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Folding forms of *Escherichia coli* DmsD, a twin-arginine leader binding protein

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Abstract

Escherichia coli DmsD interacts with the twin-arginine leader sequence of the catalytic sub-unit (DmsA) of DMSO reductase. DmsD was purified as a mixture of a number of different folding forms including: dimer (A); monomer (B); a minor thiol oxidized form; a heterogeneously folded or multi-conformational monomer form which displayed a ladder of bands on native-PAGE (D); and proteolytically degraded and aggregated forms. Polyacrylamide gel electrophoresis (PAGE), under denaturing and non-denaturing conditions, was used to examine the folding and stability of DmsD. Additionally, the biophysical methods of dynamic light scattering, circular dichroism, fluorescence, and mass spectroscopy were also used. Form D could be converted to form B by treatment with 4 M urea, which is the concentration at which form B begins to denature. Forms A/B could be converted to D by incubation at pH 5.0. Forms A/B and D all had twin-arginine leader binding activity.

Keywords: DmsD; Twin-arginine leader; Twin-arginine translocase; DMSO reductase

Until recently, most protein translocation in bacteria was believed to be carried out by the general secretory pathway or Sec pathway. This results in protein translocation across the membrane in an extended or unfolded conformation. However, the discovery of a Sec-independent pathway challenges this notion; this pathway displays the remarkable ability to transport proteins to or across the membrane in a fully folded state. Initially referred to as the membrane targeting and translocation (Mtt) system [1], this pathway is now more commonly referred to as the twin-arginine translocase (Tat) pathway [2–4].

This newly discovered Tat pathway is fundamental to the bioenergetics of many prokaryotic respiratory enzymes [3]. Examples in *Escherichia coli* include the molybdoenzymes dimethyl sulfoxide (DMSO) reductase [5] and trimethylamine *N*-oxide (TMAO) reductase [6]. Tat is a multimeric transmembrane complex consisting of the TatA, B, C, and E sub-units which are required for the export of pre-proteins with twin-arginine signal sequences [1,2]. This field has been extensively examined in several reviews [3,4,7,8].

A feature of the Tat system is that the N-terminal leaders of proteins targeted for translocation contain a conserved twin-arginine motif [9]. Numerous proteins containing the twin-arginine leader motif have been identified from a wide variety of organisms [9]. Oresnik et al. [10] hypothesized that specific leader binding proteins should exist to escort the proteins containing the twin-arginine leader to the Tat complex. Oresnik et al. [10] isolated a protein using only the DmsA leader sequence in an affinity column approach. Further research has found that this protein interacts with the twin-arginine leader from DMSO reductase (DmsA) and TMAO reductase (TorA), and was termed DmsD. Sequence comparisons illustrated that DmsD is a member of the TorD family [3,10,11].

The DmsD homologue, TorD, is considered a specific chaperone for the twin-arginine containing TorA molybdoenzyme [12,13]. TorD has been shown to bind the TorA enzyme and its precursor form [13], and to play a role in the maturation pathway providing for a molybdopterin cofactor competent form [14]. It was therefore

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hypothesized that TorD, DmsD, and like proteins act as private molecular or Mate chaperones and may be termed redox enzyme maturation proteins (REMPs) [15].

The molecular forms of *Shewanella massilia* TorD have been investigated and were shown to form multiple oligomeric states [12]. Additionally, Trainer and co-workers recently published the structure of this protein. They found the protein to contain a unique fold that is all helical, with two domains which undergo domain swapping [11]. *E. coli* DmsD has 20% sequence identity to this protein; despite several regions of dissimilarity, it is also predicted to be helical and may have the same structural architecture as TorD.

In this study we investigated the molecular properties of the major folding forms of DmsD from *E. coli*. We purified DmsD primarily as a monomer (form A), with dimer (B), and other forms present in smaller amounts. The different folding forms of DmsD were separated during purification by Ni-affinity and size-exclusion chromatography. Form C is obtained only in minor quantities and appears more susceptible to intra-disulfide bonding. Here, we used native and SDS–polyacrylamide gel electrophoresis (PAGE) to examine the folding and stability of DmsD. Form D displays the unique characteristic of a ladder of different migratory forms on native-PAGE. A Far-Western dot blot assay was developed to assess leader peptide binding activity. Using this assay both forms A/B and D had DmsA leader binding activity. The molecular characteristics of these forms of DmsD were also studied by mass spectroscopy, dynamic light scattering, circular dichroism, and fluorescence spectroscopy.

Materials and methods

Expression and purification of DmsD. DmsD with an N-terminal His_6 tag was over-expressed in *E. coli* C41(DE3) harboring pTDMS28 [10] and grown at 37 °C in Luria broth. At an OD_{600} of 0.5, the culture was induced by the addition of IPTG to a final concentration of 0.5 mM. Three hours after the addition of IPTG, the cells were harvested by centrifugation at 2500*g* for 10 min.

The purification protocol for the His_6T_7DmsD construct has been changed a number of times in our group which has resulted in the observation of different folding forms. Additionally, we observed that changing the NaCl concentration in the Luria broth from 5 to 10 g NaCl/L has resulted in increased yield.

Initial purifications re-suspended cells in: 20 mM phosphate buffer, pH 7.9; 5 mM imidazole; and 0.5 M NaCl prior to lysis by two passages through a French press at 16,000 psi. The homogenized cells were centrifuged at 14,000*g* for 10 min and the supernatant was further centrifuged at 120,000*g* for 90 min to remove the membranes. The supernatant was passed down a 7 mL immobilized metal-affinity column (Ni–NTA agarose resin, Qiagen) that had been freshly charged with $NiSO_4$ and equilibrated with 50 mL binding buffer at 4 °C. The flow-through was recycled to ensure that the maximum amount of DmsD:His_6 was recovered. Bound DmsD:His_6 was washed with 10 column volumes (CV) of binding buffer containing 5 mM imidazole followed by another 10 CV of the same buffer containing 50 mM imidazole. The His-tagged DmsD was eluted with approximately 1 CV of buffer containing 1 M imidazole.

Subsequent purification used an Akta Purifier with a PerSeptive Biosystems column containing Poros 20MC resin, with the buffers changed to Tris. A lower concentration of imidazole was used in the elution at room temperature. Cells were lysed as above but with binding buffer II (50 mM Tris–HCl, pH 7.9; 0.5 M NaCl; and 5 mM imidazole). The 1.25 mL of 20MC column was equilibrated with this binding buffer. The sample was loaded and then washed with binding buffer at 50 mM imidazole. DmsD was then eluted with 250 mM imidazole in less than 3 mL. The best method was to use the FPLC with Ni–NTA superflow resin (10 mL column) with the buffer system indicated above.

It was important to apply the sample from either method to the size-exclusion column within one day of elution to avoid precipitation.

Size-exclusion chromatography. Nickel-affinity column-purified DmsD:His_6 was further purified by size-exclusion chromatography on a calibrated Superose 12 HR 10/30 column connected to an Akta Purifier system equilibrated with 20 mM Tris, 100 mM NaCl, and 1 mM β-mercaptoethanol, pH 7.0. For each run, ~2 mg of protein was applied to the column with a flowrate of 0.5 ml/min. The different peak fractions were investigated by SDS–PAGE and native-PAGE without further treatment. The size-exclusion column was calibrated using the following standard macromolecules: blue dextran; aldolase (158 kDa); albumin (66 kDa); α-chymotrypsin (25 kDa); myoglobin (17 kDa); and aprotinin (6.5 kDa).

The concentration of DmsD:His_6 in the fractions was determined by using Bio-Rad Bradford or Lowry protein assays or by UV absorption using an estimated extinction coefficient ($\varepsilon_{280} = 5.6 \times 10^4\ cm^2\ mol^{-1}$).

Denaturing and native-PAGE. DmsD samples prepared by affinity and size-exclusion chromatography were used for denaturing and native-PAGE analysis without further treatment. For SDS–PAGE analysis, 5–10 μg protein samples were prepared in aliquots of sample buffer (5 ml sample buffer: 0.1 M DTT; 0.05 M Tris, pH 6.8; 2% SDS; 20% glycerol; 0.1% bromophenol blue; and water). The samples were run through a 5% stacking gel and resolved on a 12.5% acrylamide gel (30% acrylamide/bisacrylamide) in a discontinuous buffer system at 180 V at room temperature. The electrophoretic buffer contained 30 g/L Tris and 144 g/L glycine at pH 8.3. Protein bands were detected with Coomassie blue and compared against Bio-Rad low molecular weight standards. Twice the amount of protein used in SDS–PAGE was used for native-PAGE. Protein samples for native-PAGE were prepared in sample buffer without SDS and with/without DTT. Samples were applied directly without further treatment to non-denaturing polyacrylamide gel electrophoresis, (5% (w/v) stacking and 7% resolving gel) at either 40 or 80 V at room temperature.

Protein denaturation and renaturation. DmsD (0.2 mg/ml) was treated with urea to a final volume of 6 ml and urea concentration of 4 M for 4 h. Treated samples were refolded by dialysis against a liter of 10 mM phosphate buffer at pH 7.2. Eight micrograms of treated or untreated (control) monomer was subjected to native-PAGE to determine the effect of chaotrope treatment on its conformational forms.

Interconversion between folding form A/B to D was performed by incubating 30 μg DmsD overnight at 4 °C in 20 mM sodium acetate buffer, pH 5.0. After treatment the sample was concentrated and the buffer was exchanged by dialysis with a 10 mM Tris–HCl, 50 mM NaCl buffer at pH 8.0. Controls were incubated in the pH 8.0 buffer, and then dialyzed overnight.

Fluorescence spectroscopy. Intrinsic tryptophan fluorescence spectra were collected with a Fluorolog spectrofluorimeter equipped with a 450 W xenon lamp and controlled by DataMax version 2.0. Samples of 2.5 ml at 0.02–0.03 mg/ml were incubated in appropriate buffers for the pH range titration. Excitation was at 280 nm and emission was recorded from 290 to 500 nm with excitation and emission band-passes at 5 nm. All spectral readings were recorded using a 1-cm path length cuvette.

Circular dichroism. Measurements were carried out on a JASCO J-715 spectropolarimeter. One micromolar protein was used for CD measurements at ambient temperature using a 1-cm path length quartz cuvette. Spectropolarimeter parameters were set as follows: 1 s response time; 0.5 nm step resolution; 2 nm band width; 200 mdeg sensitivity; and 100 nm/min scan rate. All spectra were averaged over 5 scans. After the background of the sample solvent had been subtracted, spectra in mdeg units were converted to the mean residue molar ellipticity units.

Mass spectrometry. DmsD:His_6 form D and DmsD forms A/B (with the His_6 tag removed by enterokinase treatment) were prepared by size-exclusion FPLC and dialyzed against 10 mM Tris–HCl, pH 7.0, at 4 °C. Samples were concentrated using a Speed-Vac and then approximately 10–15 μg was analyzed at the Southern Alberta Mass Spectrometry Centre (Calgary, Canada) for mass spectrometry using a MALDI-TOF spectrometer.

Dynamic light scattering. Protein samples were analyzed for their hydrodynamic radius using a DynaPro-MSTC instrument (Protein Solutions) and analyzed using the Dynals software package. One milliliter 0.2–0.4 mg/mL DmsD in 10 mM phosphate buffer, pH 7.0, 100 mM NaCl, or 6 M urea was concentrated to 0.1 mL using Millipore ultrafree-centrifugal filters (10 kDa cut-off membrane). The concentrated protein solution was passed through a 0.02 μm Whatman filter into the dynamic light scattering (DLS) cuvette. One-hundred acquisitions were collected for each sample.

Activity assay. Purified DmsA-leader-GST [10], 5 μg was applied to nitrocellulose (0.2 μm) and allowed to dry for 15 min. The membrane was then washed in TBS (50 mM Tris–HCl, pH 7.5, 200 mM NaCl) for 5 min before blocking overnight at room temperature with 50 mL 10% milk in TBS/0.02% w/v azide. DmsD aliquots were diluted 20-fold into the following buffers: (1) 20 mM Tris, pH 7, 100 mM NaCl; (2) same as 1 with 10 mM DTT; (3) same as 1 with 2% SDS; (4) same as 1 with 4 M urea; (5) same as 1 with 5 M GuHCl; and (6) 20 mM Na-acetate, pH 5.0, 100 mM NaCl. The final DmsD concentration was approximately 0.1 mg/mL. The DmsD solutions were then allowed to equilibrate in these conditions overnight at 4 °C.

The equilibrated DmsD was then diluted 8-fold into 1% milk TBST (TBS + 0.05% v/v Tween 20)/azide (final concentration of DmsD = 0.01–0.02 mg/mL). This solution was added to the DmsAleader-GST dot blots and incubated at room temperature for 4 h. Membrane was washed twice, 5 min with TBS, then twice with TBST, 5 min each. The membrane was then incubated with anti-T7-HRP conjugate (1:5000) in 1% milk TBST/azide for 1 h at 20 °C followed by washing four times 5 min each with TBS, then TBST. The membrane was developed with HRP reagents (Bio-Rad).

Results

Purification of DmsD

From the purification and subsequent characterization, DmsD was found to exist in a number of different folding forms including: dimer (A); monomer (B); a minor amount of a form that had a greater propensity to form intra-disulfide (C); heterogeneous folded or ladder form (D); proteolytically degraded DmsD (E); and aggregated/precipitated DmsD (F). All forms could be separated by SEC-HPLC (Fig. 1).

DmsD was over-expressed in *E. coli* as a fusion protein carrying the hexa-histidine tag, a T_7 epitope, and an enterokinase cleavage site at its N-terminus. Its calculated molecular weight based on the amino acid

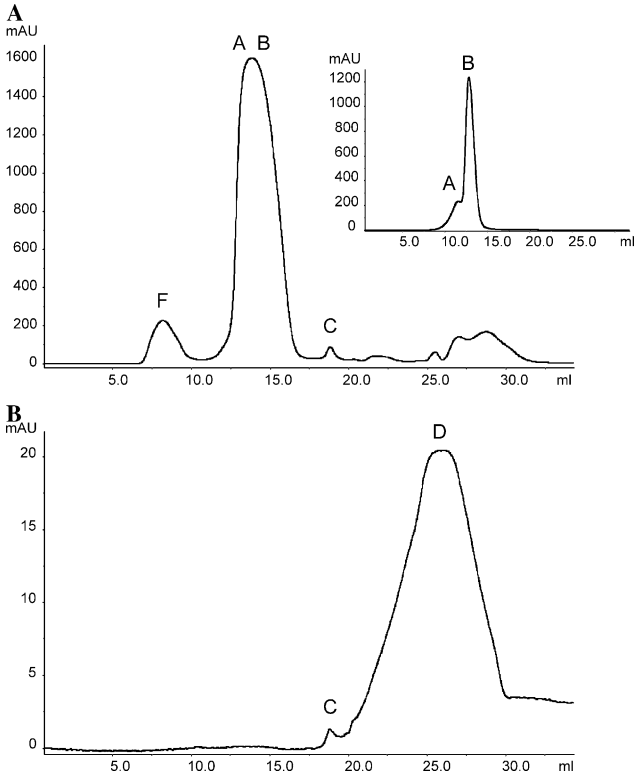

Fig. 1. DmsD:His_6 size-exclusion chromatography elution profiles. (A) Typical elution profile from protein purification method II, 2 mL injection. The inset is the re-injection of concentrated peak form A/B (100 μL). (B) Elution profile of forms A/B treated at pH 5.0. The peak labeled form D was also prominent in elution profiles from purification method I. All peaks labeled with a letter were found to be DmsD by SDS–PAGE and Western blot analysis.

sequence is 27.5 kDa. DmsD purified using the initial method was washed consecutively with binding buffer containing 5 and 60 mM imidazole until A_{280} ~0.003. The bound DmsD was eluted with 1 M imidazole and combined into two fractions: high absorbance and low absorbance fractions, which were analyzed by SDS–PAGE. The high absorbance fraction showed several protein bands, while the low absorbance fraction showed a single band (not shown). Western blotting against the T_7 epitope verified the presence of DmsD that corresponded to a single band in both fractions, with an apparent molecular weight of around 28 kDa. With this initial method the second peak gave a form of the protein that migrated as a ladder on native gels (form D). Purified with this method, the amount of A/B was typically twice that of form D. When purification protocols changed, the amount of form D decreased to trace levels. Nonetheless, form D showed activity (see below); therefore, we investigated to determine if it plays a role in the biology of the protein.

Purified with the initial protocol, forms were only stable for a few days. Both forms aggregated and precipitated out of solution when stored above 1 mg/mL. We also found that treatment by lyophilization, freezing

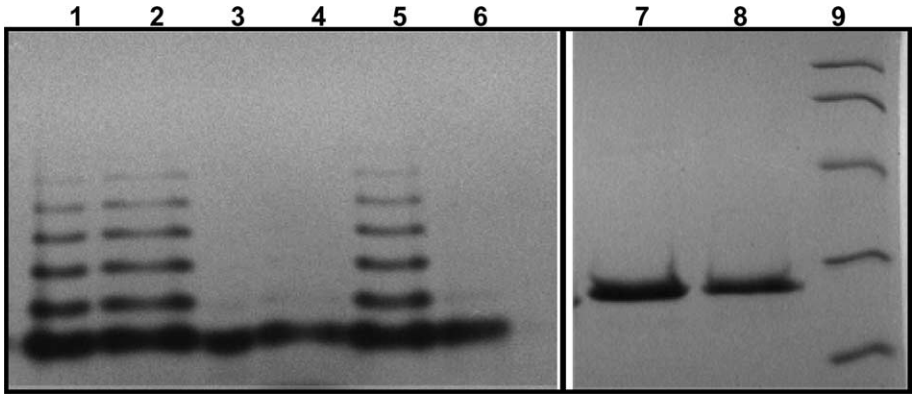

Fig. 2. Migration of different forms of DmsD:His_6 on PAGE. (A) Profile of DmsD folding forms separated by 7% native (lanes 1–6) and SDS– (lanes 7–8) polyacrylamide gel electrophoresis. Lane 1, DmsD form D without the reducing agent DTT; lane 2, DmsD form D with DTT; lane 3, DmsD forms A/B without DTT; and lane 4, DmsD forms A/B with DTT. Ten micrograms of protein per lane was resolved on a 7% native gel. Lane 5 is also form D and lane 6 corresponds to form D treated with 8 M urea and refolded by dialysis to convert back to form B. Lanes 7–9 are a 12% SDS–PAGE showing that both DmsD form D (lane 7) and forms A/B (lane 8) migrate equally and as single bands corresponding to a molecular weight of approximately 28 kDa. Lane 9 is the low range molecular weight markers.

at −20 or −70 °C (either slow or flash freeze), or ammonium sulfate precipitation resulted in low recovery of soluble protein. DmsD forms A/B purified by subsequent methods proved to be more stable and could be stored at high concentrations (~2 mg/mL) for up to 2 weeks; as well, it was more recoverable (~75%) from −20 °C storage. The different protein forms in the preparations arise from differences in ionic strength, buffer components, and how the resin was charged with Ni. The presence of different folding forms of the protein from the different protocols illustrates the sensitivity of this protein to the changes in physiochemical conditions.

Fig. 1 shows representative chromatograms from size-exclusion chromatography. Fig. 1A shows an elution from later purification protocols with the re-injection of an aliquot of concentrated form A/B peak to display the dimer (A)–monomer (B) amounts. Typically, DmsD was purified as ~95% monomer with 5% as dimer. After dilution of the dimer peak through re-injection, it migrated as monomer. Fig. 1B is an example of form D elution from treatment of A/B at pH 5.0. Although this form gives protein that migrates as a single band at ~28 kDa on SDS–PAGE (Fig. 2), this protein elutes as a very broad peak close to V_t; this indicates that this form of the protein interacts strongly with the column matrix.

The aggregates that precipitate out indicate the poor stability of the proteins; they require urea denaturation to re-solubilize. This aggregation was found to be independent of the His_6 purification tag. *E. coli* TorD purified under the same conditions was found to be more stable (unpublished results).

Characterization of the folding forms by PAGE

DmsD:His_6 forms, A, B, and D, migrate as single bands on SDS–PAGE with an apparent molecular weight of ~28 kDa (Fig. 2, lanes 7 and 8), but differ on native-PAGE (Fig. 2, lanes 1–6). On 7% native-PAGE, form B migrates as single band, whereas form D displays a ladder of uniformly separated bands which do not correspond to increasing multimer aggregates. The positions of these bands did not change when 10 mM DTT was added to the sample buffer; this indicates that disulfides are not responsible for the migration pattern.

The DmsD hexa-histidine tag could be completely cleaved at its enterokinase cleavage site as assessed by SDS–PAGE. When the tag was removed from form D and analyzed by non-denaturing gel loading conditions, it displayed the same ladder pattern of bands as the protein with the His_6 tag (not shown). Thus, the presence of the His_6 tag through the fusion system does not influence the conformations or multimeric states of the protein.

DmsD form D could be converted back to form A/B only through urea denaturation treatment (Fig. 2, lanes 5 and 6). This indicates that the form D conformations are somewhat stable once established.

Size analysis of DmsD

MALDI-TOF mass spectroscopy was employed to further investigate the DmsD complexes and the purity of samples. The samples of DmsD:His_6 forms D and B gave the same mass corresponding to monomer. Form A showed an additional minor peak with mass corresponding to a dimer. The purified forms of DmsD were also investigated by dynamic light scattering. When concentrated, the sample of the peak of form A/B returned a uniform hydrodynamic radius of 3.2 nm; this corresponds to a M_r of ~51 kDa, suggesting a dimer species is driven by concentration. DmsD in 4 M urea gave two species: one corresponded to the dimer; the other at 2.7 nm is similar to that of diluted form B and corresponds to a monomer. Form D returned a slightly

broader distribution centered around a hydrodynamic radius of ~2.6 nm, similar to that of the monomer.

Spectroscopic analysis of DmsD stability

NMR experiments were attempted with A/B and D forms. However, due to spectral heterogeneity from the D form and the line broadening from the size of form A (the result of concentrating), no useful spectra were obtained. Additionally, at the concentrations required, the protein readily aggregated.

Circular dichroism spectra of DmsD forms D and A/B, in a pH 7 phosphate buffer at 20 °C, suggest that the forms have similar secondary structure (Fig. 3). The spectra indicate that DmsD has a high α-helical content in agreement with the predicted secondary structure.

Following the fluorescence characteristics of the protein, Fig. 4 shows pH titrations of D and A/B forms. The marked inflection at pH 5 falls at the theoretical p*I* of ~4.8.

Form interconversion

The related protein *S. massilia* TorD was reported to undergo monomer/dimer interconversion at pH 3.0 [12].

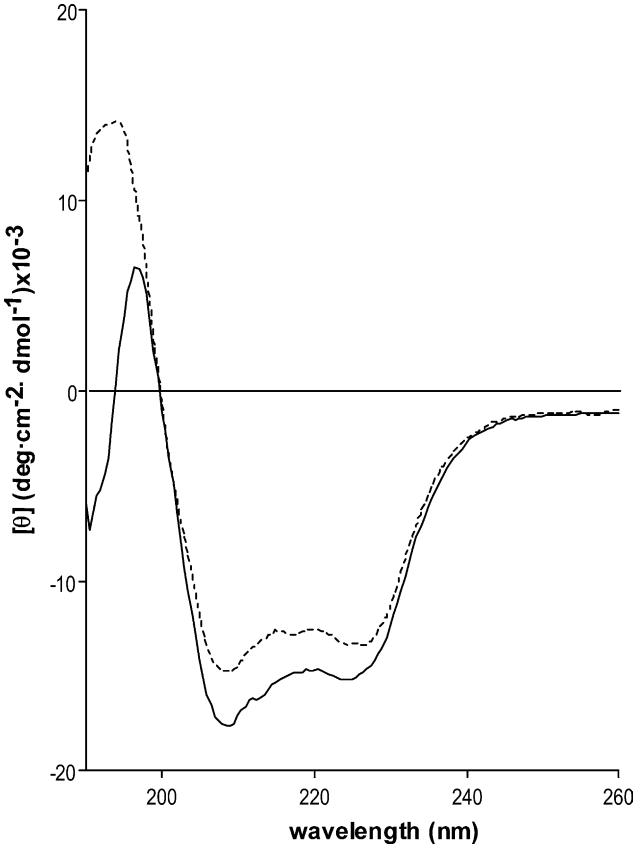

Fig. 3. Far-UV circular dichroism spectra for DmsD. Spectrum of form D (------) and forms A/B (——) in 10 mM phosphate buffer at pH 7.0.

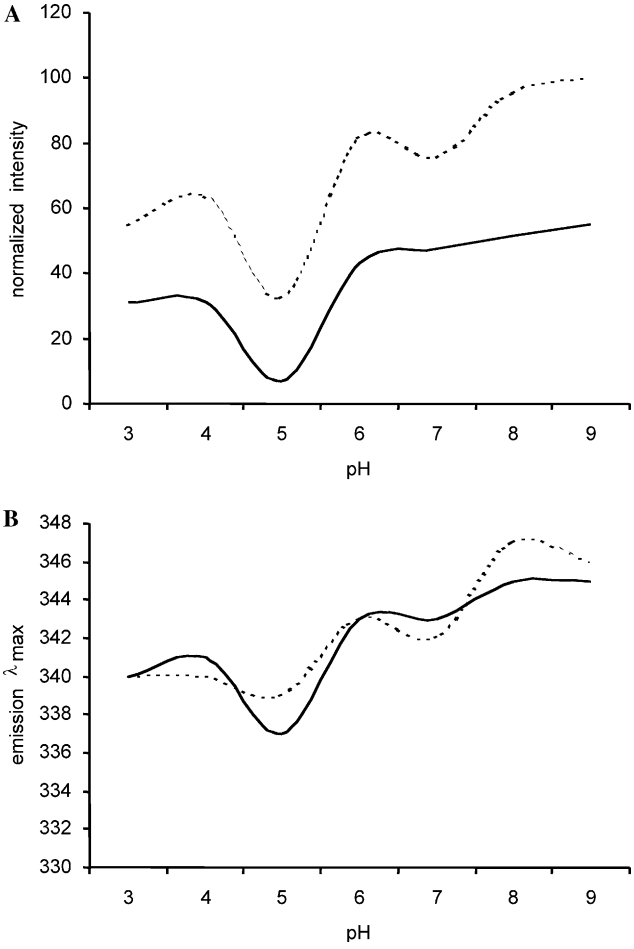

Fig. 4. pH titration of DmsD followed by fluorescence spectroscopy. DmsD at 0.015 mg/ml of form D (——) and forms A/B (------). Samples were excited at 280 nm and emission was collected at 290–450 nm for an average of 5 scans with 5 nm bandpasses. (A) Effect of pH on the fluorescence intensity of form D and forms A/B with both showing a steep decline in fluorescence intensity at pH 5. (B) Change in wavelength of maximum fluorescence emission.

Our observations indicated that DmsD form A/B can be converted to show the ladder (form D) in a native gel by overnight exposure to pH 5, which is at a pH which shows a major feature in Fig. 4. The chromatogram in Fig. 1B also reflects this conversion: forms A/B run as D in elution buffer of pH 5. However, form D did not interconvert to forms A/B by pH treatment. Only urea denaturation/renaturation treatment influenced form D conversion to forms A/B (Fig. 2, lanes 5 and 6).

Activity of folding forms

The functionality of DmsD folding forms was assessed using a style of a Far-Western blot. A chimera of the twin-arginine leader sequence from *E. coli* DmsA as an N-terminal fusion to glutathione *S*-transferase was employed. Purified DmsA leader GST chimera was blotted onto nitrocellulose, followed by incubation with

Fig. 5. Far-Western dot blots demonstrating leader peptide binding activity. (A) Sample incubated in 20 mM Tris–HCl, pH 7.0, 100 mM NaCl. (B) As in (A), but with 10 mM DTT. (C) As in (A) but with 2% SDS. (D) As in (A) but with the addition of urea to 4 M. (E) Sample incubated in 20 mM Na-acetate, pH 5.0, 100 mM NaCl.

DmsD under various conditions. The results in Fig. 5 show that DmsD forms A/B and D have leader binding activity.

Discussion

DmsD was purified using an N-terminal hexa-histidine tag on a Ni-metal interaction matrix, followed by size-exclusion chromatography. The purified protein was found to be pure by SDS–PAGE and by identification through Western blotting against the additional T_7 epitope adjacent to the His_6 tag (not shown). SDS–PAGE analysis of the peaks from the size-exclusion column found that several peaks contained DmsD. Additionally, after calibration of the column, we observed that the later eluting peaks corresponded to apparent molecular weights significantly smaller than that of DmsD, despite the fact that the protein still migrated at the appropriate position on an SDS–PAGE.

Others have reported approaches to analyzing oligomeric proteins by the interconversion of their multimeric states through changes in ionic strength [16], concentration levels of multimers [17], and pH [12,18]. In this study, we used native and denaturing gel electrophoresis to investigate the protein interconversion between form D and forms A/B. After denaturing either form D or forms A/B in 8 M urea, all forms refolded to the same monomer form.

Native-PAGE experiments demonstrate that DmsD has a number of stable folding forms. In particular, form D migrates as a ladder of distinct bands. The DLS and SEC data on form D does not show higher molecular weight species; this indicates that the ladder is not a series of increasing multimer forms. DmsD consists of 209 amino acids, with only two cysteine residues not expected to play any structural role towards the stability of DmsD. However, DmsD has a high ratio of acidic to basic amino acids, which in concert with Asn and Gln are polar groups that can promote sub-unit association. These findings suggest to us that the differential exposure of negatively charged groups on the protein surface accounts for the presence of various distinct conformational states under native gel conditions; this results in the formation of a ladder of bands shown in Fig. 2.

This assumption is supported when we compare the migration of DmsD form D by gel electrophoresis to the gel electrophoretic analyses of the gradual modification of bovine ribonuclease A with succinic anhydride [16]. The successive reduction of surface net charge of ribonuclease A produced a ladder of discrete bands of protein from a sample simultaneously containing all the modified species. This phenomenon is not only associated with unfolded proteins but with folded proteins which have groups with different pK_a values, and also associated with conformational changes or ion binding of folded proteins. Additionally, the regular ladder seen in Fig. 2 has previously been shown to be associated with unfolded or partially folded proteins [17,18].

During modification of the purification protocol we were able to maximize the purification so that the monomer form (B) was the major species purified with a small amount of dimer (A) and trace amounts of other forms. *S. massilia* TorD has also been purified as a mixture of monomer and dimer [12]. *S. massilia* TorD was purified in phosphate buffer and eluted with a low imidazole concentration. We have also purified *E. coli* TorD with our method; we obtained only trace amounts of forms C and/or D, with the majority being monomer with some dimer, as with DmsD. It is not clear if the 'monomer' form of TorD described by Trainer et al. [12] was in fact equivalent to our form D, which is also monomeric. Additionally, the closer *E. coli* DmsD homologue, YcdY, was also purified with a similar multiple molecular form distribution (unpublished results); this suggests these different folding forms may be a characteristic of this family of proteins.

Trainer et al. [12] observed that the related protein SmTorD underwent monomer–dimer interconversion at low pH. However, we considered this to be the interconversion between form D and forms A/B. In our case interconversion between the monomer and dimer appears to be only concentration-dependent. We found we could generate form D from forms A/B with a pH 5 treatment. However, treatment of form D with 4 M urea (a concentration just before denaturation begins) demonstrated a collapse of the ladder in native gels to a band similar to forms A/B.

DmsD is predicted to be a helix-rich protein. Circular dichroism analysis confirms this; forms A/B and form D have similar amounts of α-helical structure. For the

most part, all forms of the protein examined showed similar fluorescence spectral characteristics. Additionally, using a Far-Western dot blot we show that forms A/B and form D have leader binding acitivity.

DmsD was initially identified as a protein that interacts with the twin-arginine leader sequence of DmsA [10]. This protein has been classified based on sequence similarity as a member of the TorD family of proteins. The recent structure of TorD dimer from *S. massilia* shows a unique peptide fold organized into a two-domain structure [11]. DmsD is homologous to this protein and it is reasonable to assume that DmsD would at least follow a similar architecture. The solved structure of TorD displayed domain swapping or exchange that was observed in other structures [19,20]. Domain exchange, or partially formed domain swapped structures, resulting in differential exposure of charged residues through such an event, could be an explanation for the observation of the various molecular forms observed here as form D.

The proposed function of these oxidoreductase-specific chaperones is to hold the protein in a co-factor receptive competent state and to facilitate the maturation of the precursor form of the enzyme. Additionally, these proteins may play a role in targeting their substrates to the Tat [21]. It may well be that the unique folding forms and folding domain exchange may be part of the mechanism of binding and release of this class of accessory proteins.

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