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Unusual pairing between assistants: Interaction of the twin-arginine system-specific chaperone DmsD with the chaperonin GroEL

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ABSTRACT

DmsD is a system-specific chaperone that mediates the biogenesis and maturation of DMSO reductase in *Escherichia coli*. It is required for DmsAB holoenzyme formation and its targeting to the cytoplasmic membrane for translocation by the twin-arginine translocase. Previous studies suggested that DmsD also interacts with general molecular chaperones to assist in folding of the reductase subunits. Here, the interaction between DmsD and GroEL was further characterized to understand the role of GroEL in DMSO reductase maturation. The inherently weak interaction between the two was strengthened *in vivo* under growth conditions that induce DMSO reductase expression, and the DmsD–GroEL complex showed negligible change in hydrodynamic diameter by dynamic light scattering when cross-linked. Mapping the cross-linked sites on DmsD shows that the GroEL binding site is in close proximity to the previously characterized DmsA leader binding site. These findings support a role of GroEL in DMSO reductase maturation that likely involves its chaperonin function for assisting in folding of the DmsA preprotein.

1. Introduction

DMSO reductase is one of many redox enzymes that allow bacteria to respire in anaerobic environments. In the facultative model organism *Escherichia coli*, it forms a redox loop with various hydrogenases/dehydrogenases through the quinone pool (consisting of menaquinone, ubiquinone, and demethylmenoquinone) in the cytoplasmic membrane, receiving electrons to reduce DMSO to DMS while contributing to the proton motive force (reviewed in Ref. [1]). Similar to other redox enzymes involved in anaerobic respiration, DMSO reductase is targeted and translocated across the cytoplasmic membrane by the bacterial Tat system [2,3].

In *E. coli*, it has been demonstrated that the biogenesis of DMSO reductase requires the accessory protein DmsD [4,5], which is a member of the NarJ REMP subfamily of complex iron-sulfur molybdoenzyme chaperones [6]. DmsD binds the conserved twin-arginine leader peptide of the cofactor-containing catalytic subunit DmsA of the reductase [4], likely to assist in cofactor loading and folding as these are general roles described for REMPs [7]. This hypothesis is supported by a study showing *in vitro* and *in vivo* interactions between DmsD and proteins involved in general protein folding and those for catalytic cofactor biosynthesis [8]. One of the identified interactors was GroEL, the general molecular chaperone belonging to the chaperonin family with homologues found in both prokaryotes and eukaryotes. The function of GroEL is to assist in protein folding by providing a large protective and hydrophilic cavity for *ab initio* or unfolding/refolding of protein substrates (reviewed in Ref. [9]).

Here we characterized interaction between *E. coli* DmsD and GroEL. We found that the interaction between the two is weak *in vitro* and *in vivo*, but is enhanced *in vivo* under anaerobic growth conditions. We were able to trap the DmsD–GroEL complex using a photo-activatable cross-linker to study it by dynamic light scattering and found that the hydrodynamic diameter of the complex is similar to that of GroEL. By taking advantage of the label-transfer and cleavability of the cross-linker, we were able to map the binding surfaces of GroEL on DmsD and *vice versa*. Upon mapping the GroEL-binding region on the structure of DmsD, we find that this region is in close proximity to the known binding site of the DmsD

Abbreviations: DMSO, dimethylsulfoxide; Tat, twin-arginine translocase; REMP, redox enzyme maturation protein; DLS, dynamic light scattering.

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substrate DmsA leader. Our findings implicate DmsD as an adaptor to connect DmsA with GroEL for the purpose of assisting in the folding of DmsA and DMSO reductase biogenesis.

2. Methods

2.1. In vivo BACTH assay

An *in vivo* bacterial two-hybrid (BACTH) assay was performed exactly as described in Ref. [10] for DmsD, GroEL, and DmsA leader except duplicate cultures were also grown anaerobically in minimal DMSO/glycerol medium [11] for 72 h at 30 °C.

2.2. Protein procedures

Recombinant DmsD was expressed and purified as described [10]. Recombinant GroEL with an N-terminal His_6 tag was obtained from the ASKA library [12], and expressed and purified similarly to DmsD by Ni^{2+}-affinity chromatography eluting with a linear gradient of 0–250 mM imidazole. Fractions containing GroEL were pooled and exchanged into buffer A (25 mM Tris–HCl pH 7.4, 100 mM NaCl, 1 mM DTT), and then further purified by ion exchange chromatography using a HiTrap Q 1 ml column (GE Life Sciences) and eluted over 15 column volumes with buffer B (25 mM Tris–HCl pH 7.4, 1.2 M NaCl, 1 mM DTT). Fractions containing pure GroEL were pooled and exchanged into buffer A.

For cross-linking experiments, purified DmsD and GroEL were dialyzed into BupH™ PBS (Thermo Scientific) prior to labeling. Their concentrations were measured following dialysis using a Bio-Rad Protein Assay (Bio-Rad) and then adjusted to 50 μM. They were mixed with 10-fold molar excess of sulfo-SBED (Thermo Scientific) and incubated at 22 °C for 50 min, and then dialyzed into label transfer buffer (50 mM HEPES, pH 7.4, 150 mM NaCl). Prior to cross-linking, unlabeled DmsD or GroEL were dialyzed into label transfer buffer and then mixed at 12.5-to-1 M ratio DmsD-to-GroEL (assuming GroEL is a 14-mer of ~798 kDa) and incubated at 22 °C for 30 min. The aryl azide group was photo-activated using a handheld long wave UV lamp (365 nm) at 5 cm distance for 30 min on ice.

Prior to analysis by mass spectrometry, samples were dialyzed into buffer containing 50 mM ammonium bicarbonate, pH 7.8 overnight and then subjected to two treatments prior to mass spectrometry. Treatment 1 consisted of reduction and alkylation of the samples followed by tryptic digestion and affinity purification of biotin labeled peptides, whereas Treatment 2 involved tryptic cleavage followed by purification of biotinylated cross-linked peptides which were then reduced and alkylated. The working protocols of each step were the same with 500 μl of cross-linked sample. The samples were reduced with 100 mM final concentration of DTT and incubated for 1 h at 60 °C, and then brought to 22 °C and iodoacetamide added to a 50 mM final concentration and incubated for 1 h. For tryptic digestion, trypsin (Promega) reconstituted in 50 mM ammonium bicarbonate, 2.5% acetonitrile pH 7.8 was added to the samples for a final concentration of 2 ng/μl and incubated at 37 °C for 16 h. Purification of the biotinylated peptides by affinity chromatography used the supplied 1 ml column from the Pierce monomeric avidin kit (Thermo Scientific) and protocols therein.

2.3. Mass spectrometry

Samples eluted from avidin affinity chromatography were desalted using C18 tips (Pierce), dried using a vacuum concentrator and dissolved in 3% acetonitrile containing 0.1% formic acid prior to analysis by LC/MS. MS analyses were performed using a Waters Synapt G2 HDMS connected to a Nanoacquity nano-LC with a Waters BEH C18 column (75 μm × 250 mm). A gradient was run from 3% to 50% organic solvent (acetonitrile/0.1% formic acid). Spectra were acquired over the gradient in MS^E resolution mode using leucine-enkephaline as lockspray. The resulting spectra were analyzed using the Waters ProteinLynx Global Server version 2.5 and searches were performed against an *E. coli* proteome database extracted from Uniprot (release 2013_05). Parameters were: single missed cleavage, fixed modification: carbamidomethyl, variable modification: oxidation (M), false discovery rate <1. Peptides were identified with a residual mass error of <5 ppm.

2.4. Far-Western dot blot

The far-Western dot blot assay for binding of DmsD and GroEL were performed as described in [10], where 0.05 to 50 μg of purified protein was applied in duplicate onto the membrane and incubated with 20 μg/ml purified DmsD.

2.5. Dynamic light scattering

Dynamic light scattering experiments were performed on unlabeled DmsD and GroEL, and labeled DmsD either unreacted or reacted with GroEL to form a cross-link product. Samples were purified by avidin affinity chromatography as described above without any trypic digestion for DLS. Measurements of the dispersed proteins were performed in the Nanoscience lab (University of Calgary) with a Zetasizer Nano ZS (Malvern Instruments), using a He–Ne laser at 633 nm and a power of 4.0 mW. Samples of 300 μl were measured in a 10 mm path length quartz cell, at a fixed scattering angle of 173° and 25 °C. Data was averaged over three runs for each sample and the size distributions on the protein samples were obtained using the instrument software. The viscosity of water was taken as 8.9×10^{-4} Pa s and its refractive index as 1.33.

2.6. Electron microscopy and image processing

GroEL and cross-linked, avidin chromatography purified DmsD–GroEL samples were adsorbed at ~20 ng/ml to glow discharge carbon-coated copper grids for 3–4 min and negatively stained with uranyl formate as described in Ref. [13]. Samples were visualized using a Tecnai Spirit transmission electron microscope (FEI) operated at an accelerating voltage of 120 kV and images recorded at a nominal magnification of 49,000× on a 4 K × 4 K Eagle camera and then analyzed in 2D as described in Ref. [13], with the exception that 3700 particles of DmsD–GroEL and 2919 of GroEL were windowed into 72 × 72-pixel images and 50 classes of *K*-means classification was specified.

3. Results

3.1. DmsD interacts with GroEL in vitro and in vivo

A previous study targeting the interactome of DmsD identified GroEL as one of its transient interactors [8]. GroEL was identified through global cross-linking of cellular proteins to DmsD and confirmed using an *in vivo* two-hybrid assay, which does not discriminate against indirect interactions as other cellular factors could be present to facilitate the interaction. Using an *in vitro* dot-blot far Western procedure, we demonstrate that DmsD does indeed interact with GroEL in the absence of other cellular factors (Fig. 1A). Compared to the well-characterized DmsD substrate, DmsA twin-arginine leader, the interaction between DmsD and GroEL was much weaker judging by the faint intensity, indicating that only a small amount of DmsD was captured by GroEL. This interaction was specific as the signal intensity was clearly above that of the

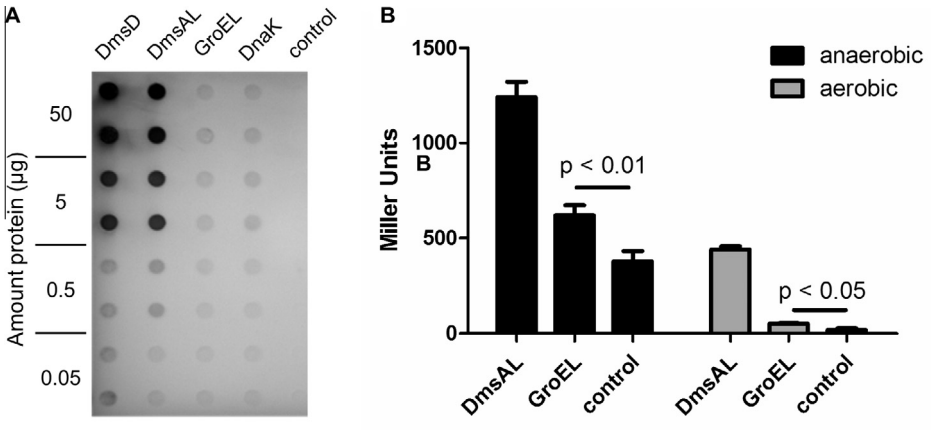

Fig. 1. Interactions with DmsD. (A) *In vitro* far-Western dot-blot between DmsD and DmsA leader (DmsAL), GroEL, DnaK, and a buffer control with duplicates of each titration. (B) β-galactosidase activity assays from *in vivo* bacterial two-hybrid screens of DmsD binding to DmsA leader (DmsAL), GroEL, and the leucine zipper protein as a negative control.

negative control. As a weak interaction positive control, DnaK, a protein that was also identified to interact with DmsD *in vitro* and *in vivo* [8] also showed binding to DmsD at levels similar to that of GroEL.

Being the system-specific chaperone for anaerobic DMSO reductase maturation, we suspected that the conditions for monitoring the interaction between DmsD and GroEL was not optimal in previous studies using aerobic conditions [8]. Under anaerobic growth conditions with DMSO supplied as the sole electron acceptor in the media, we detect a much stronger interaction between DmsD and GroEL and the DmsA leader (Fig. 1B). As a negative control, a portion of the leucine zipper protein [14] was included to rule out non-specific interactions. While this control assay also had a higher signal under these conditions, it was evident that under both anaerobic and aerobic growth the interaction between DmsD and GroEL was significant compared to the control. This interaction was approximately 2–3-fold weaker than that observed between DmsD and the DmsA leader, confirming that the interaction with GroEL is relatively weak. Unfortunately, DMSO reductase activity assays of a *groEL* mutant could not be performed as this mutant failed to grow in conditions that induce the expression of DMSO reductase (data not shown).

3.2. Biophysical characterization of the DmsD–GroEL complex

We utilized DLS to characterize the interaction between DmsD and GroEL. The data showed a single peak for both DmsD and GroEL with hydrodynamic diameter (D_h) of 9.1 nm ± 1.8 nm and 18.5 nm ± 3.6 nm, respectively (Fig. 2A). Cumulant fit D_h values were not valid due to high polydispersity, presumably because of small amounts of large sized particles detected during light scattering (Supplementary Fig. 1). We suspect that these could be aggregates (as indicated by the large error), or in the case of DmsD, dimeric assemblies of the protein that exists in equilibrium despite apparently being removed during purification [15].

DmsD and GroEL was mixed and allowed to interact for 30 min and we observed two peaks with D_h of 9.7 nm ± 1.6 nm and 18.2 nm ± 4.1 nm (Fig. 2A). The peaks were not completely resolved and the D_h values correspond closely to those obtained for DmsD and GroEL alone. The data suggest that both proteins may interact weakly each other but keep their identity as separate proteins, as we expect that the reaction equilibrium favors dissociation of the complex from DmsD – GroEL ↔ DmsD + GroEL. To overcome this, we utilized a photo-activatable biotinylated cross-linker pre-labeled onto DmsD to generate a stable complex for

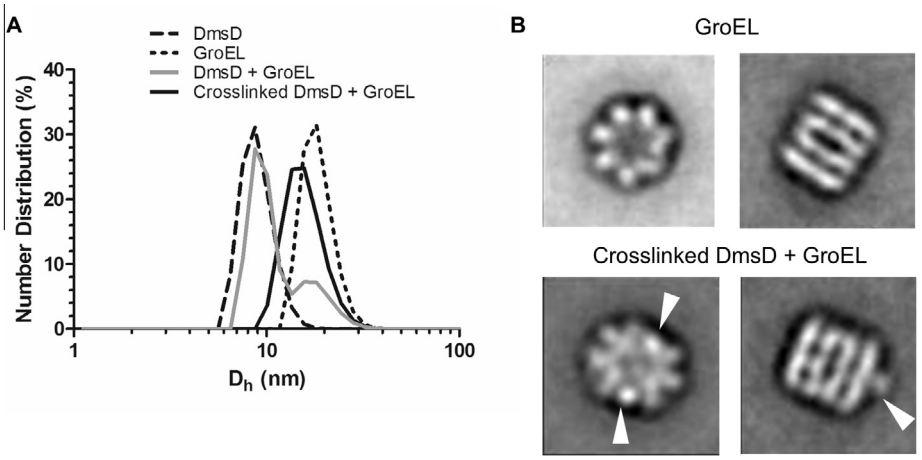

Fig. 2. Physical characteristics of DmsD binding to GroEL. (A) DLS spectroscopy of DmsD, GroEL, DmsD mixed with GroEL, and DmsD cross-linked to GroEL and their corresponding hydrodynamic diameters (D_h) are shown. (B) Representative class average images of GroEL and DmsD cross-linked to GroEL by electron microscopy. Arrow indicates the additional density that may correspond to DmsD at the periphery of GroEL.

analysis. Utilizing the biotin group on the cross-linker, we enriched for the cross-linked DmsD–GroEL complex with avidin affinity chromatography (Supplementary Fig. 2A and B). The cross-linked complex showed a single peak with D_h of 16.0 nm ± 3.8 nm, which is similar to that of GroEL alone. Again the high polydispersity of this sample prevented us from obtaining its D_h from cumulant fitting (Supplementary Fig. 1). We suspect that this is due to the other species that result from the cross-linking reaction, which the avidin chromatography step cannot separate as long as they contain the biotin label (Supplementary Fig. 2C). These include labeled but uncross-linked DmsD (DmsD*), cross-linked DmsD dimers (DmsD*–DmsD), a multimeric assembly of cross-linked DmsD [$(DmsD^*–DmsD)_n$], and cross-linked DmsD dimers associated with GroEL [(DmsD*–DmsD)::GroEL].

Attempts to characterize the cross-linked complex by electron microscopy yielded particle images that appeared to have an additional density attached to the periphery of GroEL (Fig. 2B, arrow). However, the particles with the additional density represent a minor population of total particles from our dataset, and this precluded us from further structural analysis of the cross-linked complex.

3.3. Characterization of the DmsD–GroEL binding interface

We utilized the cross-linked complex in order to further characterize the interaction between DmsD and GroEL. The cross-linker was chosen for its specific reactivity only upon UV irradiation and its ability to be reduced, leaving the biotinylated moiety to be transferred to the prey protein (Supplementary Fig. S1 illustrates the reaction using DmsD as bait and GroEL as prey). The initial strategy was to attach the cross-linker to both proteins to act as bait in two complementary reactions (i.e. labeled DmsD with unlabeled GroEL and *vice versa*) to identify binding sites on both DmsD and GroEL. However, we found that only GroEL peptides were identified by mass spectrometry when using GroEL as bait, due to the much higher cross-linking efficiency of GroEL to adjacent protomers in the homo-oligomeric assembly, and therefore did not continue with this option.

SDS–PAGE analysis of the cross-linked sample shows the appearance of a faint band at ~80 kDa (Supplementary Fig. 2, lane 8, arrow) that does not appear to be present in the other control lanes, which may correspond to the cross-linked complex. Given that the interaction between the two is relatively weak, we expected that majority of the DmsD and GroEL protein to appear unreacted, as indicated by the lack of change in migration pattern (Supplementary Fig. 2).

We also enriched for the cross-linked complex using avidin affinity chromatography but included a complement strategy to subtract 'background' peptides from bait proteins that still contained the biotin moiety but did not cross-link to any prey proteins. Given the ability to transfer the cross-linker label onto the prey protein upon reduction of the disulfide bond (Supplementary Fig. S1), the cross-linked samples were either digested into peptides prior to reduction or reduced prior to digestion. Upon analysis, fifteen GroEL peptides were found to be cross-linked to DmsD and two DmsD peptides to GroEL (Supplementary Tables 1 and 2).

We mapped the two DmsD peptides onto the *E. coli* DmsD structure and found that they were on the same 'plane' of DmsD (Fig. 3A, pink and red), suggesting that this region of DmsD is involved in binding to GroEL. Since the cross-linker only attaches to primary amines, we were able to narrow down the cross-linked residues to Arg161, Arg176, and Arg183 and found that they are in close proximity to each other (Fig. 3A, yellow). By mapping the known DmsA leader binding sites previously identified by site-directed mutagenesis or molecular dynamics simulation [10,16], we see that the GroEL and DmsA leader binding sites are adjacent to each other (Fig. 3B). In fact Cys147, Leu151, and His172 are found in the sequence of the two cross-linked peptides (Supplementary Table 2), and are in close proximity to the three Arg residues (Fig. 3C). This demonstrates that the GroEL and DmsA leader binding sites on DmsD are in close proximity.

Peptides from GroEL appeared to mostly map near the top and bottom of *E. coli* GroEL (Fig. 3D), suggesting that DmsD may bind to either ends of the GroEL opening. Binding at these two ends of GroEL is possible in this assembly as we did not identify any GroES peptides from the samples (data not shown), where GroES would normally be bound at the top of the structure. We also observed some peptides of GroEL mapping to regions that would face the inside of the cavity (Fig. 3D right is a slice-through of GroEL, Supplementary Table 1).

4. Discussion

Biogenesis of DMSO reductase is an intricately coordinated process. Following nascent polypeptide synthesis of the reductase subunits DmsA and DmsB, they must properly fold and coordinate their appropriate cofactors, which is then followed by subunit assembly into the active holoenzyme DmsAB. The holoenzyme is then targeted to and translocated across the cytoplasmic membrane, where it attaches to the anchor subunit DmsC to gain access to the quinone pool. The system-specific REMP DmsD participates in DMSO reductase biogenesis at various steps [6]. It has become apparent that DmsD does not perform these functions alone, but does so in coordination with molybdenum cofactor biosynthesis proteins and general molecular chaperones. By further characterizing the interaction between DmsD and the chaperonin GroEL, we gain further insight into the intricately coordinated DMSO reductase biogenesis pathway.

The interaction between DmsD and GroEL was weaker than the well-characterized interaction between DmsD and the DmsA leader. An overall increase in the interaction signal under growth conditions that induce expression of DMSO reductase suggests that the interaction is stabilized under relevant physiological conditions. Although the interaction between DmsD and the DmsA leader was strong enough to be measured by standard biophysical techniques such as ITC, the dissociation constant between the two is by no means considered a tight interaction in biological systems (0.2 μM, [17]). This may be due to the need for DmsD to release DmsA to the other proteins involved in the numerous steps of DMSO reductase biogenesis. Given that the interaction between DmsD and GroEL appeared to be approximately 9-fold weaker than that with DmsA leader under aerobic conditions *in vivo*, we were unable to characterize the interaction using routine techniques such as ITC or gel filtration chromatography (data not shown). By cross-linking the DmsD–GroEL complex, the biophysical properties was characterized by DLS where a single population was observed. Without cross-linking, two populations with D_h similar to that of DmsD and GroEL alone were observed, further supporting the weak interaction between the two.

GroEL is anisotropic in overall shape and is much larger than DmsD; with double-stacked hexameric rings it is ~800 kDa in size compared to the ~28 kDa DmsD. If DmsD binds to GroEL without significantly affecting the long axis of GroEL, the magnitude of change in hydrodynamic diameter will be nearly undetectable as D_h strongly depends upon the anisotropy of the dispersed particles [18].

The last possibility explaining the negligible change in D_h could be that DmsD bound inside the cavity of GroEL. This hypothesis is feasible since DmsD has dimensions of 49 × 37 × 34 Å [16] and could comfortably fit inside the 45 × 145 Å GroEL cavity [19]. The observation that more than half of the GroEL peptides

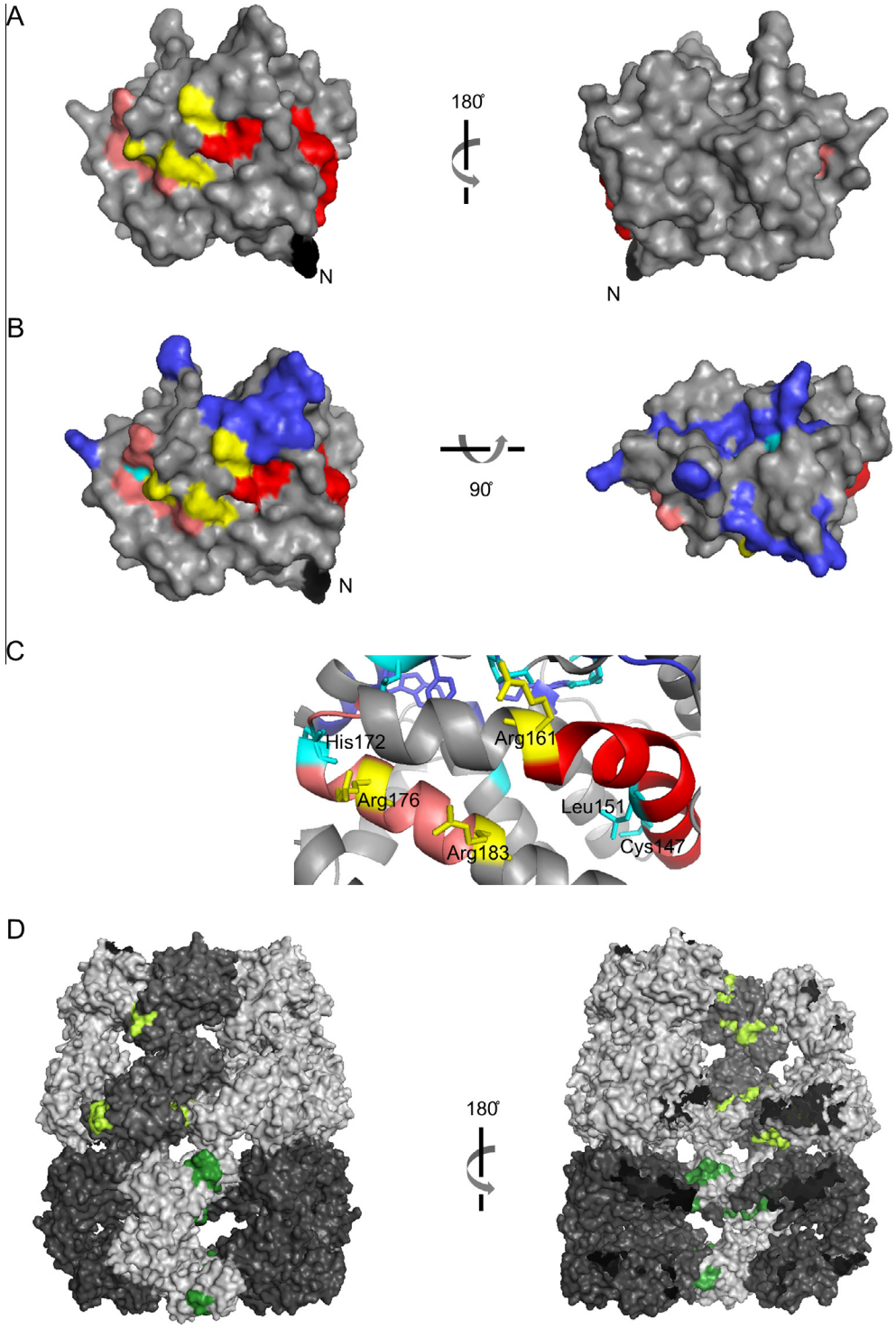

Fig. 3. Binding sites of DmsD (PDBId 3EFP) and GroEL (PDBId 1PCQ). (A) Peptides in DmsD that cross-link with GroEL were identified colored red and pink, with the three cross-linking Arg residues colored yellow. The N-terminus is labeled and colored black for reference. (B) DmsA leader-binding residues identified by mutagenesis (indigo blue, [10]) or molecular dynamics simulation (cyan, [16]) are also indicated with those that cross-linked in panel A. (C) Close-up of cross-linked and DmsA-binding region from panel B, with the respective residues labeled. (D) Regions in GroEL identified to cross-link with DmsD are colored lime or forest green on two separate subunits, which are colored an opposite shade of gray for comparison. The GroES cap is removed for simplicity along with two subunits from the top and bottom ring to give an inside view of the cavity (right panel).

cross-linked to DmsD are located on the inside of its central cavity supports this (Supplementary Table 1). Whether this is a coincidence or has biological relevance in DMSO reductase biogenesis remains to be answered. It is unlikely that DmsA-bound DmsD brings DmsA into the cavity of GroEL for folding based on the GroEL substrate cutoff size of ~60 kDa [20], with one example reporting a 86 kDa substrate [21]. The preprotein form of DmsA with its leader intact is ~90 kDa and is likely too large to fit inside GroEL for folding. It may follow a model of GroEL-assisted folding with GroEL acting as a 'holdase' with the DmsA folding in the bulk solution

while interacting with the open (*trans*) ring of GroEL (reviewed in Refs. [19,22]). Our findings that the GroEL and DmsA leader binding sites are in close proximity to each other on DmsD supports a model where DmsD brings the DmsA preprotein to GroEL for assistance in folding. It is probably more energetically favorable for DmsD to remain bound to DmsA while it interacts with GroEL for folding, so that it may also facilitate interactions with other proteins for cofactor insertion and holoenzyme assembly. Since DmsA is too large to fit into the cavity of GroEL for folding, it is possible that DmsD tucks itself into the cavity of GroEL to allow DmsA to interact with GroEL.

Our findings confirm the interaction between DmsD and GroEL and demonstrate the weak interaction strength as a contributor to the difficulty in detecting this interaction. Further characterization of the cross-linked DmsD–GroEL complex reveals the function of DmsD as a connector to bring the DmsA preprotein to the chaperonin for GroEL-assisted folding during the biogenesis pathway.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.12.046.

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