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Review

'Come into the fold': A comparative analysis of bacterial redox enzyme maturation protein members of the NarJ subfamily

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ABSTRACT

Redox enzyme maturation proteins (REMPs) are system-specific chaperones required for the maturation of complex iron sulfur molybdoenzymes that are important for anaerobic respiration in bacteria. Although they perform similar biological roles, REMPs are strikingly different in terms of sequence, structure, systems biology, and type of terminal electron acceptor that it supports for growth. Here we critically dissect current knowledge pertaining to REMPs of the nitrate reductase delta superfamily, specifically recognized in *Escherichia coli* to include NarJ, NarW, TorD, DmsD, and YcdY, also referred to as the NarJ REMP subfamily. We show that NarJ subfamily members share sequence homology and similar structural features as revealed by alignments performed on structurally characterized REMPs. We include an updated phylogenetic analysis of subfamily members, justifying their classification in this subfamily. The structural and functional roles of each member are presented herein and these discussions suggest that although NarJ subfamily members are related in sequence and structure, each member demonstrates remarkable uniqueness, validating the concept of system-specific chaperones.

Contents

Abbreviations: REMP, redox enzyme maturation protein; CISM, complex iron–sulfur molybdoenzyme; Tat, twin-arginine translocase; RR, twin-arginine

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1. Introduction

1.1. Anaerobic respiration in bacteria

Respiratory redox enzymes catalyze oxidation/reduction reactions by transferring electrons from a donor to an acceptor molecule. Many respiratory enzymes operate at the cytoplasmic membrane by forming a redox loop between periplasmic and cytoplasmic enzymes connected by the quinone pool [1,2]. In addition to oxygen, bacteria can utilize a variety of substrates as a terminal electron acceptor for respiration in anaerobic environments. A well characterized example of this is the facultative model organism, *Escherichia coli*, which has a variety of known anaerobic electron acceptors that include nitrate (NO_3^-), nitrite (NO_2^-), dimethyl sulfoxide ($(CH_3)_2SO$, DMSO), fumarate ($C_4H_4O_4$), and trimethylamine *N*-oxide ($(CH_3)_3NO$, TMAO) [2].

The respiratory enzymes that catalyze reduction of DMSO, TMAO, and nitrate are grouped under the molybdoenzyme superfamily [3–8]. They all contain a molybdenum-*bis*(pyranopterin guanine dinucleotide)

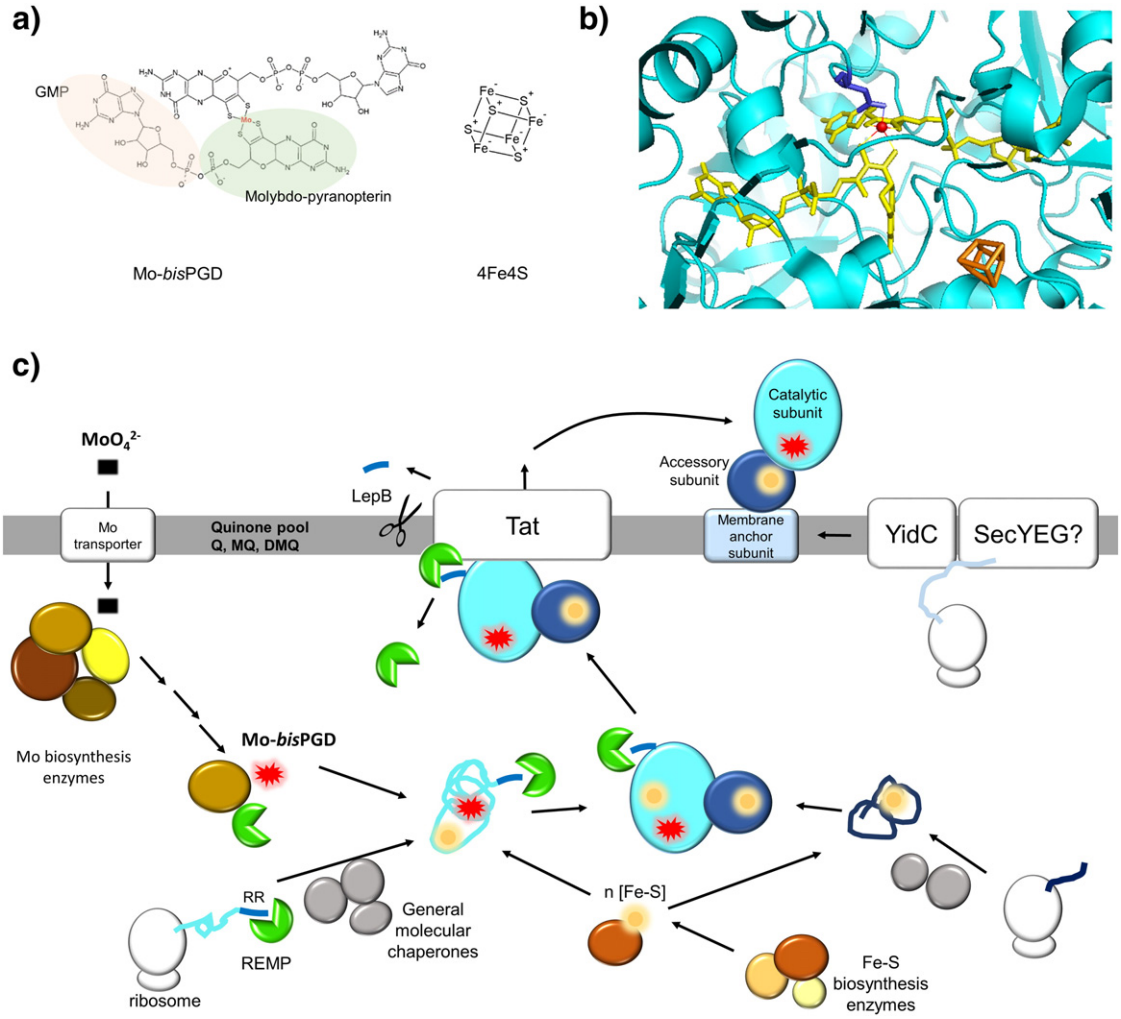

Fig. 1. Complex iron sulfur molybdoenzymes in bacteria. a) Structures of the catalytic cofactor Mo-*bis*PGD and the iron–sulfur cluster (typically [4Fe–4S]) found in a bacterial CISM. b) Example of how Mo-*bis*PGD and [4Fe–4S] is coordinated in the NarG catalytic subunit of *E. coli* nitrate reductase A (PDB ID: 1Q16). The Mo atom (red) is coordinated by the two pyranopterins (yellow) and the carboxylate group of Asp222 (blue). Proximity of the [4Fe–4S] to Mo-*bis*PGD is also shown (orange). c) Maturation pathway of a typical three-subunit CISM begins with protein translation from the ribosome. The large catalytic subunit is synthesized with a twin-arginine (RR) leader peptide and folding is aided by general molecular chaperones and likely its cognate REMP. It is bound by the REMP chaperone at the RR-leader and folding is assisted along with insertion of the Mo-*bis*PGD cofactor, which was synthesized by the molybdenum cofactor biosynthesis pathway proteins. At the same time, the small accessory subunit is translated, folded, and its [Fe–S] iron–sulfur cluster(s) are coordinated. The two subunits come together to and are targeted towards the Tat machinery by the REMP by a 'piggyback' or 'hitchhiker' mechanism. The complex is translocated across the cytoplasmic membrane and RR-leader is cleaved by leader peptidase I (LepB). The subunits attach to its membrane anchor subunit, which was inserted into the cytoplasmic membrane via the YidC pathway that may or may not involve the SecYEG translocon. The redox loop is completed through transfer of electrons via the quinone pool, consisting of ubiquinone (Q), menaquinone (MQ), and demethyl-menaquinone (DMQ) in the cytoplasmic membrane.

(Mo-*bis*PGD) catalytic cofactor in the active site of its catalytic subunit (Fig. 1a & b). In many instances this subunit also coordinates an iron–sulfur ([Fe–S]) cluster near the catalytic site. It receives electrons from ubiquinone, menaquinone, and demethyl-menaquinone within the cytoplasmic membrane via an integral membrane protein subunit, sometimes known as the anchor subunit. In many cases, an [Fe–S] cluster-containing electron transfer subunit participates as an electron conduit between the catalytic and membrane anchor subunits [6]. These enzymes are now often referred to as complex iron–sulfur molybdoenzymes (CISM) to differentiate those from iron–sulfur-lacking molybdoenzymes and have an added level of complexity in coordinating biogenesis of the iron–sulfur cluster(s) [9]. The architecture, function, biogenesis, and maturation of these enzymes have been a major topic of study (as reviewed by refs [6,8,10]).

1.2. Biogenesis of complex iron–sulfur molybdoenzymes

Biogenesis of a CISM begins with the transcription of FNR-regulated genes when oxygen-sensing regulatory proteins in the cell detect a lack of oxygen (reviewed in ref [11]). Following translation, maturation into a functional holoenzyme requires multiple steps that involve folding, cofactor insertion and coordination, and assembly with other subunits (where applicable). The active holoenzyme must then be targeted to the membrane, translocated across the cytoplasmic membrane (if necessary), and attached to its anchor subunit in order to access the quinone pool (reviewed in refs [12] and [6]). A generalized summary of CISM biogenesis is shown in Fig. 1c. During this process, proteins that participate in biogenesis include chaperones for holoenzyme formation, cofactor biosynthesis and insertion proteins, chaperones that assist in folding, the twin-arginine translocase for membrane targeting and moving the folded holoenzyme across the membrane, and the general secretory pathway (Sec) and YidC translocases for insertion of the membrane anchor subunit. The many steps during the biogenesis process are highly complex and must be intricately coordinated to produce a functional CISM.

1.3. Redox enzyme maturation proteins

Biogenesis and maturation of CISMs in the cytoplasm involve the assistance of chaperone proteins. Over the last decade it has become recognized that each enzyme has a specific chaperone protein dedicated towards its own maturation; these chaperones correlate to their CISM based on phylogenetic analyses [13–15]. Such system-specific chaperones were named redox enzyme maturation proteins (REMPs), to distinguish them from other general molecular chaperones that act globally for general protein folding [13]. Canonical characteristics of a REMP are its absolute requirement for the assembly and maturation of its cognate CISM albeit its absence in the final assembled holoenzyme (refs [16–18] for example). Since attachment of most CISMs to the membrane anchor subunit occurs in the periplasm and there have not been evidence showing extra-cytoplasmic localization of the REMPs, it is unlikely that they participate in the anchoring process. However, biogenesis of the holoenzyme and anchor subunit entities must be intricately coordinated as evidence showed that insertion of the anchor subunit DmsC of *E. coli* DMSO reductase was lethal in the absence of the DmsAB holoenzyme [19]. Despite the knowledge that REMPs are required for cofactor insertion into the catalytic subunits, there have not been any studies to address their potential in folding and cofactor insertion of heme-containing membrane anchor subunits. The REMP family of proteins in the model organism *E. coli* includes: DmsD, TorD, NarJ, NarW, NapD, FdhD, FdhE, HyaE, HybE, and YcdY [13,20].

1.4. Twin-arginine translocase

The twin-arginine translocase (Tat) system was discovered through experimental investigations into the maturation process of periplasmic localized CISMs, those which exhibited cofactor insertion events within the cytoplasm [21–23]. These enzymes contained an amino acid signal or leader sequence that differed from general secretory pathway (Sec) signal peptides, with a conserved S/T-R-R-x-F-L-K twin-arginine (RR) motif [24,25]. Polypeptide substrates are synthesized as pre-proteins containing the RR-motif in their N-terminal leader peptide sequence (RR-leader), and majority of the RR-leaders are removed upon translocation. The CISM substrates are translocated as an active and folded holoenzyme across the cytoplasmic membrane by Tat [21,26]. This implied that the translocase complex must be large enough to permit passage of large protein substrates possessing secondary, tertiary, and likely quaternary structures to coordinate their cofactors and interact with accessory proteins.

The Tat translocon consists of the TatABC subunits and the current model identifies TatA as the homo-oligomeric pore/channel subunit, whereas TatB and TatC act as the substrate reception and recognition/delivery module (reviewed in refs [27–29]). Originally described in chloroplast thylakoid membranes, investigators utilized the homologous ΔpH translocation system of plants to help identify and characterize the bacterial Tat system [22]. Gram positive bacteria have what is often described as the minimal Tat system, consisting of only TatA and TatC (reviewed in ref [30]), with the exception of some such as *Streptomyces* sp. [31,32] and *Myocobacterium* sp. [33,34] that have all three Tat subunits.

As the purported protein conducting channel, TatA is reported to oligomerize using energy from the proton motive force, which also supplies the energy for translocation [35–41]. New research suggests that the TatA entity does not exist by itself outside of the TatABC complex [42], contradicting previous studies that TatA is present in oligomeric assemblies of varying sizes independent of the TatABC complex [36,43]. Other studies suggest that the TatA oligomer is recruited to TatBC only in the presence of substrate and an intact proton motive force [38]. TatB is homologous to TatA, but is functionally distinct in its absolute requirement for translocation of Tat substrates [44]. It is found in complexes consisting of TatABC or TatBC in the cell [42,45], and has been implicated in regulating and preventing premature cleavage of the RR-leader by binding to the hydrophobic region of the signal peptide [28]. This is consistent with earlier studies that suggest a role in quality control by TatB in conjunction with TatC [46,47]. Recent research has suggested that TatC is the subunit responsible for inserting and translocating the RR-leader across the membrane [48], and it has specificity for recognizing the RR-motif region of the leader [47,49]. This suggests a model where TatC initiates translocation of the substrate, followed by complete translocation by TatA. Formation of a functional translocase requires all three components; specifically the presence of TatBC for TatA oligomerization [39]. The substrate binds to TatA and TatBC separately, suggesting that the translocon is not fully assembled until receiving a substrate [35,46,50–54]. Live cell imaging confirmed this model where association of fluorescent-tagged subunits of the translocon would only occur in the presence of a substrate-bound TatC [39]. With the numerous amounts of data available on Tat complex formation, piecing together the translocation pathway from various studies still proves to be a daunting task.

In general, Tat-targeted respiratory enzymes appear to have RR-leaders in only one subunit of the holoenzyme [24]. A notable exception is the tetrathionate reductase (TtrAB) of *Salmonella enterica serovar* Typhimurium, where both subunits contain an RR-leader sequence [55]. CISMs with two subunits in the holoenzyme are co-targeted to the membrane via a piggyback or hitchhiker mechanism by its cognate REMP [56,57]. Evidence is building that supports a role of REMPs in targeting their cognate enzymes to Tat [58–60]. In addition to leader recognition, membrane targeting has been suggested to also involve association with membrane phospholipids by the RR-leaders [61–64]. It is likely that the REMP docks at TatBC to donate the substrate to the TatBC receptor [58,59]. Unfolded proteins are rejected by the TatBC receptor complex through an undefined quality control mechanism [47]. The

substrate binds to TatBC, and then appears to be donated to the TatA multi-protomer pore unit [35,46,50–54]. The substrates are translocated through the TatA multimeric pore utilizing energy from the proton motive force for oligomerization and translocation [35–40]. Following successful translocation, the RR-leader peptide is cleaved by leader peptidase I, LepB [65].

1.5. A distinct subfamily of REMPs

Previous homology analyses of REMP proteins identified a subfamily of REMPs including NarJ, NarW, TorD, DmsD, and YcdY [13,15]. The Pfam protein family database (http://pfam.sanger.ac.uk/) classified these proteins under the nitrate reductase delta (NarJ) superfamily designation based on their significant sequence similarity [66]. This subfamily has a fascinating assortment of proteins with shared sequence similarity, warranting a closer examination to determine the extent of their relationship. This type of assessment is useful and important to explore, particularly for those wishing to study chaperone-mediated enzyme maturation processes. Towards this end, the remaining focus of this review will explore and examine members of this subfamily. Current knowledge of subfamily members including structural, biochemical, cellular and systems biology will be compared and dissected in detail. Key differences amongst subfamily members were identified and demonstrate that generalized functions based on observation with one member cannot always be assumed for other members.

2. NarJ subfamily relationship

2.1. Subfamily members

Dimethyl sulfoxide protein D, DmsD (previous *E. coli* designator YnfI), was one of the first described RR-leader binding proteins [16]. It is required for maturation of *E. coli* DMSO reductase and binds the RR-leader of DmsA [16,67], along with the RR-leaders of YnfE and YnfF [14,68]. DMSO reductase is an archetypical CISM enzyme [5], a heterotrimer comprised of DmsABC [69]. DmsA serves as the RR-leader containing catalytic subunit that coordinates the Mo-*bis*PGD catalytic cofactor in addition to one [4Fe–4S]. DmsB functions as the [4Fe–4S] electron conduit subunit, and the entire complex is anchored to the cytoplasmic membrane by the integral membrane protein DmsC.

TorD, which was known for its requirement of TMAO reductase maturation [17], was also shown to bind the RR-motif bearing TorA signal peptide [23,70]. Unlike the archetypical CISM DMSO reductase, TMAO reductase is an atypical CISM consisting of only two subunits — TorA and TorC. It is periplasmically localized with TorA being the catalytic subunit containing the Mo-*bis*PGD cofactor and TorC the pentahemic c-type cytochrome-containing anchor subunit [71].

NarJ and NarW are well-known for their requirement in cytoplasmic nitrate reductase A and Z biogenesis, respectively [18,72]. They were initially discovered to be members of the DmsD family in 2004 based on sequence homology to DmsD and TorD [13]. Despite the cytoplasmic localization of their substrate nitrate reductases, these enzymes are hypothesized to also require the Tat system [60]. This was despite the belief that all Tat dependent respiratory enzymes are extracytoplasmic [13]. Acceptance that NarJ involvement with Tat has only recently begun based on its similarity and functional characteristics as observed in the other subfamily proteins [14,60,73–75].

The final member of this subfamily in *E. coli* is YcdY. YcdY has close homology with other subfamily members, with highest sequence similarity to DmsD at 42%, followed by TorD at 34%. Its sequence is least similar to NarJ and NarW at 14% and 18%, respectively. This relationship initially guided researchers to uncover its cognate substrate by screening for binding to many RR-leaders of anaerobic respiratory enzymes, but attempts were unsuccessful [14,73]. A recent study has suggested that it is involved in swarm motility rather than respiratory enzyme biogenesis and it interacts specifically with the zinc-binding protein YcdX that appears to have phosphatase activity [76].

2.2. Updated phylogenetic analysis and naming of the NarJ subfamily

The previous phylogenetic analysis involving 56 proteins identified through an *E. coli* DmsD-based PSI-BLAST search found that DmsD, TorD, and YcdY homologues appeared to be the most closely related within the five subfamily members [13]. They were named DmsD family in that analysis. To follow-up this analysis, a greater expansion (256 protein sequences) of these subfamily members was performed here and is summarized in Fig. 2 and Supplementary Fig. 1.

tBLASTn searches were selected to include unannotated nucleotide sequence data from newly sequence organisms and allowed for the identification and inclusion of pseudogenes, which is advantageous over ORF/translation biased searches such as BLASTP and tBLASTp. Diverse archaeal and bacterial species were included to select sequences reflecting the most diversity within major phyla and classes and to obtain the greatest number of members. This provided a final total of 256 sequences including NarJ, TorD, DmsD, NarW, YcdY, and a few unnamed/unclassified sequences for phylogenetic analysis. The Neighbor Joining method [77] using two crenarchaeal sequences as outgroup estimates member relatedness and the results of this analysis are presented as a circular dendrogram (Fig. 2; Supplementary Fig. 1 presents it as a rectangular dendrogram). The chosen sequences were from *Metallosphaera yellowstonensis* and *Thermoproteus tenax* (Mye and Tte, Supplementary Table 1) and selected based on their poor sequence identity scores (<10% identity to their respective *E. coli* counterparts). Fig. 2 shows the dendrogram using Mye sequence as outgroup; the cladistic arrangements in both trees were nearly identical using either sequence as outgroup (not shown). Both Mye and Tte sequences contained a nitrate reductase delta subunit conserved domain [78], but the Tte sequence was named SreE as this gene falls within the *sre* operon and encodes for a nitrate reductase-like complex similar to the nitrate reductase chaperone NirD [79].

Based on the dendrogram, the five subfamily members clustered into 3 major clades (Fig. 2). Clades 1 and 3 were enriched almost entirely with NarJ sequences, whereas clade 2 was subdivided into 5 subclades. Subclades 2a and 2d were enriched in γ-proteobacterial TorD, 2b and 2c largely consisted of a mixture of TorD, NarJ and all of the YcdY sequences from non-proteobacterial phyla. Lastly subclade 2e was enriched in DmsD. It should be noted that the annotations of most REMP sequences in this analysis were not validated and many within the DmsD subclade (2e) may be orthologues. To represent their current annotation in NCBI, members were colored according to their GenBank annotation/description. For example, a sequence that was not only annotated to belong to the TorD chaperone family but also has a nitrate reductase delta subunit (i.e. NarJ) conserved domain grouping is colored half yellow and green to reflect this.

In clade 1, NarJ sequences appear to be monophyletic consisting of sequences from α-, β-, and γ-proteobacteria, whereas clade 3 consisted of sequences from mixed phyla including bacilli/lactobacilli, actinobacteria, and a mixture of δ-proteobacteria, clostridia, negativicutes, and Crenarchaea, although bacilli/lactobacilli sequences formed a distinct sub-branch of 3b. TorD sequences within subclades 2a and 2d were mainly monophyletic consisting of α- and β-proteobacteria, whereas in subclades 2b and 2c TorD consisted of sequences from diverse phyla. Like DmsD, many of these NarJ sequences may be mis-annotated TorD sequences. TorD (subclades 2a–2d) appears to form distal branches separate from DmsD (subclade 2e) suggesting that TorD members diverged much earlier than the more recent DmsD. The lack of DmsD homologues detected in tBLASTn searches of archaea species would support this phylogenetic observation. Interestingly YcdY appears to be more closely related to TorD and was specifically enriched in γ-proteobacteria primarily and a select number of actinobacteria. This might reflect a recent

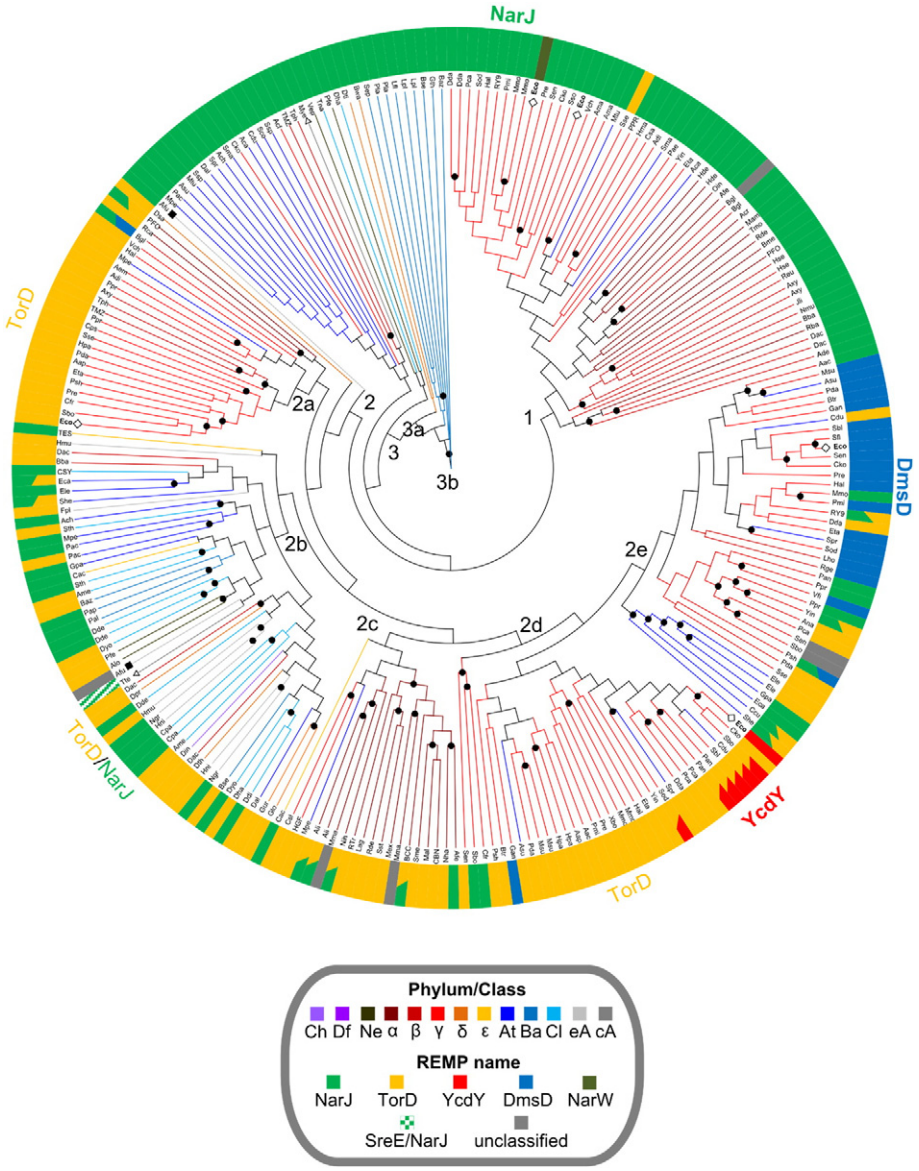

Fig. 2. Phylogenetic analysis of archaeal and bacterial REMP protein family members NarJ, NarW, DmsD, TorD, and YcdY. An unrooted circular dendrogram is provided for the Neighbor Joining analysis of 256 protein sequences. The outgroup sequence used for this analysis was the Crenarchaeal *Metallosphaera yellowstonensis* NarJ protein sequence (NCBI accession no. WP_009070307). A thousand bootstrap replicates were performed for this analysis and confidence values based on bootstrapped values above 80% are represented by closed circles at its respective node. The inner color-coded dendrogram illustrates the branching patterns between REMP proteins belonging to various archaeal and bacterial phyla/classes (refer to legend), from Chrysiogenetes (Ch), Deferribacteres (Df), Negativicutes (Ne), alpha-proteobacteria (α), beta-proteobacteria (β), gamma-proteobacteria (γ), delta-proteobacteria (δ), epsilon-proteobacteria (ε), Actinobacteria (At), Bacilli (Ba), Clostrida (Cl), Euryarchaea (eA) and Chrenarchaea (cA). The colored outer ring around the dendrogram highlights each of the five NarJ subfamily members, corresponding to each branch shown in the dendrogram. The five open diamonds between the inner dendrogram and the outer ring indicate *E. coli* REMP proteins used to seed the tBLASTn searches. The two Crenarchaeal sequences are indicated with an open triangle, and the two *Archeaglobus fulgidus* sequences with a closed square. Detailed information for each sequence such as abbreviation for genus species names and NCBI accession numbers can be found in Supplementary Table S1.

duplication event within TorD, however little functional evidence currently exists for YcdY to confirm this.

From this most recent analysis, the origins of this family appear to stem from NarJ and not DmsD or TorD. Hence, we propose that this REMP subfamily be named to the NarJ subfamily to reflect its current cladistic relationship, which is also consistent with the naming by the Pfam database [66]. The outcome of this current phylogenetic analysis of NarJ subfamily members is in agreement with previous bioinformatic analysis of the entire REMP family [13]. However, divisions between all subfamily members were apparent based on the phylogeny presented herein. The current division of members may provide clues as to why specialized functions of each chaperone were tailored for their cognate CISM. If all REMPs functioned the same then there would be no need to evolve system-specific chaperones. The greatest examples stem from the functions of NarJ and YcdY: 1) the substrate of NarJ, NarG, constitutes part of the cytoplasmic nitrate reductase A, whereas Tat-dependent respiratory enzymes were believed to be solely extracytoplasmic [80]; 2) YcdY appears to be a recently evolved member and the closest relative to TorD, so it is not surprising that the YcdY respiratory enzyme substrate remains a mystery. Whereas DmsD forms its own subclade (2e) from the TorD branches (2a–2d) indicating that was tailored towards its cognate respiratory enzyme as observed by its function as an S- and N-oxide reductase [81]. Further analyses of members in this family require an in-depth comparison of functional

and structural data to better understand the relationship of NarJ subfamily members and are discussed in sections below.

3. Structural relationship of NarJ subfamily members

3.1. Available structures

Of the NarJ subfamily members, X-ray crystal structures are available for DmsD, TorD, and NarJ from various bacterial genera. Structures for REMPs not belonging to the NarJ subfamily include HyaE, FdhE, and NapD and their differences within the REMP family as a whole are discussed in Turner et al. [20]. NarJ subfamily protein structures include two *E. coli* DmsD (EcDmsD, PDB ID: 3EFP and PDB ID: 3CW0 [82,83]), a *Salmonella typhyimurium* LT2 DmsD homologue (StDmsD, PDB ID: 1S9U [84]), and a *Shewanella massilia* TorD homologue (SmTorD, PDB ID: 1N1C [85]).

Crystal structures for putative molybdenum enzyme-specific REMPs from the archaebacterium *Archaeoglobus fulgidus* are also available (PDB ID: 2O9X, PDB ID: 2XOL, and PDB ID: 2IDG, [86,87]). Their sequences were identified within the updated phylogenetic tree (Fig. 2, black squares), thus are included here for comparison. One of the two proteins was demonstrated to bind the RR-leader containing subunit TtrA of tetrathionate reductase and was named by the authors to be TtrD [87] and has a nitrate reductase delta conserved domain, while the other remains unnamed. For simplicity we will refer to them as *Af*TtrD and *Af*REMP in this review.

3.2. Structural alignments

Proteins of similar or related function often share structural properties [88], thus structural relationships are useful measures to compare NarJ subfamily members. The available structures of NarJ subfamily members exhibit all-alpha folds, which contrast the mixed α-helix and β-strand structures of HyaE, NapD, and FdhE [20]. YcdY was previously shown by circular dichroism spectroscopy to be mainly α-helical [15]. Bioinformatic secondary structure prediction algorithms PredictProtein [89] and JPred 3 [90] performed on *E. coli* sequences of YcdY and NarW also support an all-alpha fold structural organization (data not shown).

Pairwise alignments of the available structures are provided for direct comparison in Fig. 3. *Sm*TorD was crystallized as a homodimeric structure, where its N- and C-termini appeared as two lobes connected by a center hinge region [85]. Its structure was unique in comparison to other NarJ subfamily members where the N-terminus from one monomer interacts with the C-terminus of the other as a result of domain swapping. To explore whether domain conservation is present in all NarJ subfamily proteins, the coordinates of the *Sm*TorD structure were edited to produce a 'monomer' to reflect domain swapping by choosing

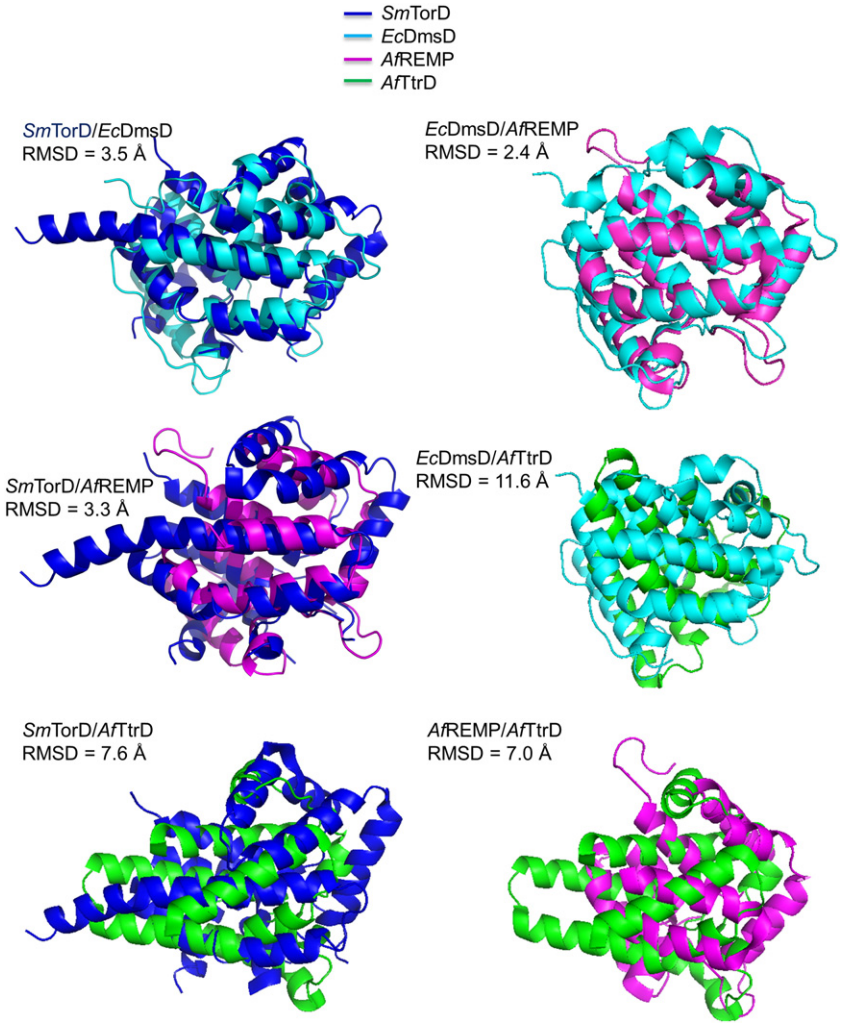

Fig. 3. Structural alignment of NarJ subfamily proteins. Protein structures of *Shewanella massilia* TorD (PDB ID: 1N1C), *Escherichia coli* DmsD (PDB ID: 3EFP), *Archaeoglobus fulgidus* REMP (PDB ID: 2O9X), and *A. fulgidus* TtrD (PDB ID: 2IDG) were aligned against each other for comparison (see legend). Alignment of backbone atoms in PyMol resulted in the corresponding RMSD values indicated. Residues from one 'lobe' of the domain-swapped TorD dimer consisting of residues 4–128 of chain A and 129–214 of chain B were chosen for alignment, whereas all residues from a monomeric assembly of the other proteins were used directly.

residues 4–128 from the N-terminus of one monomer and 129–214 from the C-terminus of the other. Of the three DmsD structures available, the *Ec*DmsD structure was chosen for this alignment as its complete structure was available, whereas the *St*DmsD homologue had a small portion of the putative DmsA RR-leader binding site unresolved. Both *Ec*DmsD and *St*DmsD structures are highly similar with backbone root mean square deviations (RMSD) of 0.71 Å after the two structures were superimposed [82].

It became apparent upon aligning the available structures that *Af*TtrD was the most dissimilar to all the other structural alignments and is reflected by backbone RMSD values of >7 Å. The remaining alignments had relatively low RMSD values, with *Ec*DmsD and *Af*REMP alignment having the lowest RMSD value of 2.4 Å. With the exception of *Af*TtrD, it is clear that the structural arrangements of proteins of this subfamily are remarkably similar. As more protein structures become available and potential REMPs being identified (as in the case of *Af*REMP), it is possible to utilize structural alignments such as this to validate whether a protein is a bona-fide NarJ subfamily REMP.

3.3. Functional insights from structural information

3.3.1. Oligomeric conformations and their implications

Many studies have attempted to elucidate the biological relevance of the multimeric forms of REMPs. Crystallization of a domain swapped *Sm*TorD homologue points towards a functional role for this unique domain organization, or at least the dimeric assembly [85]. By dissecting TorD into N- and C-terminal lobes and the connecting hinge, specialized functions important for cofactor insertion or proofreading were identified for each region [70]. The functional role of domain swapping in the dimeric assembly was unknown until studies revealed that *E. coli* TorD could only hydrolyze GTP in a homodimeric domain-swapped conformation [91].

As for the other NarJ subfamily REMPs, *St*DmsD and *Af*TtrD were crystallized as monomers, *Ec*DmsD as monomer and tetramers, and *Af*REMP as dimer [82–84,86,87]. *E. coli* DmsD was purified as a mixture of monomer and dimer and showed subtly different folding forms that would transition with pH [92]. In terms of RR-leader binding, monomeric *E. coli* DmsD and TorD interact with the RR-leaders of their cognate substrates DmsA and TorA, respectively in a 1:1 fashion [93,94]. *S. massilia* TorD could be purified as monomer, dimer, and trimer [95]. However, while both monomeric and dimeric forms of *S. massilia* TorD bind to the mature form of TorA, more appeared to interact in the dimeric form. *A. fulgidus* TtrD was purified as a monomer, but protein interaction studies performed with TtrA do not indicate whether multimeric forms exist or participate in interactions with its substrate enzyme [87]. From the literature it is apparent that of all the NarJ subfamily members that exhibit oligomeric assemblies, yet only the dimeric form of TorD has a defined biological role so far.

3.3.2. Conformation changes upon substrate binding

A key event in CISM maturation involves binding to the RR-leader peptide of its cognate substrate, presumably as the substrate exits the ribosome as a nascent polypeptide (Fig. 1). The events that occur after REMP binding to the RR-leader of its substrate are poorly understood and remain an ongoing area of interest. Dissecting the maturation process can assist in piecing together the activities that lead up to formation of the holoenzyme. This section focuses on the structural implications upon substrate binding; further discussion on the functional implications is covered in Section 4.1.

Altered conformations of DmsD upon DmsA RR-leader peptide binding were determined using molecular dynamics simulation [82]. Based on these simulations, residues in DmsD that are buried yet important for RR-leader peptide binding appear to become surface-exposed upon leader binding [82,96]. These conformations are suggestive of the multiple folding forms observed for DmsD that were all capable of binding the DmsA RR-leader [93]. However, overall secondary and tertiary conformational changes in DmsD upon DmsA RR-leader peptide binding appear to be quite small as they were undetectable by differential scanning calorimetry and circular dichroism spectroscopy [97]. The extreme C-terminus (last 173 residues) of TorD appeared to be folded differently when comparing the unbound to the apo-TorA-bound form [94]. In this study, the apo-TorA binding site was a secondary site located outside the RR-leader that is important for maturation and cofactor insertion [94,98], suggesting that this altered conformation of TorD may reflect its linkage to cofactor insertion. Conformation changes in NarJ were also observed upon binding to the NarG RR-peptide, where its two dominant conformations became restricted to one upon binding [99]. This NarJ alteration was described as a conformational selection mechanism by the authors and may reflect a general mechanism related to the multiple subtle folding forms observed for DmsD and TorD [92,95].

One of the postulated roles of NarJ subfamily REMPs is that they bind their substrates and maintain them in a state that is competent for cofactor insertion. Due to the complexity in the sequence of events during maturation, it is often difficult to discern the degree of folding by the substrates when they are bound by the REMP. Studies of the TorD/apo-TorA complex found that the amount of disordered structure in the complex was similar to that of the holoenzyme [94]. Similarly, N-terminal peptides of NarG (the first 15 to 28 residues) displayed almost no changes in structural organization upon NarJ binding, remaining mostly α-helical in solution as it was prior to the interaction [99]. Contradicting observations have been reported for the DmsD/DmsA RR-leader complex, with one study showing that it remained mostly unstructured [100], and another showing overall helical content increase [97]. Studies of NapD, a REMP that is not considered a close member of the NarJ subfamily, indicate that the NapA RR-leader had increased propensity for forming an α-helix when bound to NapD [101].

Altogether, these observations highlight the structural differences during the interaction with their substrates within the NarJ subfamily members, revealing that despite structurally similarities, they appear to have different models of binding to their cognate substrate and they may adopt different structural conformations upon binding the leader peptide.

3.4. The role of GTP

In the previous section we mentioned the importance of domain swapping for GTP hydrolysis by dimeric TorD. GTP binding and hydrolysis are hypothesized to govern the activity of TorD during certain stage(s) in its activity; the interaction with GTP decreased when TorD was allowed to bind to TorA RR-leader first [102]. Further, it was demonstrated that only dimeric TorD could hydrolyze GTP under a magnesium-dependent fashion [91]. This may reflect a general regulatory mechanism used by NarJ subfamily REMPs to control its activity. Based on this hypothesis, GTP-binding sites have been modeled onto the structures of *St*DmsD and *Sm*TorD, revealing the potential locations of GTP binding [84,102]. The residues predicted to bind GTP differed from the two studies mapped to adjacent areas on the surface of TorD (Fig. 4, yellow and green). By aligning the structures of *St*DmsD and *Ec*DmsD (not shown), the corresponding GTP-binding residues based on those from *St*DmsD [84] were identified for *Ec*DmsD and mapped onto the surface of *Ec*DmsD (Fig. 4, orange). Even with the differing putative GTP-binding sites on *Sm*TorD, it is apparent that the location of the putative GTP-binding site on *Ec*DmsD differs greatly, located at almost the opposite side of the protein compared to the *Sm*TorD sites. This suggests that while the two are structurally similar (RMSD of 3.5 Å), the mode in which they utilize GTP may be different. However, at this point it is only speculation and further biochemical investigations are required to validate a role for GTP with the NarJ subfamily REMPs and CISM enzyme maturation. The potential relationship between GTP and RR-leader binding will be discussed further in Section 4.1.3 below.

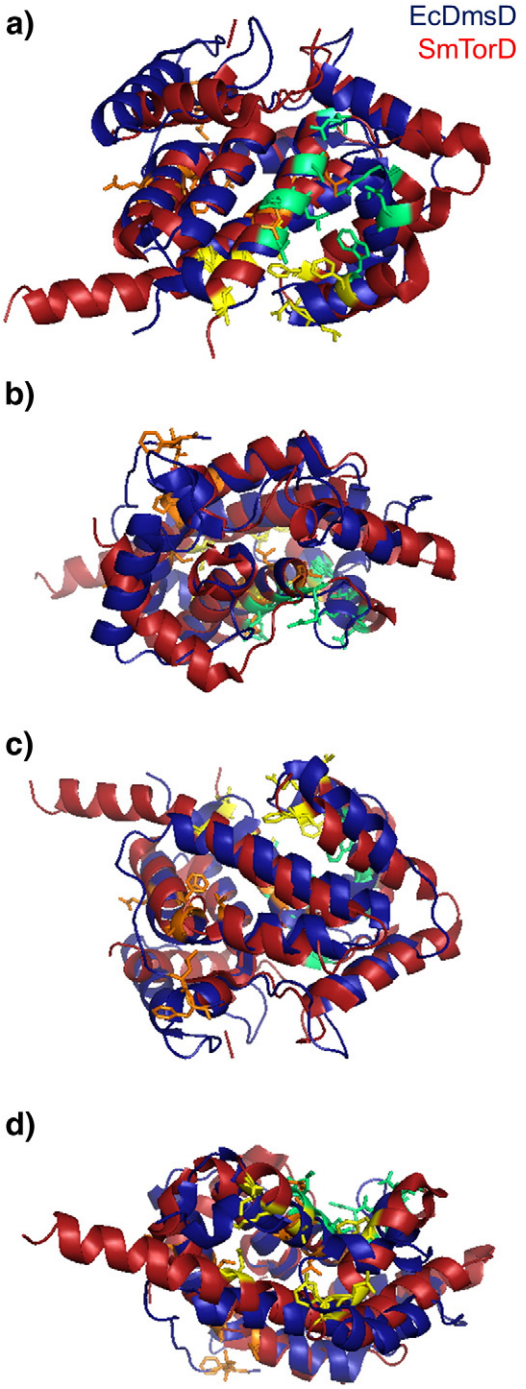

Fig. 4. Putative GTP-binding sites in DmsD and TorD. Comparison of putative GTP-binding sites in *E. coli* DmsD (PDB ID: 3EFP, blue) and *S. massilia* TorD (PDB ID: 1N1C, red). Alignment done as in Fig. 3 (*Sm*TorD/*Ec*DmsD) with residues predicted to be involved in binding GTP highlighted. Panels a, b, c, and d are 90° rotations about the x-axis of the aligned structures. Residues on *Sm*TorD predicted from Hatzixanthis et al. [102] are colored green and Qiu et al. [84] yellow. The *S. typhyimurium* (PDB ID: 1S9U) DmsD structure [84] was aligned with *E. coli* DmsD structure (not shown) to identify corresponding GTP-binding residues in the *E. coli* structure and then colored orange on *Ec*DmsD.

4. Functional relationship of NarJ subfamily members

4.1. Cognate substrates and their interactions

Twin-arginine leader peptide binding is a canonical feature of REMPs, with the sole exception of YcdY. Binding of TorD and DmsD to the TorA and DmsA RR-leader peptides respectively, was suggested to be a pre-requisite for TMAO and DMSO reductase biogenesis [16,17]. The catalytic subunits of both nitrate reductases A and Z (NarG and NarZ) contain a vestige or remnant RR-motif in their N-terminus [13]. Fig. 5a shows the identified motifs in NarG and NarZ and their corresponding REMP chaperone in relation to several other CISM substrates. Unlike all other known respiratory enzyme substrates, the N-terminal peptide of NarG is not cleaved in the final mature enzyme and it is assumed that the same occurs for NarZ [103]. Similar to other NarJ subfamily members, the N-terminal region of NarG and NarZ containing the remnant RR-motif bound their known accessory REMP chaperones NarJ and NarW [14,60,74,104]. Further characterization showed that the interactions between NarJ and NarG showed a partial dependence upon the Tat complex [60]. These findings have great implications for defying the notion that the Tat system acts strictly as a translocase, since the final respiratory complexes of nitrate reductases A and Z are cytoplasmic localized. This additional functionality was implied in the original naming of the *tat* operon itself as *mtt*, for membrane targeting and translocation [21].

4.1.1. Specificity of substrate interactions

Understanding the details of RR-leader peptide binding has been a major focus for many research groups since the discovery of REMPs. Such knowledge furthers the understanding of the maturation process of respiratory enzyme biogenesis. A comparative study found that the REMP to RR-leader interactions could be further differentiated into those with or without cross-interactions to non-cognate enzymes [14], and characterized REMPs that were highly specific from those that were less specific. The level of specificity of the NarJ subfamily REMPs is illustrated in Fig. 5a. Of the NarJ subfamily REMPs, TorD was the most specific and only bound the RR-leader of its cognate substrate TorA. NarJ and NarW had moderate specificity, binding only the RR-peptides of cytoplasmic nitrate reductases NarG and NarZ while DmsD was the least specific, binding the RR-leaders of DmsA, TorA, YnfE, and YnfF. A clear function for YnfE and YnfF (both paralogues of DmsA [105]) remains unknown in *E. coli*, but both proteins have some involvement in selenate reduction in *Salmonella* that requires DmsD for functionality [68]. A study involving a fusion chimera that replaced DmsA RR-leader with the TorA RR-leader resulted in a partly functional membrane-bound DmsABC complex [106], suggesting that this 'cross-talk' between DmsD to the TorA RR-leader in vivo is possible. Since only partial function was restored in DMSO reductase in these chimera replacement experiments, it suggests that some of the chimeras were likely targeted by TorD that could not replace DmsD in the other aspects of biogenesis. While YcdY and several 'orphan' respiratory enzyme RR-leaders were included in the aforementioned specificity study, none were shown to bind YcdY by the methods tested [14]. Overall, it is clear that all REMPs bind the RR-leaders of their specific respiratory enzymes, but for some there is a remarkable level of cross-talk.

So, what governs the specificity of cognate leader peptide binding by the REMPs? The high degree of similarity in the RR-motif is an unlikely candidate for governing specificity and was confirmed by studies targeting the DmsA or TorA RR-leaders. These studies found that the RR-motif region had only a minor involvement for tight binding to DmsD or TorD respectively [97,102]. Both studies identified the hydrophobic region immediately following the motif as important for tight binding, and another identified a conserved $_{28}$LAMA$_{31}$ motif within this region in DmsA that may be involved in conferring specificity of DmsD binding [14]. Of the four RR-leader sequences that DmsD was shown to bind, the TorA leader had the most divergent sequence ($_{21}$LTVA$_{24}$) compared to DmsA. Coincidentally, it appears that V23 of TorA was one of five residues required for specific binding by TorD [107]. The remnant RR-peptide of NarG has also been examined and mutations within the remnant RR-motif (making it a double arginine) and the pseudo-hydrophobic region (substituting many polar residues with non-polar ones) could induce translocation of NarG by Tat into the periplasm [73]. These mutations, however, interfered with the interaction with NarJ and impaired its activity. NapD, a member of the REMP

a)

Substrate	DmsA	YnfE	YnfF	TorA	NarG	NarZ
REMP \ motif	SRRGLVK	SRRTLVK	SRRQLMK	SRRRFLA	DRFRYFK	DRFRYFK
DmsD	✓	✓	✓	✓		
TorD				✓		
NarJ					✓	✓
NarW					✓	✓

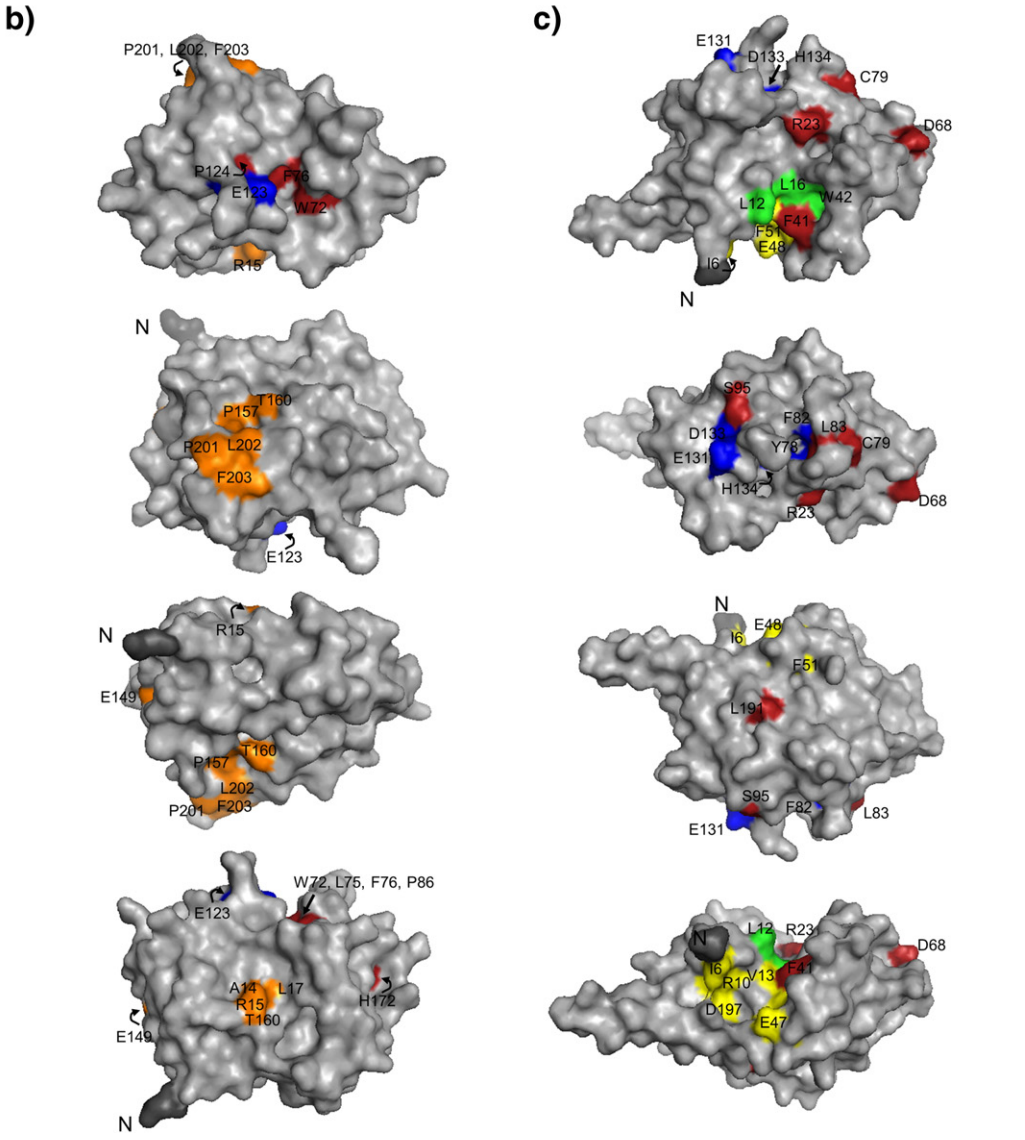

Fig. 5. NarJ sub-family substrate RR-leader and GTP interactions. a) REMP interactions with RR-motif containing peptides assessed by various techniques are summarized and indicated by a check for experimentally proven interactions. The corresponding RR-motif in each peptide is also indicated. Summarized from Chan et al. [14]. b) Comparison of DmsA RR-leader binding sites (red) in *E. coli* DmsD (PDB ID: 3EFP) determined experimentally [96] or by molecular dynamics simulation [82]. The conserved residues (blue) amongst NarJ sub-family members [13] and putative GTP-binding sites (orange) as in Fig. 4 are also indicated for comparison. Structures are 90° rotations about the x-axis. The N-terminus (N) of the protein is indicated. c) Comparison of TorA RR-leader binding sites (red) in *S. massilia* TorD (PDB ID: 1N1C) determined from its *E. coli* homologue [70,91,98,102,107], with the NarJ sub-family conserved residues (blue) and putative GTP-binding sites (green and yellow) as indicated in Fig. 4.

family but unrelated to the NarJ subfamily, was shown to have a binding site within the NapA RR-leader that included the RR-motif itself [101]. This observation suggests that the specificity of REMP binding is conserved for members of the NarJ subfamily and appears to be governed by the hydrophobic region of the RR-leaders and not the RR-motif. Additionally, the degree of hydrophobicity in this region appears to determine whether a protein is translocated across the membrane.

4.1.2. Secondary binding sites on substrate enzymes

Differences observed between NarJ subfamily members are revealed further in their variation to utilize secondary binding sites within their CISM enzyme substrates. Multiple binding sites were first suggested to influence REMP-substrate binding when TorD was shown to interact with apo-TorA and activate TMAO reductase activity in vitro [108]. Investigations into multiple binding sites by NarJ subfamily members

to its cognate substrate found that TorA and NarG had additional TorD and NarJ binding sites respectively in regions other than the RR-leader [75,98,104], whereas DmsD had no additional sites other than the DmsA RR-leader [75]. This DmsD–DmsA RR-leader interaction did not appear to have any Tat dependence, yet TorD and NarJ interactions demonstrated reduced binding when some or all of the Tat subunits were absent in the cell [75].

The existence of multiple REMP interaction sites of their substrates indicates that there may be a role in the folding and maturation of the unfolded apoenzyme into holoenzyme in these cases. The remnant RR motif-containing N-terminus in NarG appears to be important for membrane anchoring to NarI [104]. Additionally, observations from other studies suggest that the interaction between this region of NarG and NarJ has a strong Tat-dependence [60,75], and that perhaps the targeting to the Tat complex acts as an adaptor for the anchoring/attachment process. The secondary interaction site in NarG was found in a region outside the first 40 residues and was implicated in cofactor insertion [104]. Similarly, the secondary binding site in apo-TorA appears to be important for maturation and cofactor insertion [94,98]. The lack of a secondary binding site for DmsD in DmsA suggests that the method whereby DmsD assists in cofactor insertion is different from the other NarJ subfamily members. These differences highlight the specialized functions of each NarJ subfamily member towards maturation of its cognate CISM.

4.1.3. Implication of RR-leader binding sites on CISM maturation

The currently available information regarding site-specific REMP interactions is useful for dissecting their functional role during CISM maturation. Early bioinformatics studies identified two conserved (Y/F/W)xxLF and E(Px or xP)D(H/Y) motifs common to the NarJ subfamily REMPs [13]. Using this information to guide their initial studies, Chan et al. [96] targeted these residues in *E. coli* DmsD to search for the DmsA RR-leader binding site and found that four of the seven conserved residues from these motifs were important for binding. Mapping these residues onto the surface of DmsD reveals a close proximity for the DmsA leader-binding pocket with the conserved motifs (Fig. 5b, top panel). However, it does not appear that any of the putative GTP binding sites discussed in Section 3.4 map near the DmsA leader-binding site (Fig. 5b), suggesting that GTP binding and/or hydrolysis occur elsewhere on DmsD that may rely on conformational changes within DmsD to relay regulatory changes to DmsA maturation. This is supported by conformation changes observed in DmsD upon binding to a small DmsA leader peptide [109]. Similar overlap of the NarJ subfamily conserved motifs and the TorA RR-leader binding site is observed when corresponding *E. coli* residues are mapped onto the *S. massilia* TorD structure (Fig. 5c, second panel). While several of the putative GTP-binding residues fall within close proximity to some TorA leader-binding residues (Fig. 5c), residues R23 and D68 had overlapping functions binding both GTP and TorA leader (when conducted on the *E. coli* equivalent residues) [91,102], suggesting that the regulation of TorA maturation by TorD through GTP is tightly coordinated. These differences between DmsD and TorD further highlight the specificity of function for CISM maturation by NarJ subfamily REMPs.

4.2. Interactions with the translocase and membrane

Another major function hypothesized for REMPs is the targeting responsibility to guide substrates to the membrane and leads to the question of whether REMPs could interact with Tat machinery and/or the cytoplasmic membrane. In a recent study TorD demonstrated interactions with the phospholipid bilayer, suggesting that it directly binds the membrane during substrate targeting towards the Tat machinery [110]. This supports previous studies showing the requirement of phosphatidylglycerol and phosphatidylethanolamine in the membrane for successful translocation of TorA [111]. In contrast, DmsD did not demonstrate any phospholipid interactions, only showing interaction with the membrane through the TatB and TatC subunits of the translocase itself [58,59]. Additionally, DmsD appeared to impair the binding of the DmsA RR-leader to phospholipid bilayers [110], implying that it could be involved in retarding the transport of premature protein. Reduced membrane localization of NarJ was observed in a mutant lacking the entire *tat* operon, suggesting that its membrane localization is partly Tat-dependent [74]. While interactions between NarJ and phospholipids have not been investigated, studies have shown that cardiolipin is required for NarGHI activity by binding to the complex near the heme in NarI and connecting the enzyme to the quinol pool in the membrane [112].

In vivo investigations between four NarJ subfamily members (DmsD, TorD, NarJ, YcdY) with TatB and TatC demonstrate that they all can bind to TatBC (Kuzniatsova et al., unpublished). These results support those previously observed either directly [58,59] or indirectly [60,74], but is the first time reported for YcdY. This provides further support that the membership of YcdY in the NarJ subfamily is functionally legitimate. The results from these studies suggest a uniform functionality of membrane targeting through the Tat system for NarJ subfamily members, albeit differences in their Tat targeting pathways with regard to the role of membrane lipids.

4.2.1. Implications on the functional relationship of YcdY with NarJ subfamily members

Attempts to characterize the function of YcdY by several research groups have not yet revealed its functional characterization through homology analysis [13,15], biophysical characterization [15], and substrate identification [14,73,76]. Although YcdY appeared to bind a cytoplasmic protein with alkaline phosphatase activity that was involved in swarm motility [76], questions still remain as to why this protein exhibits sequence and biochemical homology to other members of the NarJ subfamily, all involved in respiratory enzyme biogenesis. In the aforementioned study, YcdY also appeared to facilitate the incorporation of zinc into YcdX. The authors of this study proposed that YcdY was an atypical member of the TorD (NarJ) family of REMPs and the common link between these members was their involvement in metal incorporation. However, this does not explain why YcdY interacts with TatBC in a similar manner as other NarJ subfamily members (Kuzniatsova et al., unpublished). In previous sections, we hypothesized that NarJ uses TatBC subunits as a scaffold to target NarGH to the membrane anchor NarI. Perhaps there is a similar role here, but membrane localization of YcdX has not yet been determined. At this point, there is still too little biochemical data on YcdX, and thus YcdY remains an enigma.

Data mining efforts of various transcriptome- and proteome-wide analyses have resulted in some new information about YcdY expression. The Protein abundance across organisms database (PaxDb) website (http://pax-db.org) compiles various 'omics' studies and computes the absolute protein abundance from six independently performed studies into one centralized database [113]. From PaxDb we found that YcdY is a highly abundant protein, as it ranked within the top 25th percentile of all proteins expressed in *E. coli*. The database indicated that NarJ and NarW were in moderately low abundance (bottom 50th percentile), but information was unavailable for DmsD and TorD. Additionally, *ycdY* and all other REMP subfamily members appear to be non-essential under aerobic conditions [114], supporting the notion that YcdY may be functionally related to NarJ subfamily members with respect to anaerobic respiration. Despite this knowledge, we are no closer to understanding its membership in the family. It is clear from previous [13] and current phylogenetic analyses of REMP subfamily members that YcdY is a close homologue of TorD and a more distant homologue of NarJ. What is certain is that four of the five subfamily members are chaperones involved in biogenesis and maturation of respiratory redox enzymes. We suggest that YcdY remains classified within the REMP family until further information and a clearer understanding of its function are demonstrated.

4.3. Interactions with other proteins and implications on their function

REMPs are purported to aid in folding and cofactor insertion into the catalytic subunits of respiratory enzymes during maturation. Positioning of the Mo-*bis*PGD cofactor within the CISM protein structure implies that the cofactor is inserted during folding, unless it is inserted post-folding via large domain movements and rearrangements, which is energetically costly. Since DmsD, TorD, and NarJ were shown to be required for assembly of DmsAB, TorA, and NarGH into mature holoenzymes [67,108,115], this supports their involvement in folding. Protein folding in the cell is normally assisted, when necessary, by general molecular chaperones, some of which are associated with the ribosome. DmsD was found to interact with ribosomal elongation factor (Ef-Tu) and trigger factor (Tig), along with other folding chaperones that include DnaJ, DnaK, GroEL, and GrpE [116]. These general chaperones (excluding GrpE) are well-known for their role in forming a complex network to assist in protein biogenesis by associating with the ribosome (as reviewed in refs [117,118]). Mining of data obtained from a global study of *E. coli* molecular chaperones revealed that DmsB solubility was enhanced by ~10-fold in the presence of DnaJ–DnaK–GrpE and 2–3 fold in the presence of Tig or GroEL–GroES using a cell-free translation system [119]. While DmsA was not included in this study, other respiratory enzyme subunits (NarG, NarH, and NarY) were included and also had enhanced solubility in the presence of all general chaperones. Other studies have shown direct interaction of Tig with the TorA RR-leader [120], and DnaK with the RR-leaders of DmsA and TorA [16,121]. Together the observations from these studies suggest that DmsD, and possibly the other subfamily members, works in coordination with general molecular chaperones for proper folding of DmsA or their cognate substrates.

Interactions of DmsD with Ef-Tu and Tig also suggest that DmsD may be recruited to the ribosome as the nascent polypeptide emerges from the exit tunnel [116]. This study also found interactions between DmsD and proteins involved in molybdenum cofactor biosynthesis, specifically MoeA, MoeB, MogA, and MobB. Interactions between TorD and MobA, another cofactor biosynthesis protein, were reported in addition to two Mo-*bis*PGD pathway intermediates Mo-molybdopterin and Mo-PGD [98], supporting that the NarJ subfamily REMPs also coordinate cofactor insertion. A summary of all known direct and indirect interactions for each of the NarJ subfamily members is provided in Fig. 6. The key difference appears to lie in the number and type of interacting partners for each NarJ subfamily REMP. Taken together, it suggests that these CISM enzymes follow similar, but distinctly different maturation pathways where its cognate REMP facilitates the interaction with general chaperones and or cofactor biosynthesis to coordinate protein folding and cofactor insertion.

4.4. Other roles in CISM biogenesis

4.4.1. Protection against cleavage

Another suggested role for REMPs is its ability to provide protection of its substrate enzyme against protease activity. TorD was shown to

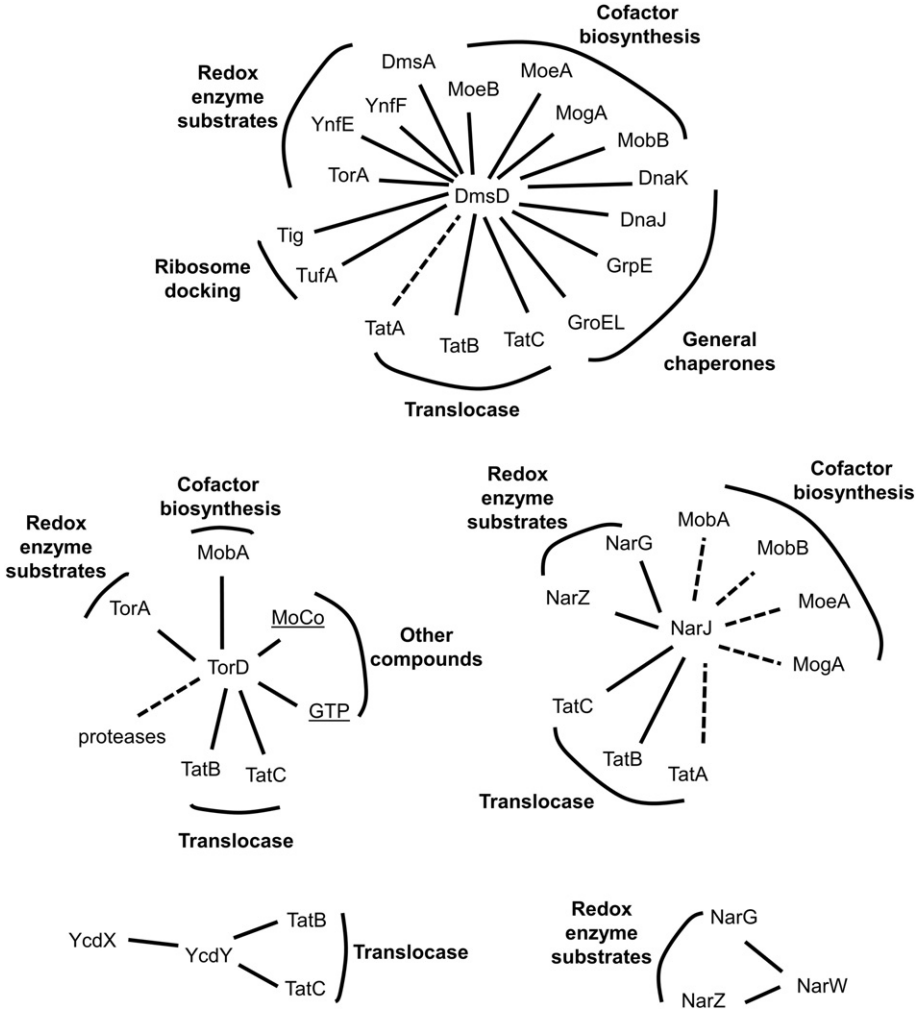

Fig. 6. Interaction network of NarJ sub-family members. A compilation of all observed interactions for members from available literature. The interaction partners are grouped by function. Dashed lines indicate indirect or inferred interactions.

have a protective role in TorA maturation, where it prevented proteolytic cleavage of the leader peptide from apo-TorA [122]. Most of the cleavage products occurred after residue R35 in the leader peptide sequence, suggesting that the protease responsible for cleavage was an enzyme like trypsin, as it almost exclusively cut after arginine or lysine residues [123,124]. This protective role is similar to that of the molecular chaperone Tig, by binding nascent polypeptides to shield them from proteases (reviewed in refs [117,125]).

The protective role has not been directly observed with the other NarJ subfamily REMPs. It does however appear that expression of DmsA leader- or TorA leader-fused GFP is vastly improved when coexpressed with DmsD or TorD, respectively [126,127]. Whether or not all members of the NarJ subfamily specifically exhibit a protective role remains to be uncovered.

4.4.2. Proofreading or quality control

A proofreading function for TorD has also been suggested based on a study using a fusion chimera, showing that it mimicked the quality control function of the unrelated REMP HybE involved in hydrogenase maturation [70,128]. This process suggests that misfolded enzymes or those lacking their partnering subunits were recognized and corrected prior to translocation. Other observations reveal that TorD may prevent misfolding and improper cofactor loading of apo-TorA at elevated temperatures and under cofactor limiting conditions, providing further support for its proofreading function [129,130]. Studies on NarJ showed that it had a role in preventing premature attachment of NarGH to NarI when Mo-*bis*PGD and [Fe–S] cofactors were absent [104,131]. Interaction of DmsD with folding chaperones has already been discussed above, and also supports the proofreading function of REMPs. Together, this data supports a widespread and essential role for quality control by NarJ subfamily REMPs in respiratory enzyme biogenesis.

5. Concluding remarks

This review has provided a detailed overview of the biochemical, structural, and systematic functions of the redox enzyme maturation proteins for complex iron–sulfur molybdoenzymes that are trafficked to the Tat system. After compilation and comparison of currently available findings it is apparent that current data supports a functional relationship between members in the NarJ subfamily of REMPs. Despite their close homology, subtle differences also exist between each REMP and their requirement in CISM biogenesis which distinguishes each from the other. We have shown that while the NarJ subfamily members (and other REMPs) share a similar biological function, they are not the same with respect to their individual biochemistry, supporting their initial classification as system-specific chaperones.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2014.08.020.

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