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Influence of GTP on system specific chaperone – Twin arginine signal peptide interaction

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ABSTRACT

Many bacterial respiratory redox enzymes depend on the twin-arginine translocase (Tat) system for translocation and membrane insertion. Tat substrates contain an N-terminal twin-arginine (SRRxFLK) motif serving as the targeting signal towards the translocon. Many Tat substrates have a system specific chaperone – redox enzyme maturation protein (REMP) – for final folding and assembly prior to Tat binding. The REMP DmsD strongly interacts with the twin-arginine motif of the DmsA signal sequence of dimethyl sulfoxide (DMSO) reductase. In this study, we have utilized the *in vitro* protein–protein interaction technique of an affinity pull down assay, as well as protein thermal stability measurement via differential scanning fluorimetry (DSF) to investigate the interaction of guanosine nucleotides (GNPs) with DmsD. Here we have shown highly cooperative binding of DmsD with GTP. A dissociative ligand-binding style isotherm was generated upon GTP titration into the DmsD:DmsAL interaction, yielding sigmoidal release of DmsD with a Hill coefficient of 2.09 and a dissociation constant of 0.99 mM. DSF further illustrated the change in thermal stability upon DmsD interaction with DmsAL and GTP. These results imply the possibility of DmsD detection and binding of GTP during the DMSO protein maturation mechanism, from ribosomal translation to membrane targeting and final assembly. Conceivably, GTP is shown to act as a molecular regulator in the biochemical pathway.

1. Introduction

Bacterial anaerobic respiration is possible through a variety of substrates acting as terminal electron donors/acceptors for respiration, permitting energy generation in anoxic environments. Oxidation/reduction reactions are catalyzed by a menaquinone electron transfer redox loop between the periplasm and cytoplasmic membrane of the host bacterium [1]. The primary respiratory molybdoenzyme superfamily utilizes dimethyl sulfoxide (DMSO), trimethylamine-N-oxide (TMAO) or nitrate as terminal electron acceptors, all containing a GTP derived molybdenum-bis(pyranopterin guanine dinucleotide) cofactor in the active site of the mature catalytic subunit [2,3]. Iron-sulfur cluster carriers are also commonly associated to facilitate electron transfer [4]. Such complexity observed in iron-sulfur molybdoenzymes subsequently led to the term CISM [5].

The transport of folded proteins across the inner membrane of bacteria requires the use of the twin-arginine translocase (Tat) system, with substrates containing an N-terminal twin-arginine (SRRxFLK) motif as the targeting signal [6–8] on one of the subunits. The CISMs are Tat substrates and their subunits are individually folded and final assembly with cofactors completed prior to translocation [9]. The CISM DMSO reductase is heterotrimeric in nature, consisting of DmsABC subunits in the final oligomeric structure [2,10]. DmsA, the catalytic subunit, contains the molybdopterin cofactor, while DmsB contains four [4Fe–4S] clusters to assist in electron transfer. DmsC acts as the membrane anchor subunit facilitating DmsAB connection to the quinone pool [11].

Redox enzyme maturation proteins, REMPs, are critical for leading a nascent polypeptide chain through a pathway from ribosomal translation to folding and cofactor insertion to membrane

Abbreviations: Bis-MGD, molybdenum-bis(pyranopterin guanine dinucleotide); CISM, complex iron-sulfur molybdoenzyme; DmsAL, DmsA leader peptide; DMSO, dimethyl sulfoxide; DSF, differential scanning fluorimetry; GST, Glutathione S-transferase; Q RT-PCR, quantitative real time-polymerase chain reaction; REMP, redox enzyme maturation protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tat, twin-arginine translocase; Tm, melting temperature.

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targeting. This described maturation of respiratory redox enzymes is required prior to Tat translocation [12]. Previous research has shown that the specificity between the REMP and its substrate is maintained and absolutely required for final assembly, translocation and insertion [13]. The NarJ- REMP superfamily (DmsD, TorD, YcdY and NarJ) has been studied the most extensively [14,15]. *Escherichia coli* DmsA contains the N-terminal RR-motif in its signal sequence region (DmsA leader (DmsAL)). This region is required for DmsD binding facilitating DMSO reductase folding and cofactor insertion [16]. The leader peptide consists of a highly conserved pair of arginines in the N-terminal region, a central hydrophobic region, and a polar, peptidase cleavage site including C-terminal region [17]. Previous research has established that the hydrophobic region of the leader peptide is the most significant module to confer binding between the Tat substrate and DmsD [18]. Further, micromolar affinity exists between DmsAL and DmsD, with amino acids crucial in binding and specificity clustered within a DmsD hydrophobic pocket (Fig. 1, green) [18–20].

The domain-swapped dimer of TorD [21], a DmsD functional homologue responsible for the maturation of the molybdoenzyme TorA [22], showed an increase in GTP affinity following TorA ligand binding [23]. Further, *in vitro* magnesium dependent GTPase activity was detected [24]. A putative GTP binding site was then modeled onto the structures of *St*DmsD [25] and *Sm*TorD [23], with further alignment mapping such residues onto *Ec*DmsD (Fig. 1, red) [25]. The predicted GTP binding sites between *Ec*DmsD and *Sm*TorD are located on opposite sides of these proteins, suggesting differential utilization or roles of GTP [15].

Currently the DMSO reductase maturation pathway comprised of cofactor insertion, final folding and assembly, consists of steps of mere speculation suggested by protein–protein interactome mapping [26]. A key question in the pathway is what is the trigger and mechanism of DmsD to DmsA leader peptide interaction release prior to handoff to Twin arginine translocon. There has also been no identified function for DmsD GTP binding to date. We therefore sought to explore the interaction between DmsD and the twin-arginine leader peptide of DmsA under a range of GNP concentrations using both a synthetic $DmsAL_{15-41}$ peptide and C-terminal Glutathione S-transferase (GST) fused DmsAL chimeric protein as an effective surrogate substrate. Here we observe a role of GTP in regulating the DmsD to DmsAL interaction.

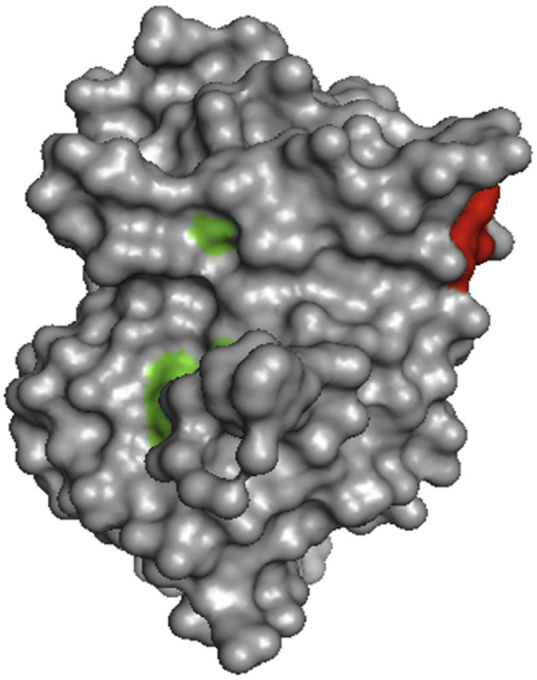

Fig. 1. DmsD structure highlighting locations of DmsAL peptide and GTP interaction sites. Location of *E. coli* DmsD (PDB ID:3EFP) DmsAL RR-binding site (green) determined experimentally [19] and putative GTP binding site (red) modeled from *S. typhimurium* DmsD by structure alignment [25]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2. Materials and methods

2.1. Protein and peptide preparation

DmsD WT, $DmsAL_{1-43}$:GST and $DmsAL_{15-41}$ peptide samples were expressed, purified and/or prepared as described previously [18,19].

2.2. Differential scanning fluorimetry

Assessment of DmsD protein melting temperature was sensed using a fluorescent probe approach. DSF technique allows for the evaluation of thermal melting of a protein, quantitatively determining Tm and corresponding thermal stability. A highly sensitive fluorescent dye detects protein denaturation by binding newly exposed hydrophobic residues upon temperature increase. Increase or decrease in thermal stability was indicated via ΔTm. Purified DmsD sample was thawed, centrifuged (10500 × g, 4 °C, 30 min), molarity determined via Bradford assay (BioRad) [27] and diluted to 50 μM. Each trial was performed as follows: Thirty-six 200 μl PCR Eppendorf tubes were filled with DSF Buffer pH 8.0 (12.5 mM Tris–HCl pH 8.0, 50 mM NaCl, 0.5 mM DTT), 10 μL DmsD (50 μM stock, 20 μM final concentration) and 5 μL synthetic $DmsAL_{15-41}$ peptide (250 μM stock, 50 μM final concentration). Assay vessels were incubated (15 min, 25 °C) and 5 μL (25×) SYPRO Orange (Sigma S5692) added directly prior to assaying. For trials in which GTP was included, DSF Buffer pH 8.0, 10 μL DmsD protein and aliquots of GTP stock solution (25 and 10 mM) was added. The pH was maintained at 8.0. Assay vessels were incubated (15 min, 25 °C) and 5 μL SYPRO Orange (Sigma S5692) added. A Rotor-Gene Quantitative Real Time – PCR (Q RT-PCR) machine (QIAGEN) was used to monitor the change in fluorescence intensity of the fluorophore during temperature ramping. The temperature was increased from 25 to 90 °C with 0.2 °C increments at 5 s intervals with a Gain of 6. Samples were excited at 470 nm and emission collected at 555 nm.

Each DSF experiment produced a thermal melt curve, with midpoint in fluorescence intensity corresponding to 50% unfolded protein sample. The first derivative of the averaged melting curve produced a peak maximum, providing the Tm. Data reported are the mean and standard deviation from the mean of at least 3 technical replicates from 3 experimental replicates.

2.3. Glutathione sepharose DmsD dissociation assay

Affinity resin was used to facilitate protein–protein interaction pull down assays. A 50% slurry of Glutathione Sepharose 4B beads (GE Healthcare Life Sciences) was prepared with GST bind buffer (50 mM Tris–HCl pH 8.0, 200 mM NaCl, 5 mM DTT). Purified DmsD and DmsAL:GST protein samples were prepared as in DSF protocol and DmsAL:GST added to saturate at 5 μg/μL and incubated (1hr, 4 °C) with gentle end-over-end rotation. Unbound DmsAL:GST concentration was determined via A_{280} measurement following sedimentation by centrifugation (500 × g, 5 min) and supernatant removal. DmsD protein was added in 2:1 ratio of leader peptide:REMP, incubated (15 min, 4 °C), sedimentation by centrifugation and released protein measured at A_{280}. DmsD was not added to a control tube, ensuring increase in A_{280} reading was not due to DmsAL:GST release from the resin.