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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