ early on, promoting insertion of FS0 and Moco within NarG, likely in a sequential manner. In contrast, insertion of the four Fe-S clusters in NarH likely proceeds through the action of one of the resident Fe-S biosynthetic machineries (Isc, Suf) (29, 30). Finally, the mature...
NarGH complex is directed to the membrane for interaction with a mature NarJ subunit. It is notable that our findings provide the first hints concerning the stepwise maturation of a transmembrane b-type cytochrome, often involved in respiratory complexes such as the succinate dehydrogenase (complex II), cytochrome b_{6}c_{1} and b_{6}f complexes, as well as in photosystem II of higher plants and cyanobacteria. Future investigations involve studies on the molecular mechanism underlying the strict requirement for NarJ in F50 cluster insertion, which will most likely lead to the elucidation of a more general mechanism of F50/Moco insertion by the NarJ protein. Another line of investigation will be to elucidate the molecular mechanism governing the release of NarJ upon redox cofactor incorporation within NarG.

Our findings not only clarify the role of the NarJ protein in nitrate reductase assembly and assign another function to this multitask protein but also provide useful insights into a mechanism of coordination of the multiple steps required for the biogenesis of complex metalloproteins. It can be inferred from comparison with other multimeric molybdenoenzymes that the global function played by NarJ in the biogenesis process of the nitrate reductase complex will be extended to other related systems. Indeed, the phylogenetic tree that can be inferred from sequence comparison of the catalytic subunit of all known molybdenoenzymes belonging to the Me_{2}SO reductase family allowed McEwan and co-workers (31) to distinguish three types. Type III is represented by monomeric enzymes (Dor, Tor, Bis) harboring Moco as the sole redox cofactor, and type I is represented by monomeric or multimeric enzymes (Nap, Fdh, Nas, Psr) harboring a [Fe-S] cluster in addition to Moco in their catalytic subunit. Finally, type II is represented by multimeric enzymes (Nar, Dms, Ebd, Ser, Ddh). Later, the x-ray structures of two members of the type II class, namely the nitrate reductase (13) and the ethylbenzene dehydrogenase (32), revealed the presence of a [4Fe-4S] cluster in close proximity of the Moco in their catalytic subunit. Accordingly, type I and II enzymes need to be grouped into a single class. A new classification can be made. Class 1 is represented by monomeric enzymes (Dor, Tor, Bis) harboring Moco as the sole redox cofactor. Class 2 represents those enzymes (Nar, Fdn, Dms, Nap, Ebd . . . ) that are mono- or multimeric and harbor a [Fe-S] cluster in addition to Moco in their catalytic subunit. Genomic analyses indicate that most of prokaryotic molybdenum enzymes fall into the class 2. Accordingly, their enzyme-specific chaperones are markedly different. Chaperones specific for class 1 enzymes facilitate Moco insertion (with TorD as the prototype), whereas those specific for class 2 enzymes (with Nar as prototype) are required for incorporation of both the [Fe-S] cluster and Moco into the catalytic subunit. In the particular cases of the periplasmic nitrate reductase (Nap) and of the periplasmically oriented formate dehydrogenase (Fdn, Fdo), both members of class 2, two accessory proteins are required for their biogenesis instead of one (Napo and NapF or FdhD and FdhE, respectively) (33–38). In the case of the Nap system, it has been shown that NapF is involved in Fe-S cluster insertion within the NapA catalytic subunit (34). A clear understanding of the respective function of each of these accessory proteins must await further experiments.

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Addendum—During the course of our work, Kirilova et al. (39) reported the crystal structure of the AF0173 protein from Archaeoglobus fulgidus, a putative molybdoenzyme-specific chaperone sharing some similarities with DmsD, TorD, or NarJ. Interestingly, a cavity on the AF0173 surface has been suggested to constitute the binding site for the twin arginine signal peptide harbored by the AF0176 catalytic subunit. Therefore, it is tempting to speculate that NarJ will interact similarly with the N-terminal tail of NarG.

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