Effect of Signal Peptide on Stability and Folding of Escherichia coli Thioredoxin

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Abstract

The signal peptide plays a key role in targeting and membrane insertion of secretory and membrane proteins in both prokaryotes and eukaryotes. In E. coli, recombinant proteins can be targeted to the periplasmic space by fusing naturally occurring signal sequences to their N-terminus. The model protein thioredoxin was fused at its N-terminus with malE and pelB signal sequences. While WT and the pelB fusion are soluble when expressed, the malE fusion was targeted to inclusion bodies and was refolded in vitro to yield a monomeric product with identical secondary structure to WT thioredoxin. The purified recombinant proteins were studied with respect to their thermodynamic stability, aggregation propensity, and activity, and compared with wild type thioredoxin, without a signal sequence. The presence of signal sequences leads to thermodynamic destabilization, reduces the activity and increases the aggregation propensity, with malE having much larger effects than pelB. These studies show that besides acting as address labels, signal sequences can modulate protein stability and aggregation in a sequence dependent manner.

Introduction

In E. coli two distinct pathways exist for the export of proteins across the cytoplasmic membrane. The majority of protein translocation across the cytoplasmic membrane occurs in the unfolded state via the Sec translocon [1,2]. Another pathway is the twin-arginine translocation (Tat) pathway, so called because of the signature Arg-Arg motif found near the N terminus of the leader peptide of proteins that are engaged in this mode of export from the cytoplasm. Tat pathway translocates folded proteins post-translationally via the Tat translocon [3,4].

Proteins which are exported through the Sec translocon contain a hydrophobic signal sequence at their N termini [5]. Transloca- tion can be co-translational or post-translational. The former pathway is used for proteins with hydrophobic transmembrane segments or secreted proteins which have more hydrophobic signal sequences than those employed in the latter, post-translational pathway [6]. In the co-translational pathway, upon emerging from the ribosome, the N-terminal signal sequence binds to the signal recognition particle (SRP). Following interaction with the membrane receptor FisY, the complex of nascent chain and ribosome is transferred to the SecYEG translocase. In the latter post-translational pathway, upon emerging from the ribosome, proteins bind first to trigger factor, then SecB and SecA. Binding of SecA bound preproteins to the SecYEG translocase initiates the process of translocation [7,8]. Prior to translocation, pre-proteins must be maintained in an export competent conformation in the cytoplasm which is thought to be a loosely folded, protease-sensitive structure [9]. The export competent conformation is maintained by chaperone proteins SecB, GroEL, DnaK, and DnaJ, which also aid in preventing aggregation and improper intramolecular interactions of the exported proteins [10–15]. In addition, the presence of non-optimal codons in the signal sequence have been shown to play an important role in export for both SecB and SRP dependent export [16,17]. Recombinant proteins in E. coli can be targeted to the periplasmic space via the Sec-dependent pathway by fusing naturally occurring signal sequences to their N-terminus. Signal sequences can also be present within a protein or at its C-terminal end. For some proteins, periplasmic expression is advantageous over cytoplasmic production in a number of ways. For instance, an authentic N-terminal Methionine can be obtained after removal of signal sequences by leader peptidases. The periplasm is also conducive to disulfide formation, has fewer proteases compared to the cytoplasm and many have their own specific substrates. Finally, there are fewer proteins in the periplasm than the cytoplasm and its content can be selectively released by osmotic shock or other strategies thereby facilitating protein purification [18,19].
The signal peptide plays a key role in targeting and membrane insertion of secretory and membrane proteins in both prokaryotes and eukaryotes [20,21]. After membrane insertion, signal sequences are cleaved off by the membrane bound signal peptidases. Signal sequences have a common tripartite structure consisting of a positively charged N-terminus (N-region), a stretch of 7–15 hydrophobic residues (H-region) and a more polar region containing helix breaking Proline and Glycine residues as well as the signal peptide cleavage site (C-region) [5,22].

E. coli signal sequences are distinct for proteins that are periplasmic, inner and outer membrane-bound, and secreted outside the cell [23]. Sequence differences are also observed between mycoplasmas and other gram-negatives such as E. coli [24]. E. coli signal peptides can replace the native signal peptide of heterologous proteins for efficient expression in E. coli [25].

We have previously shown that the maltose-binding protein containing its native the N-terminal 26-residue malE signal peptide is substantially less stable and more aggregation prone than the corresponding mature protein [14,15]. We now explore the effects of two different signal peptides, pelB and malE on protein stability and aggregation in a smaller protein, E. coli thioredoxin. pelB refers to the 22 N-terminal leader sequence of pectinatease B of Erwinia carotovora CE [26]. The pelB leader sequence when attached to a protein, directs the protein to the bacterial periplasm, where the sequence is removed by a signal peptidase. pelB has been used to direct the coat protein-antigen fusions to the cell surface in engineered bacteriophages used for the purpose of phage display [27]. Both pelB and malE signal peptides utilize the post-translational translocation pathway. This has been experimentally shown for malE [28,29] and inferred for pelB based on the hydrophobicity of the signal sequence [27].

E. coli thioredoxin is a 108 amino acid long, heat stable and redox active polypeptide containing 2 cysteine, 5 proline and 2 tryptophan residues [30] whose folding pathway has been well characterized. The two tryptophans are at positions 28 and 31. Trp31 is conserved throughout the known thioredoxin sequences, while Trp28, is conserved only in prokaryotes and is replaced by serine in eukaryotes [31]. The active site disulfide is located close to the two tryptophans in the sequence (Trp28-Ala-Glu-Trp31-Cys32-Gly-Pro-Cys35-) as well as in the three dimensional structure [32–34]. This proximity results in strong quenching of tryptophan fluorescence in the native protein that is relieved upon reduction of the disulfide or denaturation of the protein [35,36]. The single disulfide bond bridges the first and fourth residue of a type III reverse turn involving residues [32–35] that likely persists in the denatured protein and could in principle direct the folding of polypeptide into its native confirmation [36,37]. Thioredoxin has also been used as a fusion partner to facilitate folding of other proteins [37,38]. Thioredoxin is well characterized in terms of its structure, stability and folding. For these reasons it is a useful model system to study the effects of signal peptides on protein stability and folding. In the present study, we compare properties of WT Trx with those of fusions of Trx with signal peptides of malE (malE Trx) and pelB (pelB Trx) respectively.

![Figure 1. Superdex 75 gel filtration elution profiles for Thioredoxin derivatives. Profile indicates that all proteins are monomeric. The inset shows the calibration curve using the marker proteins albumin, ovalbumin, chymotrypsinogen and ribonucleaseA with molecular weights of 67, 43, 24 and 13 kDa respectively, pelB Trx and malE Trx have identical elution profiles. The gel filtration column used was an analytical Superdex–75 (GE Healthcare, Column volume, Vt = 24 ml, void volume = 8 ml) column. doi:10.1371/journal.pone.0063442.g001](http://www.plosone.org/doi/abs/10.1371/journal.pone.0063442.g001)
Materials and Methods

Plasmids Used

Two leader sequences involved in directing proteins to the periplasmic space, pelB and malE were used for the experimental studies. These sequences are indicated below.

MKYLLP TA EAGLLLLLA APQIA (pelB).
MKKTGAR ILALS ELTTM MFASALA (malE).

Figure 2. CD spectra of WT Trx (-), pelB Trx (-) and malE Trx (-). (A) Far UV CD spectra of WT Trx (-), pelB Trx (-) and malE Trx (-) were obtained with 10 uM protein solution in CGH-10 buffer, pD 7.4 at 25°C with a 0.1 cm path-length cuvette. (B) Near UV CD spectra were obtained using protein concentrations of 600 uM, 400 uM and 250 uM for WT Trx, pelB Trx, and malE Trx respectively. Measurements were done in CGH-10 buffer, pD 7.4 at 25°C with a 0.2 cm path-length cuvette.

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The A to E mutation in each sequence introduced to prevent translocation and signal peptide cleavage is highlighted in bold. pelB (A9E)Trx and malE (A14E)Trx fusions were constructed by overlap PCR and cloned into pET22b(+) and pET20b(+) vectors respectively between the NdeI and HindIII sites.

Protein Expression and Purification

WT Trx and its derivatives were transformed into *E. coli* BL21 (DE3) cells and expressed under the control of the T7 promoter at 37°C. WT Trx was isolated following chloroform shock and purified using Q-Sepharose ion-exchange chromatography as described previously [40]. The cells were grown in 1L of Luria-Broth (LB) at 37°Ct oD600 = 0.6, induced with 0.4 mM IPTG and pelleted by centrifugation after 4 hours. Chloroform was added to the resuspended pellet in an equal volume and incubated at room temperature under shaking condition. This was followed by addition of 100 ml of buffer (20 mM Tris, 25 mM NaCl, pH 7.4). The resulting mixture was spun at 4000 rpm at 4°C to separate the chloroform layer from the aqueous layer. The aqueous layer was loaded onto a Q-Sepharose column pre-equilibrated with 20 mM Tris (pH 7.4) followed by washing with 20 mM Tris, 25 mM NaCl (pH 7.4). A linear gradient of 25–500 mM NaCl in 20 mM Tris, pH 7.4 was used to elute the protein. Thioredoxin elutes between 100 and 125 mM NaCl. Purified protein fractions were dialyzed into CGH-10 buffer (10 mM each of citrate, glycine, and HEPES) (pH 7.4) and concentrated to a final concentration of 6 mg/ml. pelB Trx was purified from the soluble lysate in a similar fashion on a Q-Sepharose column, following cell lysis by sonication. The proteins were dialyzed and concentrated to a final concentration of 4 mg/ml. Tricine-PAGE confirmed the purity of proteins. Purified proteins were stored in aliquots at −70°C.

Mass Spectrometry

Prior to mass spectrometry, protein samples were desalted into water using a PD-10 column (GE Healthcare). ESI-MS was performed on a Micro mass machine in positive ion mode with the desolvation temperature set at 150°C.

Analytical Gel Filtration

Proteins were subjected to gel filtration chromatography using an analytical Superdex-75 (GE Healthcare, Column volume, Vt = 24 ml, void volume = 8 ml) column on a Duo Flow FPLC system (BioRad). The column was equilibrated with CGH-10 buffer, pH7.4. 25–40 μg of protein in 250 μl was loaded on to the column and eluted at a flow rate of 0.4 ml/min.

CD Measurements

Far UV CD spectra were acquired on a JASCO J715 spectropolarimeter. A protein concentration of 10 μM was used in a 1 nm path length quartz cuvette. Measurements were done at

Figure 3. ANS Fluorescence emission spectra. The data points are shown as closed circle (●) for WT Trx, open circle (○) for pelB Trx, closed triangle (▲) for malE Trx, and open triangle (△) for MBP. Curves for WT Trx, pelB Trx and malE Trx are not distinguishable due to overlapping data. Protein concentration was 3 μM and ANS concentration was 300 μM. Sample excitation was at 388 nm. All Trx spectra were acquired in CGH 10 buffer pH 7.4 and the MBP spectrum was acquired in CGH 10 buffer pH 2.5.
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25°C over a wavelength range of 200 to 250 nm at a scan rate of 50 nm/min. Data was collected with response time of 4 s, 2 nm bandwidth and an average of four scans. Buffer scans were acquired under similar conditions and subtracted from the protein spectrum before analysis. Mean residue ellipticity was calculated as described [41].

Near UV CD spectra were acquired on a JASCO J715 spectropolarimeter. Protein concentrations of 600 μM, 400 μM and 250 μM for WT Trx, pelB Trx, and maIE Trx respectively were used in a 2 mm path length quartz cuvette. Measurements were done at 25°C, over a wavelength range of 250 to 300 nm, at a scan rate of 10 nm/min. Data was collected with response time of 8 s, bandwidth of 2 nm and an average of four scans was taken. Buffer scans were acquired under similar conditions and subtracted from the protein spectrum before analysis. Mean residue ellipticity was calculated as described [41].

Fluorescence Measurements
ANS fluorescence measurements were done at 25°C on a JASCO FP-6300 spectrophluorometer in a 1 cm water-jacketed cell using an excitation wavelength of 388 nm and an emission wavelength range of 420–550 nm. Spectra were averaged over four consecutive scans. As a positive control, a previously characterised molten globule of maltose-binding protein (MBP) at pH 2.5 was used [42].

Proteolytic Digestion (WT Trx, pelB Trx and maIE Trx)
Controlled proteolysis was carried out in CGH-10 buffer, pH 7.4 containing 150 mM sodium chloride at 37°C using the proteases Papain and Proteinase K. The enzyme-substrate ratio used was 2% (w/w). Final protein concentrations used in the reaction were 1 mg/ml, 1 mg/ml and 0.5 mg/ml for WT Trx, pelB Trx and maIE Trx respectively. The proteolytic mixture was kept for either 0 minute (control) or for 30 minutes at 37°C. The reaction was quenched with 1 mM Iodoacetic acid (Papain) and 5 μM Phenylmethanesulfonic acid, PMSF (Proteinase K). Subsequently, SDS-PAGE loading dye was added (2% SDS, 0.1% Bromophenol Blue, 10% Glycerol and 5% β-mercaptoethanol).

Samples were subjected to 15% Tricine-PAGE after boiling for 10 min followed by staining in Coomassie Brilliant Blue R250.

Isothermal GdmCl Denaturation Studies
Chemical denaturation studies were carried out by monitoring the Trp fluorescence signal at 340 nm in a JASC0 FP-6300 spectropolarimeter, using an excitation wavelength of 280 nm. Excitation and emission bandwidth used were 2.5 nm and 5 nm respectively. Protein concentrations used were 12 μM, 16 μM and 16 μM for pelB Trx, WT Trx and maIE Trx respectively. Proteins were incubated in different concentrations (0 to 6 M) of GdmCl in CGH-10 buffer (pH 7.4) at 25°C, overnight for equilibrium to be established prior to fluorescence measurement. Data were fit to a two-state N to U model [43].

Thermal Denaturation Studies
Thermodynamic parameters for thermal denaturation were measured by differential scanning calorimetry (DSC). Proteins (0.2 mg/ml) were subjected to a thermal gradient from 30°C to 110°C at a scan rate of 60°C/h in CGH-10 buffer (pH 7.4). DSC for maIE Trx at a higher concentration (2.5 mg/ml) was also carried out. Data were analyzed as described previously [41].

Refolding in Buffer
The proteins were denatured in 6 M GdmCl for at least 6 to 8 hours and refolding was initiated by rapid dilution (10-fold) into CGH-10 buffer (pH 7.4). Refolding was measured by monitoring the intrinsic Trp fluorescence. The spectra were obtained after 1 hour over a range of 300 nm to 400 nm using an excitation wavelength of 280 nm. The emission and excitation bandwidths used were 5 and 2.5 nm respectively. Protein concentrations used for CD and fluorescence were 10 μM and 5 μM respectively.

Insulin Reduction Assay
The activity of the Trx proteins was assessed by the insulin reduction assay described by Holmgren [44]. Protein concentration used was 5 μM.

Refolding in the Presence of Crowding Agent
The proteins were denatured in 4 M GdmCl in CGH-10 (pH 7.4) for about 6 hours before refolding studies were carried out. Aggregation propensity of the proteins was studied by following the scattering at 320 nm during refolding. Refolding was initiated by 10-fold rapid dilution of 100 μM denatured proteins in 4 M GdmCl (CGH-10 buffer, pH 7.4) into CGH-10 buffer (pH 7.4) without or with the crowding agent, 30% Ficoll-70. Proteins were refolded to final concentrations of 2, 5, 7.5 and 10 μM.

Analysis of Signal Peptide Aggregation Propensities
Aggregation propensity profiles for various signal sequences were calculated using three different servers, namely Zyggregator [45], PASTA [46], AGGRESCAN [47]. The Zyggregator server outputs the aggregation propensity score, Zagg for every amino acid in the query protein sequence. All the calculations were done at pH 7.4. A stretch of amino acid sequence having Zagg values in the range of 0.05–0.06 is considered to have high aggregation propensity. The region with high Zagg values is considered to be involved in intermolecular pairing, resulting in aggregation. The amyloidogenic regions of the human amyloid β-peptide (APβ-40) possess high Zagg values in the range of 0.05–0.06. AGGRESCAN

Figure 4. Tricine-PAGE analysis of proteolytic digests of WT Trx, pelB Trx and maIE Trx. These were performed at 37°C for 30 min and demonstrate that the signal peptides are protease accessible. Lanes 1–3 show undigested, papain and Proteinase K digested WT Trx. Lanes 4–6 show undigested, papain and Proteinase K digested pelB Trx. Lanes 7–9 show undigested, papain and Proteinase K digested maIE Trx respectively. Proteolysis was stopped after 30 min by the addition of 1 μM Iodoacetic acid for Papain and 5 μM Phenylmethanesulfonic acid (PMSF) for Proteinase K. Samples were boiled with SDS-PAGE gel loading dye (2% SDS, 0.1% bromophenol blue, 10% Glycerol and 5% β-mercaptoethanol) prior to loading on the gel. Following electrophoresis, proteins were visualized by staining with Coomassie brilliant Blue R250. The relevant bands are enclosed by a box.
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predicts hot-spots of aggregation in the query amino acid sequence based on a propensity scale derived from in vivo aggregation experiments. The amino acids with propensity values greater than 0.02 are considered as hot-spots of aggregation. Window width of 5 was used for these calculations. In addition, average hydrophobicity of the query amino acids, as a possible probe for aggregation, was also calculated using the program PREDBUR [48]. The width of the sliding window was set to 7. All four algorithms were applied to the pelB and malE signal sequences. To assess the prediction accuracies of Zagg, a control set of phoA, treA, and pcoE signal peptides, previously studied as soluble Trx fusion systems, was also used.

**Results**

**Protein Expression**

The malE A14E mutation has been described previously [49] and contains an A to E mutation in the hydrophobic central region of the signal peptide. This mutation renders the signal peptide export defective. We therefore hypothesized that a similar mutation in the pelB signal sequence (A9E) would also have a similar effect. To confirm this hypothesis the variants of Trx having mutations in the leader sequences, pelB Trx and malE Trx were cloned into vector pET22b(+) and pET20b(+) respectively. pelB Trx and malE Trx contains mutations A9E and A14E in pelB

![Figure 5. Isothermal GdmCl denaturation.](image-url)
and malE signal sequences respectively, to prevent translocation and signal peptide cleavage of the corresponding Trx fusions. Although, Trx is a cytoplasmic protein, it is released into the periplasm following chloroform shock [50]. WT Trx and pelB Trx could be isolated in soluble form and purified on a Q Sepharose column with yields of 70 mg/l and 20 mg/l respectively. malE Trx was refolded from inclusion bodies with a yield of 10–15 mg/l. As expected signal sequence remain uncleaved in pelB Trx and malE Trx. Tricine-PAGE confirmed the purity of the proteins. Molecular weights of the proteins were determined accurately by ESI-MS. Representative ESI-MS traces are shown in Figure S1A-C. For WT Trx, the expected and observed MW’s are 11673.3 and 11672.8 Da respectively (Figure S1A). Both signal peptide containing proteins, pelB Trx and malE Trx contain intact signal peptide. For pelB Trx, the expected and observed MW’s are 14075 Da and 14073.5 Da (Figure S1B), while for malE Trx the expected and observed MW’s are 14571Da and 14573.8 Da respectively (Figure S1C).

Analytical Gel Filtration

The aggregation state of the proteins was examined by performing gel filtration studies. The elution volumes were 15.1 ml for WT Trx and 12.5 ml for both pelB Trx and malE Trx. malE Trx and pelB Trx elute between chymotrypsinogen and RNaseA and appear to be monomeric (Figure 1).

CD Measurements

Far UV CD (200–250 nm) serves as a good reporter of the secondary structural contents of proteins under different conditions [51]. The mean residue ellipticity (MRE) in the far UV range as a function of wavelength is shown in Figure 2 quantitatively, MRE values are similar for all the three proteins. From WT Trx to malE Trx, respectively. Similar values of unfolding free energies have been previously determined for WT Trx [52,53]. A similar destabilization of malE MBP relative to mature MBP lacking the overall structure of the protein.

Near UV CD (200–250 nm) serves as reporter of the tertiary structure of proteins. The mean residue ellipticity (MRE) in the Near UV range as a function of wavelength is shown in Figure 2B. Quantitatively, MRE values are similar for WT Trx and pelB Trx but malE Trx shows somewhat less structure than WT Trx and pelB Trx.

ANS Binding

ANS binding of the proteins was monitored at pH 7.4 for WT Trx, pelB Trx and malE Trx (Figure 3). As compared to the control molten globule of MBP at pH 2.5, none of the proteins show binding to ANS which indicates an absence of molten globule formation at neutral pH.

Proteolytic Digestion

Proteolysis was carried out using Proteinase K and Papain for 30 minutes at 37°C. Under these conditions, the signal peptide in pelB Trx and malE Trx is rapidly cleaved. Following cleavage of signal peptide, pelB Trx and malE Trx which now lack the major portion of signal peptide are resistant to further digestion like WT Trx (Figure 4). This suggests that in the folded state, the signal peptide is accessible to proteases and does not significantly perturb the overall structure of the protein.

Isothermal GdmCl Denaturation Studies for WT Trx, pelB Trx and malE Trx

The unfolding transition was monitored using the Trp fluorescence signal at 340 nm (Figure 5A). The continuous solid line through the data is a fit to a two-state NU model (Figure 5A and 5B) [43] and yields a Cm of 2.60 M for WT Trx, 2.50 M for pelB Trx and 2.20 M for malE Trx. The free energies of unfolding at zero denaturant concentration (ΔG°) were found to be 8.9±0.1, 8.7±0.1 and 7.5±0.1 kcal/mol for WT Trx, pelB Trx and malE Trx, respectively. Similar values of unfolding free energies have been previously determined for WT Trx [52,53]. A similar destabilization of malE MBP relative to mature MBP lacking the malE signal peptide was seen previously [49].

Thermal Denaturation Studies

The energetics of thermal unfolding was characterized by examining the thermal stability of each protein employing high-sensitivity differential scanning calorimetric (DSC) measurements. Figure 6 shows buffer-corrected partial molar excess heat capacity data. The thermal stability (Tm) of pelB Trx was lower by ~6°C compared to WT Trx (Figure 6A and Table 1), while malE Trx shows considerably lower thermal stability with an unfolding transition at 72°C (Figure 6B and Table 1). While thermal unfolding of WT Trx and pelB Trx were reversible and two-state, thermal unfolding of malE Trx was completely irreversible and non-two-state, suggesting that unfolding is followed or accompanied by aggregation. When thermal unfolding of malE Trx was carried out at an approximately tenfold higher concentration (Figure 6C) there is an observable decrease in the thermal stability (of ~4°C) and broadening of the transition (Figure 6C and Table 1), also consistent with increased aggregation at higher concentrations of malE Trx. Since the thermal unfolding at both the concentrations is irreversible, detailed thermodynamic analysis

| Table 1. Thermal denaturation parameters at pH 7.4 for WT Trx, pelB Trx and malE Trx obtained from DSC. |
|----------------------------------|-----------------|-----------------|
| Protein                         | ΔH° (Tm) (kcal/mol) | Tm (°C) |
|----------------------------------|-----------------|-----------------|
| WT Trx                           | 112±0.41        | 87.3±0.03       |
| pelB Trx                         | 102±0.31        | 81.1±0.02       |
| malE Trx*                        | 16±0.15         | 71.9±0.04       |
| malE Trx (Concentration 2.5 mg/ml)| 18±0.14        | 68.4±0.05       |

The protein concentration was 0.2 mg/ml, unless mentioned otherwise.
*Since thermal unfolding is irreversible for this protein, these thermodynamic parameters are apparent values.

Enthalpies are calorimetric enthalpies.

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Figure 6. Representative DSC scans of WT Trx, pelB Trx and malE Trx. Scans were carried out in CGH-10 buffer (pH 7.4). The scan rate was 60°C/h and protein concentration was 0.2 mg/ml. Baseline subtracted excess heat capacity data as a function of temperature are shown. The data indicate that protein stability increases in the order malE Trx < pelB Trx < WT Trx. The data points are shown as closed circle (•) for WT Trx, open circle (○) for pelB Trx and closed triangle (▼) for malE Trx, all at 0.2 mg/ml concentration and open triangle (△) for malE Trx at a concentration of 2.5 mg/ml (A) Representative DSC scans of WT Trx and pelB Trx, the line shows the fitting to a non-two state model with one peak. (B) Representative DSC scans of malE Trx at 0.2 mg/ml concentration, the line shows the fitting to a non-two state model with a single peak. (C) Representative DSC scans of malE Trx at 2.5 mg/ml concentration; the line shows the fitting to a non-two-state with a single peak.
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![Graphs showing fluorescence intensity vs. wavelength for A, B, and C conditions.](image-url)
is difficult. However, the lower thermal stability of malE Trx is
clear from the data.

Refolding in Buffer

CD spectra before and after refolding were virtually identical for
all three proteins (data not shown). Fluorescence spectra for WT
Trx and pelB Trx, before and after refolding were similar
(Figure 7). However, for malE Trx there was an appreciable
decrease in the intensity of peak for the refolded protein (Figure 7).
The fraction refolded was 95, 88 and 68 percent for WT Trx, pelB
Trx and malE Trx respectively.

Refolding in the Presence of Crowding Agent

The synthetic polysaccharide Ficoll 70 [54] is used to mimic
intracellular crowded environment in vitro, since it is inert, highly
soluble and has an average molecular mass of ~70 kDa. The
refolding efficiency of the proteins in the presence of Ficoll 70 was
determined by measuring the aggregation propensities of the
proteins by following absorbance at 320 nm during refolding.
Aggregation was not observed when the proteins were refolded in
buffer lacking Ficoll. However, in the presence of Ficoll-70 (30%),
both malE Trx and to a lesser extent pelB Trx were prone to
aggregation (Figure 8). This shows that signal peptide increases the
propensity of thioredoxin towards aggregation while refolding.

Insulin Reduction Assay

Thioredoxin A is an oxidoreductase that has previously been
shown to catalyze the reduction of insulin disulfides by DTT [44].
A quantitative assay was developed which measures the rate of
insulin reduction spectrophotometrically at 650 nm as turbidity
formation from free insulin B chain [44]. Thioredoxin at 5 μM
concentration accelerated the reaction between 0.13 mM insulin
and 0.33 mM DTT. malE Trx showed a marked decrease in
insulin reduction activity while pelB Trx showed intermediate
activity relative to WT Trx (Figure 9). This suggest that the
presence of the hydrophobic signal peptide interferes with protein
activity, even though the signal peptide is accessible to proteases
and does not perturb the secondary structure of the protein.

Prediction of Aggregation Propensities of pelB and malE
Leader Sequences

The average hydrophobicites of the leader sequences were
calculated with a sliding window size of 7 as described previously
[48]. Plots of average hydrophobicity along the protein sequence
have been used to predict the locations of buried and exposed
regions. As shown in Figure 10, both malE (residues 7–14 and 16–
20) and pelB (residues 9–17) show similar hydrophobicity profiles.
Hence, using the hydrophobicity index it is difficult to infer that
the malE leader sequence is more hydrophobic than pelB. In an
alternative approach we calculated aggregation propensities using
the Zyggregator [45], PASTA [46] and AGGRESCAN [47]
algorithms. The $Z_{agg}$ score for pelB and malE sequence were
computed from the Zyggregator server (http://www-vendruscolo.
ch.cam.ac.uk/zyggregator.php). For malE Trx the intrinsic
aggregation propensity profile $Z_{agg}$ reveals one region of high
aggregation propensity (residues 15–22, Figure 10). In contrast
pelB Trx, does not exhibit any region above $Z_{agg} = 1$. This result is
consistent with the observation that the malE sequence can be
more aggregation prone as compared to pelB. The PASTA and
AGGRESCAN algorithms did not show any clear differences
between aggregation propensities of the pelB and malE signal sequences. The AGGRESCAN calculations were also repeated for the entire protein. Here too, no substantial difference was observed between malE Trx and pelB Trx (Figure S2). As a further test we examined the predictions of the Zyggregator and average hydrophobicity algorithms for three other signal sequences, phoA, treA, and pcoE. Previous studies have shown that Trx fusions to each of these signal sequences are soluble when expressed in E. coli. However the fusions are not efficiently translocated to the periplasm [55,56]. As can be seen from Figure 10, for treA and pcoE signal sequences both procedures give very similar results. However, for phoA, the Zyggregator shows a somewhat lower overall aggregation propensity than the average hydrophobicity calculations. For treA, Zyggregator predicts the C-terminal region of the sequence to have high aggregation propensity. However, this may be offset by the very low aggregation propensity of the N-terminal half of the sequence. For pcoE, the overall Zagg profile is similar to that of malE, yet malE Trx is insoluble while pcoE Trx is soluble when expressed in E. coli. The overall conclusion from the above analysis on a limited number of sequences is that of the various approaches for predicting aggregation propensity of signal sequences, Zyggregator does better than the other three programs, though there is still only partial agreement with experimental results. One caveat to the above analysis is that it assumes the signal peptide self-aggregates. However, it is likely that aggregation during folding/unfolding is mediated by interactions between the signal sequence and other hydrophobic stretches that are exposed in the unfolded or intermediate states.

**Discussion**

Signal peptide as a target recognition motif plays a key role in protein translocation and secretion [57,58]. However, its role in directly modulating the properties of pre-proteins and hence in export is not well characterized. In the present study, we compared the effects of two different signal peptides on the folding and stability of thioredoxin relative to WT Trx lacking signal sequence. In such a case, differences in the properties of the three proteins can solely be attributed to the effect of signal peptide on the protein. The current work provides a detailed comparison among WT Trx, pelB Trx and malE Trx in terms of their thermodynamic stabilities, aggregation propensities and activities.

WT Trx and pelB Trx were isolated as soluble proteins from the periplasm while malE Trx was refolded from inclusion bodies, which is an indication of significant destabilization of malE Trx relative to pelB Trx.

ANS is a dye which binds to exposed hydrophobic patches and is used to identify partially folded protein [42]. ANS binding studies with both pelB Trx and malE Trx (Figure 3) indicate that signal peptide fusion does not result in molten globule formation. Gel filtration studies under denaturing conditions did not show any appreciable differences in elution volume amongst the three proteins. However, given the conformational diversity and dynamic nature of the denatured state, it may be difficult to detect transient interactions between signal peptide and the rest of the protein using this technique. Under native conditions, both malE Trx and pelB Trx eluted slightly before WT Trx, consistent with the higher molecular weights of these two proteins.

The resistance of Trx to various proteases used in the current study is consistent with the earlier proteolytic digestion studies of preMBP and MBP which showed rapid removal of signal peptide to yield mature protein [49]. Proteolysis of pelB Trx and malE Trx results in rapid digestion of the signal peptide, suggesting that it is accessible to protease and has only transient interactions with the rest of the protein in the native state. Despite this, both signal peptides affect the stability and aggregation propensity with malE showing larger effects than pelB. pelB Trx shows $\Delta C_m$ and $\Delta T_m$.

![Figure 9. Insulin reduction assay for redox activity.](https://doi.org/10.1371/journal.pone.0063442.g009)
values of $\pm 0.1\ M$ and $-6\ C$ relative to WT Trx. The malE Trx shows low reversibility of unfolding for both chemical and thermal denaturation, with apparent $A_{279}$ and $\Delta T_m$ values of $-0.4\ M$ and $-15\ C$ respectively relative to WT Trx. These data strongly suggest that malE Trx is substantially destabilized relative to pelB Trx and WT Trx. The molecule is also much more aggregation prone.

All three proteins at 10 $\mu$M final concentration can be refolded after chemical denaturation in buffer though the reversibility decreases in the order WT Trx$>$pelB Trx$>$malE Trx. In the presence of the crowding agent Ficoll-70, WT Trx still refolds without aggregation. In contrast, both malE Trx and to a lesser extent pelB Trx show a tendency to aggregate in a concentration dependent manner. This is consistent with malE Trx being targeted to inclusion bodies in vivo. The redox activity of three proteins also decreases in the same order.

Overall, the data suggest that the presence and identity of the signal peptide can modulate thioredoxin stability, aggregation propensity and activity. We have previously shown that the malE signal peptide results in thermodynamic destabilization of its fusion partner, MBP [49] by about 2-6 kcal/mol. Since the signal sequence did not appear to affect the structure of the native state,

Figure 10. Aggregation propensity profiles of various signal peptides. Panels A–C show aggregation propensity profiles for pelB (empty circles,○) and malE (empty triangles,△) sequences calculated using (A) Zyggregor, (B) PASTA, (C) AGGRESCAN. (D) Average hydrophobicity calculated using PREDBUR. Amino acid regions with $Z_{agg}>1$ are considered to be aggregation prone, whereas regions with $Z_{agg}<0$ were assumed to have low aggregation propensities. These upper and lower cut-offs are indicated by dashed line (–) and by dash-dot lines (–) respectively in panels A,E,F,G. The regions with aggregation propensity values above $-0.02$ are considered as hot-spots for aggregation by AGGRESCAN algorithm. The cut-off value is indicated by a dashed line (–) in C. Panel E-G show aggregation propensity profiles calculated using Zyggregor and average hydrophobicity calculated using PREDBUR for three previously studied soluble Trx fusion systems with phoA, treA, and pcoE signal sequences. Here, $Z_{agg}$ is shown in filled circles (●), and average hydrophobicity is indicated with filled triangles (▲). The amino acid sequences for all the signal peptides are given in H. The locations of the AE mutation in pelB and malE are underlined.

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Effect of Signal Peptide on E. coli Thioredoxin

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it was suggested that it might stabilize the unfolded state through hydrophobic interactions. In the present study, the pelB signal sequence led to a decrease in ΔG° of unfolding of Trx by ~0.2 kcal/mol (Figure 5) and a decrease of ΔC in T_m. For malE Trx it was not possible to get accurate thermodynamic data because of the low reversibility thermal denaturation, although there was a substantial decrease in the apparent T_m by 15°C and a decrease in ΔG° of unfolding by ~1.4 kcal/mol with respect to WT Trx. It therefore appears that upon either partial or complete unfolding, the malE signal sequence causes destabilization and irreversible aggregation of the unfolded protein.

In E. coli, proteins are exported to the periplasm by the secretory (sec) pathway, which requires them to be in a translocation competent unfolded state to pass through the membrane-embedded secretion machinery [59]. However, many of these proteins are exported only after much of the polypeptide chain has been synthesized (i.e., post-translationally) [28]. The chaperone SecB in E. coli specifically recognizes a subset of proteins and holds them in an unfolded state until they are transferred to the rest of the secretion machinery [60].

In earlier studies, efforts were made to efficiently export E. coli thioredoxin to the periplasm by a post-translational pathway [29,55] by attaching the post-translational alkaline phosphatase (PhoA) signal sequence to the thioredoxin N-terminus. However, this results in a very small amount of the protein reaching the periplasm. The poor export of thioredoxin is thought to be due to rapid folding of the protein in the cytoplasm, preventing its post-translational translocation across the cytoplasmic membrane [29,61]. Thioredoxin is known to be efficiently exported by the co-translational pathway, when fused to appropriate signal peptides such as dhaA [29]. Thioredoxin fusions with various signal peptides have been used to identify peptides that promote co-translational translocation [53,56]. It has been assumed when Trx fusions are not translocated, this is because of rapid folding of the thioredoxin in the cytoplasm. Consequently, the corresponding signal sequences are believed to mediate SecB dependent post-translational translocation rather than SRP dependent co-translational translocation. The present study demonstrates that the signal peptides can potentially have profound effects on the stability and aggregation propensity of the protein, especially in crowded environments. These factors may also impair translocation. Hence lack of translocation of a signal peptide-Trx fusion can either be due to rapid folding of the Trx reporter or to aggregation of the fusion during cytoplasmic folding. Thus, besides maintaining substrates in an unfolded conformation prior to export, an important function of chaperones such as SecB, may be to prevent signal peptide mediated protein aggregation. In previous studies [14,15], we showed that the malE signal peptide significantly affected MBP folding and aggregation. MBP is a large 370 amino acid multidomain protein with complex folding kinetics. Thioredoxin is a relatively small and stable protein with well characterized folding kinetics, making it an excellent system to understand the perturbing effects of signal peptide on protein folding and stability. Despite having similar hydrophobicity, the malE and pelB signal sequences have significantly different effects on thioredoxin activity, stability and aggregation propensity. Future studies will examine the kinetics of folding, unfolding and aggregation of the malE Trx and pelB Trx fusion proteins.

**Supporting Information**

*Figure S1* ESI-MS spectra of WT Trx, pelB Trx and malE Trx. (A) WT Trx, expected and observed masses of 11673.3 and 11672.8 Da respectively. (B) pelB Trx, expected and observed masses of 14075 and 14073.5 Da respectively. (C) malE Trx, expected and observed masses of 14571 and 14573.8 Da respectively. (TIF)

*Figure S2* The AGGRESCAN profile for the full length proteins, pelB Trx and malE Trx. (TIF)

**Author Contributions**

Designed and executed cloning strategies: RSP PSSA BK. Conceived and designed the experiments: RV. Performed the experiments: LS PS SRK. Analyzed the data: LS PS SRK BVA. Wrote the paper: RV LS PS SRK.

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