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Twin-arginine translocase may have a role in the chaperone function of NarJ from *Escherichia coli*

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Abstract

NarJ is a chaperone involved in folding, maturation, and molybdenum cofactor insertion of nitrate reductase A from *Escherichia coli*. It has also been shown that NarJ exhibits sequence homology to a family of chaperones involved in maturation and cofactor insertion of *E. coli* redox enzymes that are mediated by twin-arginine translocase (Tat) dependent translocation. In this study, we show that NarJ binds the N-terminal region of NarG through Far Western studies and isothermal titration calorimetry, and the binding event occurs towards a short peptide sequence that contains a homologous twin-arginine motif. Fractionation experiments also show that the interaction of NarJ to the cytoplasmic membrane exhibits Tat-dependence. Upon further investigation through Far Western blots, the interactome of NarJ also exhibits Tat-dependence. Together the data suggest that the Tat system may play a role in the maturation pathway of nitrate reductase A.

Keywords: Nitrate reductase A; NarJ; Chaperone; Twin-arginine translocation; Redox enzyme; Redox enzyme maturation protein

Nitrate reductase A of *Escherichia coli* is a membrane-bound redox enzyme that allows for anaerobic respiration using nitrate as a terminal electron acceptor. It is a heterotrimer consisting of the NarG, NarH, and NarI subunits, which are encoded by the *narGHIJ* operon in *E. coli* [1–3]. NarG is the catalytic subunit that uses a molybdenum cofactor for its activity [4], while NarH serves as the 'electron conduit' subunit using its three [4Fe–4S] and one [3Fe–4S] clusters [5]. The NarGH complex is anchored to the cytoplasmic side of the membrane through NarI, which contains a *b*-type cytochrome [6]. A fourth component encoded by the *narGHIJ* operon is NarJ, a subunit that has been shown to be a chaperone required for proper cofactor insertion and biogenesis of the NarGH complex [7–9].

The twin-arginine translocase (Tat) is a newly discovered translocation mechanism used by many bacteria for membrane protein targeting and translocation in their folded forms [10,11]. Primary sequence analysis of Tat-dependent proteins has been shown to contain a conserved SRRxFLK "twin-arginine" motif in their leader peptide sequence at the N-terminus of the protein [10]. While a wide variety of proteins involved in varying functions have been discovered to be Tat-dependent, the majority of them are cofactor-containing redox enzymes involved in respiration [12,13]. These enzymes have similar functional architecture where a catalytic subunit(s) is anchored to the membrane through an integral membrane subunit [14]. In the present model, the catalytic subunits are targeted to and translocated across the cytoplasmic membrane by the Tat translocon [12,13]. In the cases where two subunits exist, the motif is only present in one of the subunits, while the other subunit is believed to be targeted via a co-dependent "hitchhiker" mechanism [15,16].

NarG does not contain the typical signature motif nor does it have a cleaved leader sequence, but was identified by Turner and colleagues [17] to contain a vestige motif of DRFRYFK in its N-terminus, which appears to be a

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remnant of the Tat signature. They also noted that NarJ exhibits sequence homology to DmsD and TorD, the chaperones of dimethyl sulfoxide and trimethylamine oxide reductase, respectively. Such observations have led them to suggest that NarJ is part of the redox enzyme maturation protein (REMP) family of chaperones that include DmsD, TorD, and YcdY [17]. We sought to investigate whether NarJ had further functional similarities to the REMP family of proteins such as binding towards the N-terminal region of NarG in a similar manner to those of DmsD and TorD towards DmsA and TorA, respectively [18,19].

Through Far Western experiments, we found that NarJ binds the N-terminal peptide region of NarG in both its native and denatured form. This binding event was confirmed to be towards residues 1–15 of NarG, which contains the twin-arginine remnant motif. We also show that NarJ exhibits Tat-dependence in terms of its cellular localization where fractionation experiments show that its membrane association is partially disrupted in a Δ*tatABCD/E* strain. Upon further investigation using Far Western blots where membrane fractions from wildtype (WT) and Δ*tat* cells were probed with NarJ acting as the "primary antibody," differences in the binding pattern were observed. Based on the above findings, we hypothesize that NarJ may be acting in a similar fashion as DmsD and TorD for nitrate reductase A maturation.

Materials and methods

Constructs and growth conditions. The gene sequence corresponding to the first 50 amino acids of the N-terminus of NarG, $NarG_{50}$, was isolated through amplification of *E. coli* HB101 [20] genomic DNA using the primers TDMS-76 (5′-ATATCCATGGCTAGTAAATTCCTGGACC-3′) and TDMS-77 (5′-ATATGGTACCAATGGATCCGTGGGTAGAGCG GACG-3′) where the underlined sequences correspond to restriction enzymes sites *Nco*I, *Kpn*I, and *Bam*HI, respectively. The PCR product of $narG_{50}$ and the vector pBEc-SBP-SET1 (Stratagene) were digested with *Nco*I and *Kpn*I (Invitrogen) for 1 h at 37 °C. Digested $narG_{50}$ was then ligated into pBEc-SBP-SET1 at an insert-to-vector ratio of 3:1 using T4 DNA Ligase (Invitrogen) at 4 °C overnight. The resulting recombinant plasmid, pTDMS66, was then transformed into *E. coli* C41(DE3) [21] competent cells via heat shock [20] and then verified by sequencing at the University Core DNA and Protein Services (University of Calgary, Calgary, AB, Canada).

pTDMS43, NarJ containing a N-terminal His_6 and T_7 epitope fusion, was generated into pRSET(A) in a similar fashion as before [18] using the primers TDMS-11 (5′-ATATAAGCTTAAGGAGTTGATCAATGATC GAACTCGTGATTGTAT-3′) and TDMS-12 (5′-ATATCTGCAGGAA TTCTTAGTGCTGTCCTCCGGT-3′) where the underlined sequences correspond to restriction enzyme sites *Hind*III, *Bcl*I, *Pst*I, and *Eco*RI, respectively.

All aerobic cultures were done in Luria–Bertani media containing 100 μg/mL ampicillin where 1% v/v overnight sub-cultures were used for inoculation. In cases where protein expression was required, cultures were grown at 37 °C until they reached an OD_{600} of 0.4–0.6, then induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and allowed to grow for a further 3 h. Anaerobic cultures were grown by adding 0.2% v/v aerobic starter culture to either glucose peptone fumarate media or glycerol nitrate media [22] supplemented with 0.3% w/v glucose or 0.5% v/v glycerol, 100 μg/mL ampicillin, and 0.002% w/v vitamin B1, and then allowing the cultures to grow at 37 °C for 48 h.

Cell harvest and subcellular fractionation. Cells were harvested by centrifugation at 2700*g* and then resuspended in 2:1 volume:wet pellet weight of appropriate buffer (see sections below). Cells were lysed either by two passes through a French pressure cell at 16,000 psi or sonication (5 × 5 s pulses) following treatment with 10 mg/mL lysozyme. The cell lystate was clarified by centrifugation at 4000*g* for 30 min to remove unlysed cells and debris, and then the cell-free extract was subjected to ultracentrifugation at 120,000*g* for 1.5 h to generate cytoplasmic and membrane fractions. Solubilization of membranes was done through incubation with 2% w/v CHAPS on ice for 2 h while periodically vortexing, followed by ultracentrifugation at 250,000*g* for 40 min to remove insoluble material to generate a solubilized membrane fraction.

Protein methods. Protein concentrations were determined by a modified Lowry method [23]. Protein separation was done by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with 12% T separating and 5% stacking components. Transfer of proteins to nitrocellulose was done through electroblotting and blocked with skim milk. Western blot detections were done by incubation of the specified antibody at room temperature for 1 h. Far Western blots were incubated with the specified amount of His_6-T_7:NarJ purified as in [24] for 2 h followed by incubation with 1:5000 T7 Tag horseradish peroxidase (HRP) Conjugate (Novagen) for 1 h. The detection of HRP-conjugated antibodies was done through colorimetry using a HRP Conjugate Substrate Kit (Bio-Rad).

Growth rate dependence on media and Tat translocon. Anaerobic cultures of *E. coli* MC4100 [25] or DADE [26] were grown in either glucose peptone fumarate or glycerol nitrate media. Cultures were grown in sealed 9 mL glass tubes on a rocker placed inside a 37 °C incubator while monitoring the OD_{600}.

NarG N-terminal peptide binding to NarJ. Aerobic cultures of *E. coli* C41(DE3) cells and cells carrying pTDMS66 or pBEc-SBP-SET1 were harvested and resuspended in buffer A (25 mM NaH_2PO_4, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), and 0.1 mM phenylmethylsulfonylfluoride (PMSF)) and then lysed by sonication. Twenty-five micrograms per millilitre of total protein from the clarified cell-free extract was tested for binding through a Far Western in its denatured form through separation by SDS–PAGE then transferring to nitrocellulose membrane. Binding to the native form of $NarG_{50}$ was done by applying cell extracts directly onto nitrocellulose membrane through the use of a Bio-Dot Microfiltration Apparatus (Bio-Rad). The blots were incubated with 15 μg/mL His_6-T_7:NarJ and then with T7 Tag HRP Conjugate.

Purified His_6-T_7:NarJ was dialyzed against buffer B (25 mM MOPS and 100 mM NaCl) overnight and its concentration was determined immediately prior to performing isothermal titration calorimetry (ITC). Ligand peptide corresponding to amino acids 1–15 of NarG, $NarG_{1–15}$, was custom synthesized by University Core DNA and Protein Services (University of Calgary, Calgary, AB, Canada). $NarG_{1–15}$ peptide was dissolved into buffer B and used at a working concentration of 200 μM. All samples were de-gassed for 5 min at room temperature in a thermovac before loading. Titrations were performed on a VP-ITC titration calorimeter (MicroCal) with injections of peptide from syringe with a volume of 293 μL while stirring at 310 rpm at 23 °C. Curve fitting and thermodynamic parameters were calculated with the ORIGIN software provided by MicroCal.

Cellular localization of NarJ. Uninduced anaerobic cultures (in glucose peptone fumarate media) of *E. coli* MC4100 [25] or DADE [26] carrying pTDMS43 were harvested and resuspended in buffer C (20 mM Tris-HCl, pH 7.9, and 1 mM DTT) and then lysed through French press. Twenty-five micrograms of total protein from cytoplasmic and membrane fractions was separated by SDS–PAGE, transferred to nitrocellulose membrane and then incubated with T7 Tag HRP Conjugate. For comparisons, the pixel densities of each lane were determined and compared using KODAK Gel Logic 100 Software and calculated as in [27].

Interactome of NarJ. Aerobic and anaerobic cultures (in glucose peptone fumarate) of *E. coli* MC4100 [25] and DADE [26] were harvested and resuspended in buffer D (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM DTT, and 0.1 mM PMSF) and then lysed through French press. Thirty micrograms of total protein from solubilized membranes was separated by

SDS–PAGE, transferred onto nitrocellulose and then incubated with 40 μg/mL His_6-T_7:NarJ and then with T7 Tag HRP Conjugate.

For Ni^{2+}-affinity experiments, a similar protocol to [28] was followed where 45 μg of purified His_6-T_7:NarJ per μL of resin was immobilized to Ni^{2+}–NTA agarose (Qiagen). The untreated or treated resin was then incubated with 12 mg total protein per mL resin from solubilized membranes isolated from anaerobically grown (in glucose peptone fumarate media) *E. coli* HB101 [20]. Eluted proteins were separated via SDS–PAGE and proteins determined to bind NarJ specifically were cut out of the gel and then subjected to proteolytic digestion with trypsin overnight. The resulting peptide fragments were identified by surface-enhance laser desorption ionization time-of-flight (SELDI-TOF) mass spectrometry using SwissProt database from Mascot [29].

Results

NarJ binding towards the N-terminal region of NarG

If NarJ was indeed a chaperone of similar functions with DmsD, then one would expect that NarJ interacts with the N-terminal region of NarG, which contains the vestige twin-arginine motif, in a similar manner that DmsD interacts with the leader peptide of DmsA [18]. A previous study from our laboratory has shown far Western blotting to be a useful assay in demonstrating the binding of DmsD. Purified recombinant DmsA-leader:GST was spotted onto membranes, which was subsequently incubated with purified His_6-T_7:DmsD or cell extracts containing overexpressed His_6-T_7:DmsD, then detected using an antibody to the T_7 epitope on DmsD [24]. As no cleaved leader sequence exists in NarG, the first 50 residues at the N-terminus were designated as the N-terminal peptide of NarG, $NarG_{50}$ (**MSKFLDRFRYFKQKG**ETFADGHGQ LLNTNRDWEDGYRQRWQHDKIVRSTH, where the vestige twin-arginine motif is underlined and the 15 amino acid peptide used in the following ITC experiment is bolded). The gene sequence corresponding to $NarG_{50}$ was cloned with a C-terminal fusion to a streptavidin-binding peptide (SBP) and a Solubility Enhancement Tag (SET1) to generate $NarG_{50}$:SBP–SET1. To determine binding of $NarG_{50}$ to NarJ, a far western similar to that described above was performed using cell extracts containing overexpressed $NarG_{50}$:SBP–SET1 and purified His_6-T_7:NarJ. The interaction between the two was then detected using an antibody against the T_7 epitope on NarJ, while purified His_6-T_7:NarJ was included as a binding signal control against T7 Tag HRP Conjugate. The far western from separation using SDS–PAGE shows that NarJ binds $NarG_{50}$ in its denatured form and that binding is not due to interaction with the SBP–SET1 tag or a side effect of the *E. coli* expression strain used (Fig. 1A). We also tested the binding against the native form of $NarG_{50}$ by repeating the experiment using cell extracts spotted directly onto the membrane and the results were similar to that of the denatured form (Fig. 1B). Additionally a weak interaction between NarJ and a ~32 kDa protein from extracts expressing only the vector was observed (Fig. 1A, lane 2), suggesting this may be the reason for the weak interaction observed in the Native dot blot (Fig. 1A, spot 2). This observation suggests that the interaction between NarJ and NarG observed in previous experiments [7] is due to the binding of NarJ towards the N-terminal peptide region of NarG and confirms recent observations made in Vergnes et al. [30].

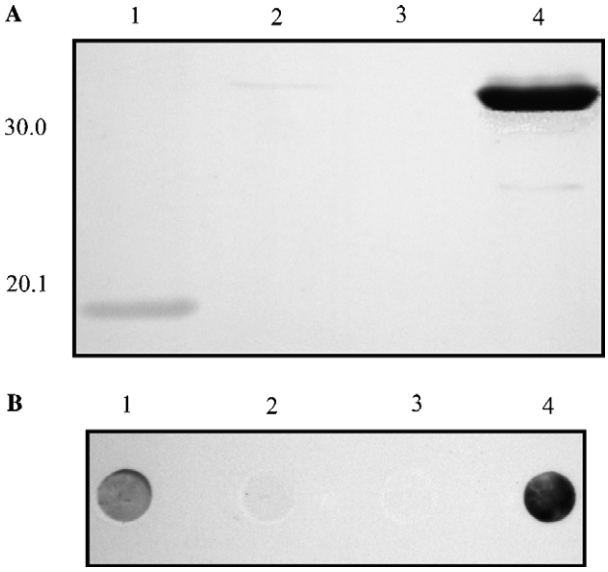
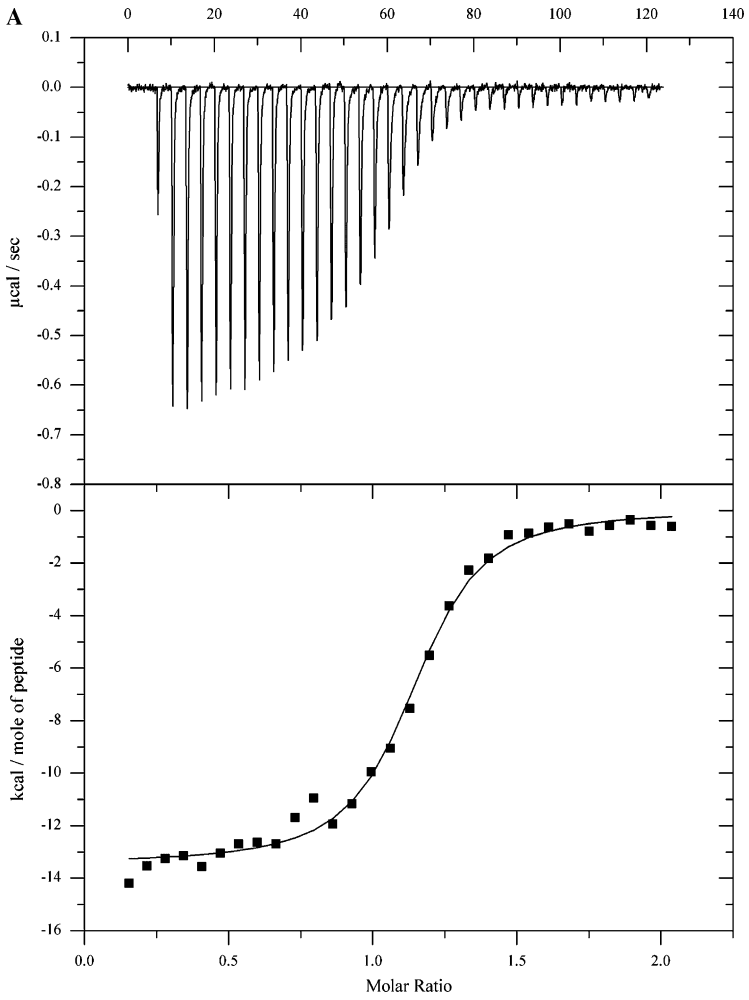


Fig. 1. NarJ binds the N-terminal peptide of NarG, $NarG_{50}$. Far Western blot of clarified cell-free extracts probed with His_6-T_7:NarJ. Lane/spot 1, cells overexpressing $NarG_{50}$:SBP-SET1; lane/spot 2, cells overexpressing SBP–SET1; lane/spot 3, cells with no vector/plasmid; lane/spot 4, 1.5 μg of purified His_6-T_7:NarJ. (A) Far western on denatured proteins from extracts separated by SDS–PAGE. Sizes of protein markers (in kDa) are indicated to the left. (B) Far Western dot blot on native proteins from extracts.

A recent study by Sargent et al. [31] showed that binding of the TMAO reductase chaperone, TorD, towards a variety of N-terminal peptides of the catalytic subunit, TorA, was dependent on the presence of the "SRRRFLA" twin-arginine motif. As NarJ was predicted to be a member of the REMP family consisting of DmsD, YcdY, and TorD previously by our group [17], we wanted to use similar ITC experiments to show binding of NarJ towards a short peptide consisting of residues 1–15 of NarG, $NarG_{1–15}$. The synthetic peptide $NarG_{1–15}$ was injected into purified His_6-T_7:NarJ and the resulting thermogram showed that the peptide binds NarJ (Fig. 2A). An average of 3 runs gave a dissociation constant (K_D) of 0.164 ± 0.024 μM with an n value of 0.92 indicating 1:1 binding (Fig. 2B). The $NarG_{1–15}$ peptide contains the sequence "MSKFLDRFRYFKQKG" (the remnant twin-arginine motif as identified by Turner et al. [17] is underlined), suggesting that the binding of NarJ towards the N-terminus of NarG observed above and in [30] is likely due to binding towards this homologous motif. The binding observed here appears to be tighter than that observed for TorD towards the TorA N-terminal peptide of 1.7 ± 0.3 μM [31], yet still suggesting that NarJ is of the same family of chaperones encompassing DmsD, TorD, and YcdY.



B

K_D (µM)	n	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})	ΔG (kcal mol^{-1})
0.16 ± 0.02	0.9 ± 0.2	-17.5 ± 3.7	-28.0 ± 12.4	-9.2 ± 0.1

Fig. 2. Isothermal titration calorimetry of $NarG_{1–15}$ peptide injected into NarJ. (A) Representative titration obtained with 8 µL injections into a 1.43 mL cell containing either 14 µM NarJ or buffer. Heats of dilution were subtracted from the heats collected for the corresponding experiment. The resulting integrated heats were plotted as a function of molar ratio and from this curve the thermodynamic parameters were calculated. (B) Thermodynamic data obtained from three runs in total.

NarJ dependence on the Tat translocon

As the *narGHIJ* operon is preferentially expressed under anaerobic conditions and is induced in the presence of nitrate [32,33], we compared the growth rates of WT and Δ*tat E. coli* strains grown anaerobically in glucose peptone fumarate and glycerol nitrate media. Cells grown in glucose peptone fumarate would rely on fumarate reductase for respiration, which is not expected to be dependent on the Tat system [22]. The cells grown in glycerol nitrate media would rely on nitrate as a final electron acceptor, and since high levels of nitrate were included in the media, this would promote the expression of the *narGHIJ* operon rather than the *napFDAGHBC* operon [34]. In comparison, the doubling time of WT was approximately 2.7 times faster than the Δtat strain in glycerol nitrate media whereas growth rates were approximately equal in glucose peptone fumarate media. These data suggest a dependence on the Tat translocon in media conditions that promote the expression of the *narGHIJ* operon.

Cellular localization of NarJ and the effect of the Tat translocon on its localization were investigated using the plasmid pTDMS43 (generating His_6-T_7:NarJ). WT and *Δtat* cells harboring this plasmid were grown anaerobically under uninduced conditions allowing for the accumulation of His_6-T_7:NarJ at low levels occurring only from the leakiness of the promoter. Following cellular fractionation, cytoplasmic and membrane fractions were examined for the presence of His_6-T_7:NarJ through a Western blot against the T_7 epitope. The Western blot showed that NarJ was solely localized to the membrane fraction in WT cells, yet ~26% of it was lost to the soluble fraction in the Δ*tat*

strain when the image pixel densities were compared (Fig. 3), indicating that the cellular location of NarJ is partly dependent on the Tat translocon.

To further explore if NarJ has a dependence on the Tat translocon, we also investigated the ability of NarJ to interact with different proteins in the presence and absence of the Tat proteins and under varied oxygen availability. This was done through Far Western blotting where solubilized membrane fractions isolated from WT and Δ*tat E. coli* grown aerobically and anaerobically were incubated with His_6-T_7:NarJ and then detected using an antibody against the T_7 epitope on NarJ. Upon comparison, it was evident that the binding profile of NarJ in the membrane fractions were dependent on the Tat translocon and oxygen availability (Fig. 4). We then sought to determine the identities of the unique proteins observed in the above experiments using an in vitro Ni^{2+}-affinity-based chromatography technique where recombinant His_6-tagged proteins were used as "bait" to fish out potentially interacting proteins [28]. Purified His_6-T_7:NarJ was immobilized to Ni^{2+}–NTA resin and interacting proteins were "fished out" by passing CHAPS-solubilized membranes isolated from anaerobically grown *E. coli* HB101 cells and compared via SDS–PAGE analysis to a control where no NarJ was immobilized (results not shown). The proteins determined to be specifically interacting with NarJ were cut out of the gel and subjected to tryptic digestion, and then peptide masses were determined through SELDI-TOF mass spectrometry and identified by Mascot [29]. One protein was significantly identified to be NarG from *E. coli* with a *p*-value less than 0.05, while the other interacting proteins did not return any significant identities. This observation shows that NarJ is able to bind NarG from membrane fractions in vitro and that NarG must be in its mature and form because the membranes used were from WT *E. coli* where all of the essential holoenzyme-forming components were present.

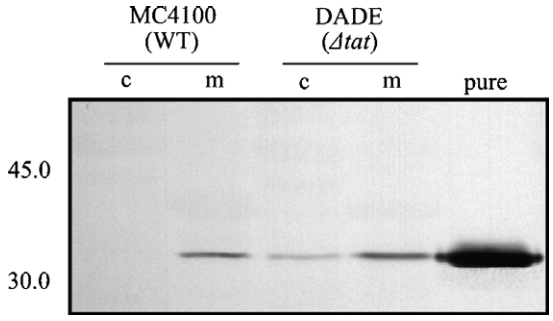


Fig. 3. Cellular localization of NarJ. Western blot of cytoplasmic (c) and membrane (m) fractions isolated from *E. coli* MC4100 and DADE expressing the recombinant His_6-T_7:NarJ. 1.5 μg of purified His_6-T_7:NarJ was also included as a control (pure). Blots were probed with T7 Tag HRP Conjugate against the T_7 epitope on NarJ. Sizes of protein markers (in kDa) are indicated to the left.

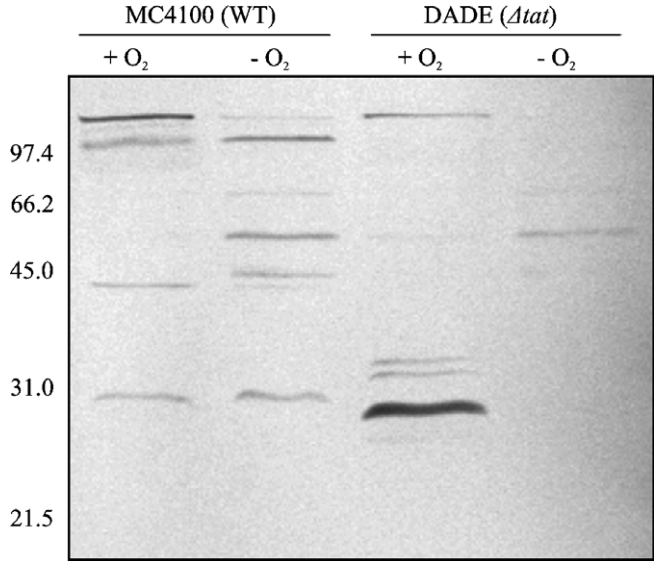


Fig. 4. The interactome of NarJ. Far Western blot of solubilized membranes from anaerobically grown *E. coli* MC4100 and DADE cells in the presence and absence of oxygen probed with His_6-T_7:NarJ, followed by T7 Tag HRP Conjugate against the T_7 epitope on NarJ. Sizes of protein markers (in kDa) are indicated to the left.

Discussion

It was demonstrated in this study that binding of *E. coli* NarJ towards the N-terminal region of mature NarG is towards the first 15 amino acids of its sequence. This region contains a homologous sequence towards the twin-arginine motif, and was described by Turner et al. [17] as a 'vestige' motif. The findings here support the observations seen here and in [30], where the first 50 or 40 amino acids of NarG, respectively, were found to bind NarJ, and that the minimal binding sequence consists of the first 15 residues. This could potentially explain our observation that NarJ binds mature/active NarG at the membrane, because this N-terminal sequence is not cleaved in the mature protein. While previous experiments have shown NarJ binding to NarG, these were done using soluble fractions indicating that it occurred in the cytoplasm where the protein is not in its holo-form or from crude extracts meaning that the maturity state of the protein cannot be distinguished [7,30]. Nonetheless, the binding towards the twin-arginine homologous motif-containing region suggests that NarJ behaves in a similar manner to the REMP chaperones that are involved in targeting and maturation of a family redox enzymes that are dependent on the twin-arginine translocation system.

The observation that NarJ exhibits localization dependency on the Tat translocon was similar to that of DmsD [27], further suggesting that it is related to the DmsD/TorD family of chaperones. In the above-described study, DmsD was found to exhibit Tat-dependence in its membrane association and this interaction was dependent on the TatB and TatC subunits of the translocon. Due to the relatedness in function, it is possible that NarJ associates with the membrane through similar interactions shown with DmsD. The cellular localization of NarJ observed here is opposite to that observed previously,

where similar Western blotting experiments were performed on recombinantly over-expressed NarJ in a wildtype strain of *E. coli* which showed its localization was mostly in the cytoplasm [8]. Since this previous study was performed by loading equivalent volumes of the cytoplasmic and membrane fractions rather than equivalent amounts of total protein as in our study, the volumes loaded by their study may have been too dilute to detect NarJ in the membrane fraction. Another explanation could be a difference in experimental conditions where cellular harvest was earlier in the growth phase in [8] whereas ours were later at 48 h. Together with the Tat dependence of the NarJ interactome observations, it appears that the presence of the translocon has a definite but not absolute role in the functions of NarJ.

The homologous $NarJ_T$ from *Thermus thermophilus* [35] was found to be required for membrane attachment to the $NarCI_T$ complex along with its requirement for the maturation of $NarG_T$. While there does not appear to be experimental proof that *E. coli* NarJ is involved in membrane attachment to date, the difference observed with the $NarJ_T$ could be attributed to the fact that membrane attachment is required prior to molybdenum cofactor insertion in *T. thermophilus*, a sequence that appears to be reversed in *E. coli* [7]. Since active NarGH containing its molybdenum cofactor results in active NarGHI holocomplex at the membrane, it is likely that NarJ's involvement in membrane attachment to NarI has been overlooked or is unable to be tested with the knowledge to date as one would need to develop a method to retard maturation/targeting following cofactor insertion. As it appears from our studies, NarJ seems to behave similar to other members of the same chaperone family of DmsD and TorD, and likely has similar functions towards the maturation of the NarGH complex. Upon further investigation, we also noticed that the N-terminal 15 residues of NarG remain exposed and face the membrane bilayer in the mature holoenzyme (Fig. 5), further supporting the idea that NarJ can remain bound to NarGH at the membrane. While the current dogma defines Tat as a system to be utilized for targeting and translocation of periplasmically localized proteins, a previous study has suggested that the translocon also plays a role in membrane protein insertion [36]. Furthermore, despite the fact that DmsAB of DMSO reductase have been suggested/shown to be periplasmically facing [11,37], previous studies have maintained that it is located at the cytoplasmic side of the membrane [38,39].

From the observations made here, we propose the hypothesis that NarJ potentially utilizes the Tat system to aid NarGH attachment towards NarI. As mentioned previously, our findings that the N-terminal 15 residues of NarG are involved in the binding towards NarJ, confirming recent results by Vergnes et al. [30]. Interestingly, the results shown by Vergnes and colleagues also show that there is at least one addition-binding site towards NarJ as binding was still observed with a mutant where the N-terminal 40 residues of NarG were deleted. It is possible that the other binding site(s) are involved in a complex interaction that links NarJ and NarG with the molybdenum cofactor biosynthetic proteins as described in [40], whereas the interaction with the N-terminus twin-arginine homologous motif-containing region is for membrane targeting.

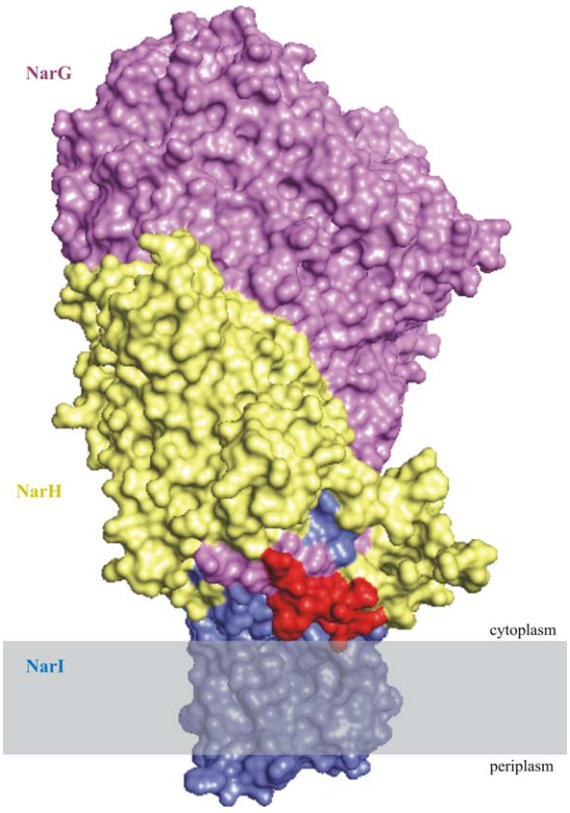


Fig. 5. Structural model of the NarGHI complex in the membrane bilayer (PDB No. 1Q16). The individual subunits are colored as violet (NarG), yellow (NarH), and blue (NarI), with residues 1–15 of NarG highlighted in red. The model demonstrates that even in the folded, mature form, the region containing the homologous twin-arginine motif remains exposed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

A potential model can be proposed from this where NarJ interacts with the undefined binding site(s) of NarG during cofactor insertion to hold it in a cofactor-accepting state. Following insertion, it then binds to the twin-arginine homologous motif-containing N-terminus of NarG and guides NarGH towards NarI at the cytoplasmic membrane through the aid of the Tat translocon. NarJ then donates the complex to NarI through an undefined mechanism after membrane attachment, allowing for the formation of the holoenzyme complex.

Acknowledgments

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