Review

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Assembly pathway of a bacterial complex iron sulfur molybdoenzyme

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Abstract: Protein folding and assembly into macromolecule complexes within the living cell are complex processes requiring intimate coordination. The biogenesis of complex iron sulfur molybdoenzymes (CISM) requires use of a system specific chaperone – a redox enzyme maturation protein (REMP) – to help mediate final folding and assembly. The CISM dimethyl sulfoxide (DMSO) reductase is a bacterial oxidoreductase that utilizes DMSO as a final electron acceptor for anaerobic respiration. The REMP DmsD strongly interacts with DMSO reductase to facilitate folding, cofactor-insertion, subunit assembly and targeting of the multi-subunit enzyme prior to membrane translocation and final assembly and maturation into a bioenergetic catalytic unit. In this article, we discuss the biogenesis of DMSO reductase as an example of the participant network for bacterial CISM maturation pathways.

Keywords: complex iron sulfur molybdoenzymes; dimethyl sulfoxide reductase; DmsD; maturation pathway; protein folding; system specific chaperone; twin-arginine translocase.

Introduction

It was proposed in the early 1970s that the information required for a correctly folded protein is contained inclusively within its primary sequence (1). This concept was later challenged in the mid-1980s with the observation that *in vivo* a heat-shock protein assisted as a 'chaperone' in protein folding (2). Non-productive folding routes, such as those leading to aggregation, degradation, or abortive maturation of inactive apoenzymes, must be prevented in order to ensure correct subcellular targeting of cofactor-containing enzymes. Thus, the 'protein-folding problem' presents a myriad of interrelated questions: What controls the protein folding process *in vivo* in addition to the peptide chain searching the thermodynamic landscape for a conformational energetic minimum? How are folding and targeting coordinated with cofactor insertion? How are proteins targeted to their correct subcellular locations? What controls the assembly of large multimeric complexes?

Bacteria are resilient organisms that can subsist in diverse environments. In addition to oxygen, bacteria can exploit a myriad of substrates as terminal electron acceptors for respiration in anaerobic environments. Respiratory redox enzymes catalyze these oxidation/reduction reactions by transferring electrons from a donor to an acceptor molecule, most often operating at the cytoplasmic membrane by forming a redox loop between periplasmic and cytoplasmic enzymes connected by the quinone pool (3, 4). The facultative anaerobic model organism, *Escherichia coli*, has a variety of characterized anaerobic electron acceptors that include nitrate (NO_3^-), nitrite (NO_2^-), fumarate ($C_4H_4O_4$), trimethylamine *N*-oxide [$(CH_3)_3NO$, TMAO] and dimethyl sulfoxide [$(CH_3)_2SO$, DMSO] (4).

The twin-arginine translocase (Tat) system is the purported protein-conducting channel used by enzymes that exhibit cytoplasmic cofactor insertion and subsequent membrane localization (5–7). Proteins that utilize the Tat pathway are characterized by two defining features: (i) they contain a consensus S/T-R-R-x-F-L-K twin arginine (RR) motif in their N-terminal leader peptide sequence (RR-leader); and (ii) they are usually translocated across the cytoplasmic membrane as an active and folded holoenzyme (8–10). The Tat translocon consists of the TatABC subunits and the current model identifies TatA as the homo-oligomeric pore subunit, whereas TatB and TatC act in substrate recognition and delivery (11, 12).

Respiratory oxidoreductases that contain a molybdenum-*bis*(pyranopterin guanine dinucleotide) (Mo-*bis*PGD) catalytic cofactor are grouped under the molybdoenzyme superfamily (13–17). In many instances, the catalytic

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subunit also coordinates an iron-sulfur cluster ([Fe-S]) near the catalytic site, an electron transfer subunit that typically contains typically four [Fe-S] clusters of various Fe:S ratios, and a membrane anchor subunit required to connect the [Fe-S] cluster electrical conduit to the quinone pool (16, 18) which may also contain hemes. These enzymes are now described as a complex iron-sulfur molybdoenzyme (CISM) to distinguish from those iron-sulfur-lacking molybdoenzymes (19). The architecture, function, biogenesis, and maturation of CISMs have been a major topic of recent study (16, 17, 20).

When oxygen-sensing regulatory proteins in the cell (e.g. Fnr) detect a lack of oxygen the biogenesis of a CISM begins (21). In many instances, the induction of CISM maturation too requires the presence of a substrate (e.g. in *Rhodobacter capsulatus*, the substrate DMSO is induced 150 fold upon commencing DMSO reductase biogenesis) (22). Maturation of a CISM into a functional holoenzyme requires numerous stages that involve initial translational ribosome integrated folding, cofactor insertion and coordination, subsequent folding and assembly with other subunits that may have had similarly complex folding pathway. The many steps comprising the cytoplasmic biogenesis processes are highly complex and must be intricately coordinated by numerous assistant proteins to produce a functional CISM. Of special interest to CISM maturation are system specific chaperones that help mediate the complete final folding and assembly (23, 24). Such chaperones were termed redox enzyme maturation proteins (REMPs) and are essential for the proper assembly of CISMs albeit absent in the final assembled holoenzyme (25–28).

The importance of REMPs in CISM maturation is becoming accepted (17, 29). A key mechanistic finding is that REMPs do not chaperone in isolation. Rather, they 'escort' their CISM substrates though the entire maturation process as implied by their interactome (30) (Figure 1). Accordingly, many potential roles for REMPs have been proposed, including functioning as: (i) foldases to ensure correct secondary and tertiary structure (25, 31); (ii) unfoldases to correct folding mistakes (25); (iii) avoidance chaperones to prevent incorrect membrane targeting during folding and assembly (32); (iv) cofactor-assembly chaperones to maintain apoenzymes in a cofactor-binding competent conformation (30, 31); (v) cofactor-binding proteins, which bind the cofactor prior to its transfer to the apoenzyme (30, 33); (vi) targeting proteins directing substrates to specific cellular locations (34, 35); (vii) escort chaperones to promote transmembrane transport of enzyme complexes (30, 31, 34); (viii) proofreading chaperones to suppress transport until essential prior steps in the assembly process are complete (33, 36, 37); and (ix) protease protection chaperones to prevent degradation during assembly (38). Yet despite these proposed roles, a detailed path of actions for REMPs has yet to be defined.

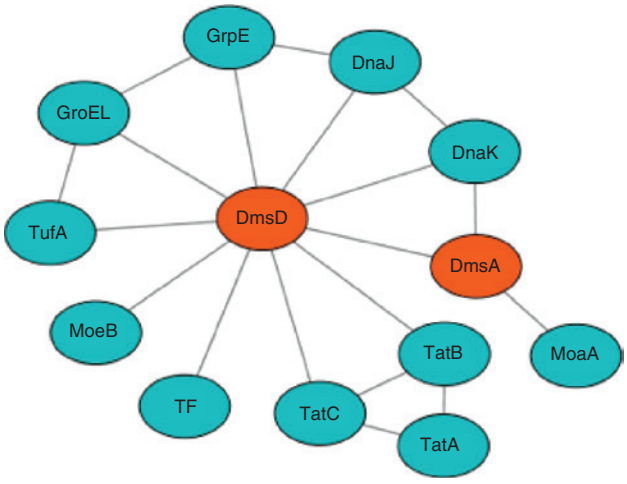

Figure 1: Interactome for DmsD.
The interaction web for DmsD derived from pair wise protein-protein interaction experiments (30, 51). The proteins identified to interact with DmsD through various biochemical studies are listed below.

Protein	Class/group	Reference
TatB	Translocase	Papish et al. (52); Kostecki et al. (51)
TatC	Translocase	Papish et al. (52); Kostecki et al. (51)
GroEL	General molecular chaperone	Li et al. (30)
DnaK	General molecular chaperone	Li et al. (30)
DnaJ[a]	General molecular chaperone	Li et al. (30)
GrpE[a]	General molecular chaperone	Li et al. (30)
TufA/Ef-Tu	Ribosome-associated	Li et al. (30)
TF	Ribosome-associated	Li et al. (30)
MoeA	Mo*bis*PGD biosynthesis	Li et al. (30)
MoeB	Mo*bis*PGD biosynthesis	Li et al. (30)
MogA	Mo*bis*PGD biosynthesis	Li et al. (30)
MobB	Mo*bis*PGD biosynthesis	Li et al. (30)

TufA-Ef-Tu translation elongation factor, TF trigger factor.
[a]These co-chaperones work concurrently with DnaK in the DnaK-DnaJ-GrpE chaperone assembly.

DMSO reductase is an archetypical heterotrimer CISM enzyme comprised of three modular subunits, DmsABC (15, 39). DmsA, the RR-leader containing subunit of the enzyme, functions as the catalytic subunit that coordinates the Mo*bis*PGD catalytic cofactor in addition to one [4Fe-4S] (40, 41). DmsB serves as the electron conduit subunit coordinating four [4Fe-4S] clusters through conserved Cys residues, and is essential for anchoring to the cytoplasmic membrane via the integral membrane protein

DmsC (42). Importantly, insertion of DmsC into the cytoplasmic membrane in the absence of DmsAB is lethal or suppresses growth (43, 44). DmsC is the subunit responsible for subunit anchoring to the membrane and is involved with binding and oxidation (44, 45).

An important component of DMSO reductase is the REMP dimethyl sulfoxide protein D, DmsD (17). Twin-arginine leader peptide binding is a canonical feature of REMPs, and as such, binding of DmsD to the DmsA RR-leader peptide is an absolute prerequisite for DMSO reductase biogenesis (26, 27). It is clear that DmsD binds the DmsA RR-leader at 1:1 stoichiometry and that the entire hydrophobic region is required for binding (46) although specificity is more difficult to define (47). Phylogenetic analyses found DmsD to be related to system-specific chaperones of other Mo*bis*PGD-containing oxidoreductases that included TorD for TMAO reductase and NarJ for cytoplasmic nitrate reductase A, both of which are CISM enzymes related to DMSO reductase (19, 25). Regardless of whether the RR-leaders from each substrate have a final teleological purpose of targeting to the Tat system, the structure of the signal peptides must be fine-tuned for a singular REMPs specificity and unique CISM maturation pathway. Here we discuss the proposed schematic network (Figure 2) of *E. coli* DmsD in the participant pathway for DMSO reductase biogenesis.

Stages of DMSO reductase biogenesis

As a result of research on the system specific chaperones over the past 15 years allows the opportunity to conceptualize an assembly pathway of CISM maturation. This pathway is illustrated in Figure 2.

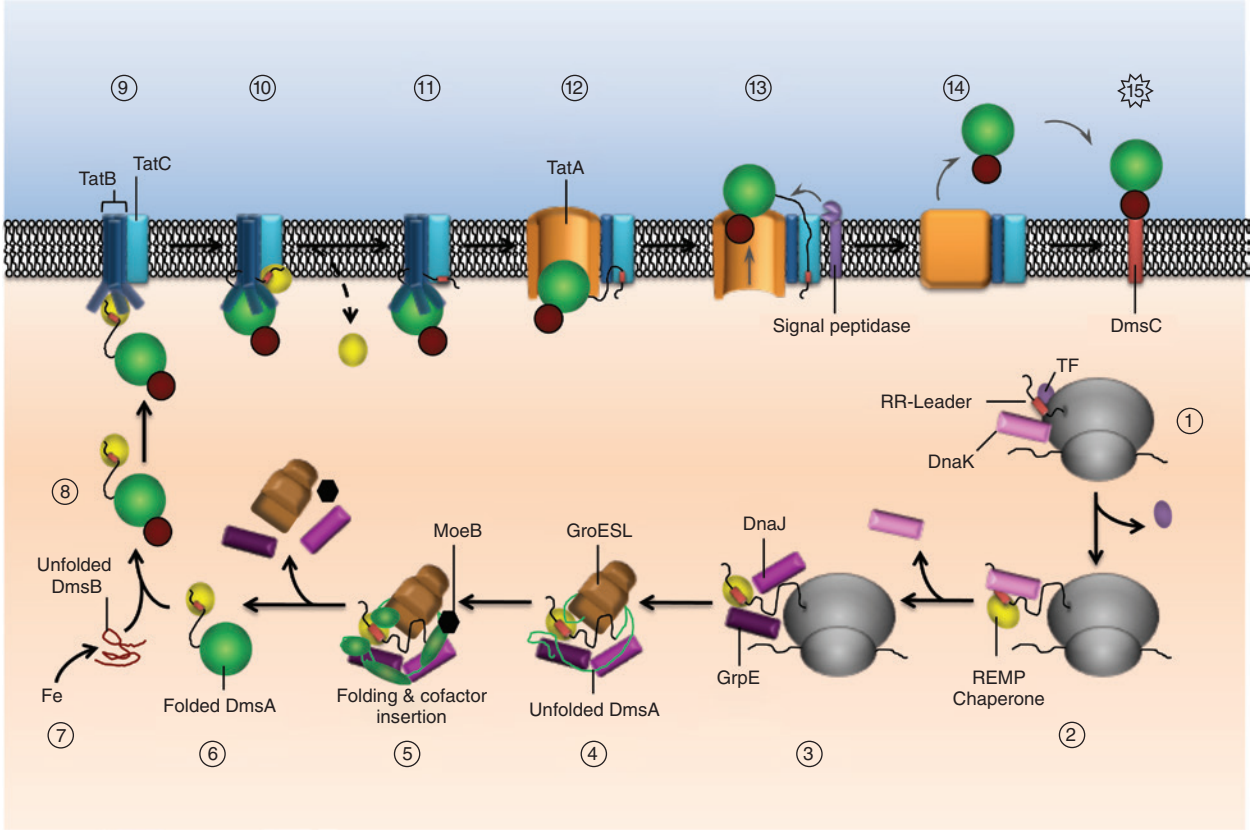

Figure 2: Maturation pathway model for DMSO reductase.
The cartooned pathway shows stages (10–15). The nascent chain exiting the ribosome and the RR-leader interacts with DnaK and trigger factor (TF) (1 and 2). The REMP chaperone DmsD (2), in addition to DnaJ and GrpE (3), join the interactome. DmsD interaction with GroEL (4) allows insertion of the molybdopterin cofactor through contact with MoeB (5). After DmsA is fully folded (6), the 4[4Fe-4S] center containing DmsB must fold and interact with DmsA (7 and 8). With DmsAB assembled, it is targeted to the Tat translocon (9–11) and translocated (12–14). Finally, DmsAB docks to the membrane anchor subunit DmsC to complete the accomplishment of securing a fully functional respiratory enzyme (15).

Stages 1 and 2: the ribosome, trigger factor (TF) and DnaK

A key event in CISM maturation involves binding of the RR-leader peptide as the nascent polypeptide exits the ribosome. TF and DnaK are promiscuous chaperones that assist in protein folding and have a combined substrate pool of over 1000 cellular proteins (48). Studies have demonstrated that TF and DnaK interact with Tat system substrates, and both chaperones have an apparently similar substrate specificity: a stretch of hydrophobic amino acid residues flanked on either end by positively charges residues (38, 49, 50). Indeed, DmsD has some form of association with the general chaperones, trigger factor (TF) and DnaK, which implicates DmsD as a central connector for all processes required for DMSO reductase maturation (30). We have established an interaction web with differential affinities for the DmsA RR-leader in the following order: DmsD > TF > > DnaK (29), suggesting that an initial chaperone cascade from ribosome to TF to DnaK could be involved in DMSO reductase maturation with each chaperone having overlapping and distinct roles in folding modulation thereby promoting productive folding and avoiding proteolytic degradation (51, 52).

Stage 3: DnaJ and GrpE

Many of the DnaK functions appear to rely on the presence of the co-chaperone partners, DnaJ and GrpE. The DnaK-DnaJ-GrpE assembly is a well-studied chaperone cascade machine in *E. coli* initially identified for their roles in heat shock rescue (53). Our interactome data suggests that there is a DmsD-DnaJ interaction; however, it is unclear whether this interaction is direct or detected due to the presence of DnaK or another effector molecular (30).

Stage 4: the DmsD-GroEL interaction

A large number of targets in the interaction proteome of DmsD have been identified that include general molecular chaperones, ribosomal components, and cofactor biosynthesis proteins. Figure 1 demonstrates the possible processes of these non-substrate proteins that were identified in the DmsD interactome from several studies (30, 51, 52). These interactions implicate DmsD as a hub of the DMSO reductase maturation pathway for connecting the nascent DmsA polypeptide to proteins that would assist in active holoenzyme maturation. Interaction of DmsD with the general chaperone, GroEL, is an example of one such downstream connection (30, 54). As GroEL provides a protective cavity for newly synthesized or misfolded polypeptides to fold or refold (55), it is plausible to hypothesize that DmsD having received DmsA from TF, directs it towards GroEL which then further assists in the folding of DmsA (56). Further folding of DmsA outside of the GroEL cavity would allow for subsequent insertion of [4Fe-4S] and Mo*bis*PGD during maturation.

Stage 5: molybdenum cofactor (Moco) insertion

A critical step to procure an enzymatically active DmsABC holoenzyme is the insertion of the Mo*bis*PGD cofactor (42, 57). Studies involving another Mo*bis*PGD-containing CISM, TMAO reductase, showed that mutants deficient in molybdate-uptake and cofactor biosynthesis were improperly localized within the cell (7). Accordingly, Mo*bis*PGD could be considered a chemical chaperone for DMSO reductase folding and stabilization of the tertiary structure of DmsA for cofactor coordination (58), further implicating the secondary importance of Mo*bis*PGD in CISM maturation. Two enzymes that catalyze terminal steps in the Moco biosynthesis pathway, MoeB and MoaA, have been demonstrated through both i*n vivo* and *in vitro* protein-protein interaction methods to interact with DmsD as well as the RR-leader peptide (30). However, complete processing of the RR-leader peptide is not dependent on cofactor availability, as *moeB* chromosomal mutation does not prevent maturation and membrane insertion of the apomolybdoenzyme (59). Although the highly-labile and complex Mo*bis*PGD cofactor is an essential component for DMSO reductase activity, understanding its insertion in the maturation pathway poses as a challenge to understanding CISM folding. Currently, it is postulated that MoeB and MoaA are involved in the final cofactor hand-off during CISM maturation (60–63).

Stages 6 to 8: DmsA and DmsB interaction

The importance of [Fe-S] clusters is recognized in all kingdoms of life because they can bind electron-rich enzymatic substances, accept or donate single electrons, and can also stabilize protein conformations (64). The biogenesis of [Fe-S] clusters in *E. coli* is a highly conserved and complex process mediated by several dedicated coordinated systems (65, 66). The proposed roles of the assembly components include: iron donors (67, 68), intermediate [Fe-S] cluster scaffold proteins (69–71), carrier proteins (72), and

putative [Fe-S] cluster repair proteins (73). The biogenesis system may have evolved to protect and enshroud [Fe-S] clusters during the majority of the biogenesis and transfer process, as the [Fe-S] species are notoriously vulnerable to oxidative stress (74, 75). Recently, the biosynthesis and distribution of [Fe-S] clusters has been linked to the maturation of molybdoenzymes and in particular to the synthesis of molybdenum cofactor (76, 77). While the four [4Fe-4S] clusters of DmsB are essential to receive and conduit electrons from the cytoplasmic quinone pool, defining the exact molecular mechanism of [Fe-S] cluster assembly in DmsB insertion is still a challenge due to the spontaneous chemical nature of the species. Yet it is considered that the fully [Fe-S] holo DmsB needs to fold with cofactors prior to interaction with holo DmsA (16).

It is suggested that RR-leader bearing oxido-reductases with more than one subunit are targeted to the translocase via a 'piggy-back' mechanism, as all identified multi-subunit oxidoreductases contain a RR-leader in only one of the subunits (78). In this way the non RR-leader containing subunit folds and interacts with the RR-leader containing subunit in order to catch a ride across the cell membrane. While recent studies have shown that the loss of several general chaperone genes *in vivo* leads to loss or retardation of respiratory growth on DMSO, the final assembly of the DmsAB catalytically active holoenzyme is an important step in the maturation pathway that remains to be investigated. However, it is known that most of the CISM enzymes will fold a catalytically active form of the enzyme prior to interaction and subsequent translocation across the cell membrane (28, 79).

Stages 9 to 11: targeting to the Tat translocon

The Tat translocon in *E. coli* is comprised of Tat A, B, and C subunits of which multiple copies of each are involved in the translocon (80, 81). Tat-targeted respiratory enzymes appear to have RR-leaders in only one subunit of the holoenzyme (8) that differs from general secretory pathway (Sec) signal peptides (82). Specifically, the RR-leader peptide is recognized at the membrane by a receptor complex formed from copies of TatB and TatC (80, 81). It has been established by both *in vivo* and *in vitro* experiments that DmsD interacts with both TatB and TatC (34, 51, 52). It is likely that the associated REMP docks at TatBC to donate the substrate to the TatBC receptor, with unfolded proteins rejected by the TatBC receptor complex through an undefined quality control mechanism (83). In addition to leader recognition, membrane targeting has been suggested to also involve association with membrane phospholipids by the RR-leader (35, 84, 85).

The events surrounding Tat targeting likely include substrate handover in conjunction with DmsD dissociation. Undoubtedly, DmsA can interact with the Tat translocon independent of DmsD, further supporting the existence of a handover or dissociation step (30). Moreover, DmsD association with the cytoplasmic membrane occurs only in the presence of TatB or TatC (51, 52). Although a transient tripartite interaction is too complicated to analyze by most biochemical techniques, an interaction between DmsD, TatB/C, and the RR-leader suggests that an intermediate complex involving DmsA(B)/DmsD/TatB(C) is highly probable.

Stages 12 to 14: crossing of the cytoplasmic membrane

The most remarkable feature of the Tat system is that its substrates are usually fully-folded prior to membrane translocation (10). Substrate protein recognition by the TatBC complex subsequently results in the recruitment and oligomerization of TatA protomers to form the active translocon pore using energy from the proton motive force (86, 87). As the purported protein-conducting channel, the TatA multi-protomer pore unit must be large enough to permit passage of large protein substrates possessing secondary, tertiary, and in many cases quaternary structure with sizes ranging from ~10 kDa to ~140 kDa (88–90). Following translocation initiation from the TatBC recognition unit, substrates are translocated through the TatA multimer pore across the cytoplasmic membrane utilizing the electrochemical gradient (86, 88, 90–93). Several studies have found that the substrate protein interacts with TatA and TatBC separately, suggesting that the translocon is not fully assembled until receiving a substrate (88–90, 94). Currently, it is proposed that both TatA and TatB use the same binding site on TatC, and since TatB occupies this site in the resting TatBC complex, TatA must displace TatB form the site at some stage in the translocation cycle (95). Despite the various data available on Tat complex formation, piecing together the translocation pathway from various studies still proves to be a daunting task. To elucidate the mechanism of Tat transport, it is critical to better define how the multiple Tat components are arranged within the translocation complex.

Following successful translocation, the RR-leader is assumed to be cleaved off by leader peptidease I, LepB (96). However, mutagenesis to the LepB recognition and cleavage site does not appear to totally inhibit RR-leader

processing of DmsA (97). To date no study has shown LepB interacting with TatABC and studies suggest that the RR-leader containing substrate is released from the Tat translocon prior to cleavage (98). This study suggests that the uncleaved RR-leader could anchor the protein to the membrane until the leader is cleaved.

Stage 15: anchoring to DmsC

The final stage of DMSO reductase assembly involved attachment of DmsAB to the DmsC membrane anchor subunit. As DmsC is a polytopic integral membrane protein with eight transmembrane segments, it is likely translocated and inserted into the membrane by one of the two known pathways in bacteria as opposed to spontaneous insertion (99, 100). Truncation studies have demonstrated that the C-terminus of DmsB is indispensable for anchoring to DmsC, suggesting that the direct docking of DmsAB to DmsC is likely solely through interactions with DmsB (42) in agreement with the overall structural architecture of CISM systems. While much is known on the mechanism of electron transfer between the DmsABC subunits, exactly how the final complex is assembled remains to be elucidated.

Molecular regulation of the system

Proteostasis

Protein quality control ensures that proteins are correctly folded and functional at the right place and time and is essential in cellular life (101). This regulation is mediated by chaperone and protease systems, in accordance with cellular clearance mechanisms (102). The most intensively studied folding chaperones, such as the GroEL-GroES and DnaK systems, facilitate substrate protein folding through ATP- and cofactor-driven conformational changes that convert the chaperones from a state of high substrate affinity to low substrate affinity, thereby allowing substrate proteins to be released (103). An emerging functional feature of ATP binding chaperones is their highly dynamic behavior, and it seems that their massive domain movement is only weakly coupled to their nucleotide states. Rather, these ATPases are in a continual state of rapid flux (104). A mechanism for GroEL-assisted folding of large proteins involves their binding to the open (*trans*) ring, and subsequent folding in the bulk solution outside of the cavity (55). If folding does not succeed, the cycle then repeats after the substrate is released by hydrolysis of ATP in the GroEL *cis* ring. Additionally, 'adaptor' proteins may be utilized to connect GroEL with protein substrates. Such an interaction was recently discovered between DmsD and GroEL, in that DmsD was shown to function as a connector to carry the DmsA substrate preprotein to GroEL for assisted folding (54). Although the numerous known folding chaperones have a fairly broad range of substrates, each chaperone family has a distinct mode of ATP binding (104). Currently, a major aim in the field is obtaining high-resolution structures of chaperone complexes acting on misfolded or unfolded proteins to better define the energetic regulation of protein quality control.

Additionally, degradation of protein in *E. coli* is generally executed by ATP-dependent proteases (105). The proteases form large multisubunit machines with an internal proteolytic chamber accessible to unfolded proteins only. The denatured polypeptides are translocated into the proteolytic chamber in an ATP-dependent manner (106). It was demonstrated that TorD (the REMP for the CISM TMAO reductase) binding to the core of apoTorA prevents proteolytic attack of the Lon protease and also the proteolysis of the N-terminal extremity by an additional, still unknown protease (50, 107). Although the region of TorD involved in the recognition of apoTorA is well defined, the region and energetic mechanism responsible for the protection of the apoTorA signal sequence is still under debate (49, 108).

Guanosine 5′-triphosphate (GTP)

Molybdoenzymes are present in all domains of life and they catalyze critical steps in carbon, sulfur, and nitrogen metabolism. A large diversity of organic and inorganic substrates are utilized for catalyzing oxidation-reduction reactions, and as such, molybdoenzymes play a crucial role in fundamental biogeochemical cycles (109, 110).

The biosynthesis of the molybdenum cofactor (Moco) is a highly conserved and complex pathway (60, 111). For instance, in *E. coli*, nine proteins with known function are directly involved in Moco biosynthesis (35, 112). The biosynthesis of Moco begins from guanosine 5-triphosphate (GTP) and is catalyzed by the two proteins MoaA and MoaC in bacteria (76), the former containing two [4Fe-4S] clusters (113). The different final forms of Moco are inserted into molybdoenzymes that are categorized based on the ligands at the molybdenum atom (14). The proteins of the DMSO reductase family in bacteria contain the *bis*PGD form of the cofactor (14), and the synthesis of which has been shown to occur in a two-step reaction

that requires hydrolysis of Mg-GTP (114). As *bis*PGD is not stable in its free form, it is immediately bound by Moco-binding chaperones, which insert the cofactor specifically into its target enzymes. The system specific chaperone of Nitrate reductase A, NarJ, has been shown to interact with Moco biosynthesic machinery and to facilitate final complex assembly prior to Tat translocation (48, 115, 116). Moreover, TorD, induces a conformational change of apoTorA to allow competency for Moco insertion, as well as interaction with the MobA protein involved in *bis*MGD formation (49). The maturation steps associated with the Moco biosynthesis involve a myriad of interactions with enzymes, chaperones and cofactors alike.

GTP binding and hydrolysis are hypothesized to govern the activity of CISM maturation during the final stages of protein folding (33). It has been suggested that GTP binding by REMP proteins themselves may play a role in the REMP/substrate maturation process (33). Accordingly, the domain-swapper dimer of TorD, showed an increase in GTP affinity following TorA ligand binding (27, 117, 118), and it was suggested to in fact be binding to the mature molybdopterin-guanine dinucleotide (MGD) form of Moco as a step in the cofactor insertion event (49). Recent investigation has illustrated strong cooperativity in the DmsD binding to both GTP and the RR-leader, suggesting that GTP binding at one site on DmsD alters affinity at additional binding sites (other protomers of a multimeric state of DmsD). Oddly, similar dissociation constants for DmsD release of the RR-leader were determined with all guanine nucleotides (GTP, GDP, and GMP), implying that hydrolysis is not involved and that the recognition is directed via the guanosine moiety rather than attached phosphate groups (119). Overall, GTP is crucial factor in CISM maturation both as a component of the molybdopterin cofactor as well as potentially regulating the REMP's protein-protein interactions.

Protein translocation energetics

Two transport mechanisms have evolved to facilitate passage of proteins across the cytoplasmic membrane. In bacteria, secretory proteins are translocated either via the general secretion (Sec) pathway (120, 121) or the Tat pathway (8). The fundamental functional difference between the two pathways is that the Sec system is involved in the secretion and insertion of unfolded proteins, while that Tat system is implicated in the secretion of folded and/or cofactor containing proteins (122, 123). It has been proposed that the Tat system is utilized only when cytoplasmic folding of the protein substrate therefore omitted the use of the Sec system (124). There may be specific folding-related motives for a Tat system preference, such as labile cofactors in the example of CISM (125, 126). If cytoplasmic folding is not required for a Tat substrate, the question arises why the Tat system is still preferred (122). Although the Sec and Tat pathways translocate proteins by distinctive mechanisms, many common fundamentals can be recognized.

The bacterial Sec translocase is composed of a membrane embedded protein conducting channel (PCC) that consists of three integral membrane proteins, SecY, SecE, and SecG, and a peripheral associated ATPase, SecA, which functions as a molecular motor to drive membrane translocation (120, 123, 127). SecA associates peripherally to the PCC, where it accepts secretory proteins from chaperones to subsequently thread the unfolded protein through the narrow PCC transmembrane channel in an ATP dependent fashion. Secretory proteins can be targeted to the Sec translocase by two different mechanisms, the co-translational and the post-translational targeting (128), both of which occurring through a step-wise process wherein a catalytic turnover of the SecA ATPase is couple to the translocation of the unfolded protein. Comparatively, as described earlier, the Tat translocase consists of three membrane integrated subunits, TatA, TatB, and TatC, which together form a receptor (TatBC) and a protein conducting channel (TatA) for protein translocation (122).

A hypothesized possible role of the REMP chaperones was for Sec system avoidance. It was noted that Tat dependent proteins could default to the Sec translocase in a Δ*tatABC* mutant, albeit not leading to a folded functional protein. Following this premise, it was observed that SecA ATPase activity *in vitro* was activated in the presence of a RR-leader containing substrate, but inhibited in the presence of its cognate REMP (unpublished results).

Protein translocation requires the input of an energy source. In general, the two main sources are chemical energy (ATP, GTP) and electrochemical energy (PMF) (129, 130). Whereas GTP is the main driving force during Sec co-translational protein translocation, ATP is the main source of energy utilized during the Sec post-translational route. Quantitative estimates of nucleotide requirements for ATP-driven Sec translocation indicated that about 500 molecules of ATP are required per translocated polypeptide of 25 kDa in length (131). Interestingly while ATP is essential for initiation of Sec mediated translocation, depending on the substrate, translocation may be further stimulated by the PMF (128, 131). Although the PMF cannot initiate translocation, PMF-driven translocation is highly efficient in the absence of SecA, and it is suggested

that the PMF acts as a driving force possibly involving vectorial proton movements (129, 130).

In contrast, Tat transport is triggered and driven by the PMF. Specifically, transport is driven by the PMF of which the pH is the dominant component (132), and there is no requirement for ATP (133, 134). Only a small ΔpH is required for Tat transport *in vitro* (132). Measurement with bacterial Tat systems *in vivo* indicate that nearly 80 000 protons pass the membrane per translocated protein (132), which render Tat mediated translocation energetically more costly than the Sec system. Furthermore, TatA oligomerizes and associates with the TatBC complex only in the presence of a ΔpH, which highlights the absolute ΔpH requirement for successful protein translocation (88). Recent studies suggest that DmsA RR-leader release from DmsD is reduced below pH 6.0 (135), a range that corresponds to a transition in DmsD folding forms (136). Collectively, these results suggest that a ΔpH is imperative for not only successful protein translocation, but H^+ may also serve as a molecular regulator in protein maturation prior to Tat system targeting.

Moreover, an important mechanistic question is whether protonation and deprotonation events are required for the conformational changes during Tat transport. In other PMF dependent motors, such as the ATP synthase, there are acidic residues located within the membrane that couple proton transfer to molecular movements and conformational changes. To date, no such acidic residues have been identified for TatA, TatB, or TatC (137), which raises the question as to the direct coupling of proton flux to Tat translocation. While numerous aspects of Sec-dependent protein translocation have been functionally reconstituted with purified translocase components, it still remains a significant challenge to address the many remaining mechanistic questions regarding Tat translocation.

Expert opinion

The process by which nascent polypeptides fold into functional proteins *in vivo* may be simple or complex. Dissecting the biochemical dance involved for a given biomolecular complex is typically poorly understood and/or studied, presenting researchers with a myriad of interrelated questions that fall under the umbrella of the 'protein-folding problem'. Our research has demonstrated that multimeric bacterial respiratory protein complexes frequently require protein chaperones for functional assembly. The working hypothesis for the maturation of CISMs is that a system-specific chaperone, is necessary to pilot its cognate substrate through the numerous folding, cofactor loading and protein-protein interaction targeting steps.

The past few years have seen the development of novel quantitative genetic interaction (GI) screens aimed to elucidate the relationship between gene function and higher-level protein complexes. The group of M. Babu has applied this approach to *E. coli* (138, 139), and their findings support our proposed DMSO reductase maturation pathway. Specifically, the work suggests that protein homeostasis groups together the roles of chaperoning, protein folding, and proteases. Thus, in our maturation cascade, stages 1 through 4 may be intertwined. Additionally, Babu et al. identified a new gene responsible for molybdopterin biosynthesis, which was then linked with genes involved with the [Fe-S] cluster assembly. This information implies that the assembly of the molybdopterin and the [Fe-S] cluster of DmsA may be coupled, and that the coordination of stages 5 through 7 may be more intimate than our pathway suggests.

Bettering our understanding of *in vivo* folding, targeting, and assembly mechanisms of large multi-subunit CISMs will have a huge biological impact. It will provide insights into how large multi-subunit respiratory enzymes assemble in all organisms. Considerable effort has been expended to define how these complexes function, but there is paucity of information available on how they come to be. A comprehensive model of how respiratory enzyme subunits are guided from the ribosome, to their final subcellular destinations, sheds light on the temporal regulation of critical maturation steps, and on how multiple subunits are brought together to form active enzyme complexes.

Outlook

Modeling bacterial chaperones and their cognate nascent polypeptides as tractable archetypal systems address a fundamental biological question that applies to the bioenergetic systems of organisms across all domains in the tree of life. Investigation of REMP interactomes illustrates participation in a complex cascade of interactions, and the study of which will provide insights into the fundamental area of protein-protein interactions. This is particularly relevant in cases of transient, but highly specific, interactions that are necessary to coordinate complex multi-step enzyme maturation pathways.

Moreover, approximately one third of all proteins contain metal ions. Of these, transition metal irons, such

as iron and molybdenum, are assembled into complex redox-active cofactors. Mo-containing enzymes exist in almost all organisms and catalyze key redox reactions that are critical to the global carbon, nitrogen, and sulfur cycles. As well, a subset of bacterial membrane-bound Mo-enzymes play a critical bioenergetic role, as their catalytic turnover contributes directly to the transmembrane proton gradient across the cytoplasmic membrane (20). The maturation and targeting of Mo-enzymes frequently depends on system-specific chaperones, most notably the REMPs and multi-cofactor containing multimeric enzymes. As such, investigation into REMP chaperones and the respiratory enzyme maturation processes in bacteria have the potential to reveal novel antimicrobial targets, and could provide broad-spectrum antibiotics against bacterial pathogens. Therefore, it is of utmost importance to better understand the exquisite coordination of cofactor insertion, translocation, multimeric subunit association, and membrane insertion in bacterial Mo-enzymes.

Highlights

- Many proteins require assistance of system specific chaperones to achieve their native fold, including correct cofactor insertion and subcellular localization.
- The twin-arginine translocase system transports full-folded proteins across the bacterial cytoplasmic membrane, and proteins that utilize the Tat pathway contain a twin-arginine motif leader peptide sequence at the N-terminus.
- Anaerobic respiratory redox enzymes exploit the use of alternative terminal electron acceptors to subsist in anoxic environments.
- Respiratory oxidoreductases that contain a molybdenum-*bis*(pyranopterin guanine dinucleotide) (Mo-*bis*PGD) catalytic cofactor are characterized as molybdoenzymes, and are further classified as complex iron its sulfur molybdoenzymes (CISMs) when iron-sulfur groups are also included in the final enzyme fold.
- DMSO reductase is a CISM that utilizes DMSO as a terminal electron acceptor, and requires the use of various general chaperones as well as its system specific chaperone DmsD, for cytoplasmic folding and assembly prior to Tat translocation.
- A large number of targets in the interaction proteome of DmsD have been identified, and recent studies has illustrated strong cooperativity in binding of DmsD to both GTP and the RR-leader.
- Next steps are to better understand exactly how the DmsAB enzyme is recognized by TatBC, the translocation across the membrane by TatA and how the final DMSO reductase complex (DmsABC) is assembled following Tat translocation.

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