Cloning, Expression, Purification and Characterization of a DsbA-like protein from *Wolbachia pipientis*

Mareike Kurz[1], Iñaki Iturbe-Ormaetxe[2], Russell Jarrott[1], Nathan Cowieson[1], Gautier Robin[1], Alun Jones[1,3], Gordon J. King[1,3], Patrick Frei[4], Rudi Glockshuber[4], Scott L. O'Neill[2], Begoña Heras[1*] and Jennifer L. Martin[1*]

[1]Institute for Molecular Bioscience, [2]School of Integrative Biology, [3]ARC Special Research Centre for Functional and Applied Genomics, The University of Queensland, Brisbane QLD 4072, Australia, [4]Institute for Microbiology, ETH Zürich, CH-8092 Zürich, Switzerland

* Joint Corresponding Authors:

Email: b.heras@imb.uq.edu.au, phone: +61 7 33462020, fax: +61 7 33462101

Email: j.martin@imb.uq.edu.au, phone: +61 7 33462016, fax: +61 7 33462101

ABSTRACT

Wolbachia pipientis are obligate endosymbionts that infect a wide range of insect and other arthropod species. They act as reproductive parasites by manipulating the host reproduction machinery to enhance their own transmission. This unusual phenotype is thought to be a consequence of the actions of secreted *Wolbachia* proteins that are likely to contain disulfide bonds to stabilize the protein structure. In bacteria, the introduction or isomerization of disulfide bonds in proteins is catalysed by Dsb proteins. The *Wolbachia* genome encodes two proteins, α-DsbA1 and α-DsbA2, that might catalyse these steps. In this work we focussed on the 234-residue protein α-DsbA1; the gene was cloned and expressed in *E. coli*, the protein was purified and its identity confirmed by mass spectrometry. The sequence identity of α-DsbA1 is similar to that for both dithiol oxidants (*Escherichia coli* DsbA, 12%) and disulfide isomerases (*E coli* DsbC, 14%). We therefore sought to establish whether α-DsbA1 is an oxidant or an isomerase based on functional activity. The purified α-DsbA1 was active in an oxidoreductase assay but had little isomerase activity, indicating that α-DsbA1 is DsbA-like rather than DsbC-like. This work represents the first successful example of the characterization of a recombinant *Wolbachia* protein. Purified α-DsbA1 will now be used in further functional studies to identify protein substrates that could help explain the molecular basis for the unusual *Wolbachia* phenotypes, and in structural studies to explore its relationship to other disulfide oxidoreductase proteins.

INTRODUCTION

Disulfide bond formation is a crucial yet rate limiting step in protein folding [1, 2]. All organisms, ranging from bacteria to humans, encode catalysts that introduce disulfides into secreted proteins and membrane proteins [3]. In eukaryotes, protein folding occurs in the endoplasmic reticulum and disulfide formation is catalysed there, primarily by PDI [4]. For most prokaryotes, the introduction of disulfide bridges occurs in the periplasm and requires proteins of the Dsb (disulfide bond) family [5] that have disulfide/dithiol redox active sites.

Escherichia coli encodes at least six Dsb family members (DsbA, B, C, D, E and G). These proteins catalyze the oxidation (introduction) of disulfide bridges, isomerization (shuffling) of incorrectly introduced disulfide bonds and reduction (removal) of inappropriate disulfide bonds [2, 3, 6]. The oxidative pathway involves two proteins, DsbA and DsbB [3, 7, 8]. DsbB is an inner membrane protein that generates disulfide bonds *de novo*, and transfers these to the key oxidant DsbA. DsbA then introduces disulfide bonds indiscriminately into newly synthesized proteins via a thiol-disulfide exchange reaction. As a consequence, DsbA becomes reduced and must be reoxidized by DsbB. The isomerase pathway of DsbC/DsbG-DsbD isomerizes disulfides that are incorrectly introduced by DsbA [9]. The isomerization is catalyzed by DsbC and DsbG, and these two proteins are maintained in an active dithiol (reduced) form through their interaction with the membrane protein DsbD [3, 8]. Finally, DsbE (CcmG) is a specialized disulfide reductant in the cytochrome c maturation pathway [10].

Dsb homologues are encoded in bacteria other than *E. coli,* and are important for pathogenicity because they help to fold secreted toxins and virulence factors [11, 12]. However, studies on *Mycobacterium tuberculosis* DsbE suggest that extrapolation of the role of Dsb proteins from the *E. coli* model may not be appropriate [13]. Moreover, some bacteria encode Dsb "hybrid" proteins that have features characteristic of both *E. coli* disulfide

oxidants and *E. coli* disulfide isomerases. These findings raise questions regarding the conservation of the Dsb pathway in other organisms and the role of Dsb hybrid proteins.

We are interested in the Dsb pathway in the bacterium *Wolbachia pipientis*, a very common and widely distributed Gram-negative obligate intracellular symbiont. *Wolbachia* is estimated to infect 15-75% of all insects and a wide range of other arthropods and filarial nematodes [14-16]. A characteristic feature of this bacterium is its ability to manipulate its host's reproduction by inducing a series of phenotypes (cytoplasmic incompatibility, feminization, male killing and parthenogenesis among others) that favour infected female reproduction, thus enhancing its own maternal transmission [17]. In filarial nematodes, *Wolbachia* is a mutualist, in that treatment of *Wolbachia* infection with antibiotics leads to developmental arrest and death of the host nematode [18]. Consequently, *Wolbachia* is a target for therapeutic intervention to combat filariasis [19] and may represent a useful biocontrol agent for insect pests and vector-borne parasites [20].

Little is known about the molecular mechanisms mediating the reproductive phenotypes induced by *Wolbachia* or its symbiotic interaction with host cells, though both are thought to involve secreted proteins, which need to be folded correctly to be functional. We have therefore focused our studies on the identification, expression and purification of *Wolbachia* Dsb proteins, that may be important for the oxidative folding of secreted *Wolbachia* proteins and thus for inducing the phenotype and/or mediating communication between both partners in the association. Our analysis of the *Wolbachia pipientis w*Mel genome [21] identified two DsbA-like homologues (WD1055 and WD1312) that we have named α-DsbA1 and α-DsbA2, respectively. These two proteins share 21% sequence identity with each other. Here we describe the methods used to clone, express and purify α-DsbA1 and its characterization as a DsbA. We plan to use the purified protein for structural and functional studies aimed at investigating the role of α-DsbA1.

MATERIALS AND METHODS

Cloning Wolbachia pipientis α-DsbA1

*Wolbachia pipientis w*Mel strain was maintained in *Drosophila melanogaster* yw67c23 flies reared on standard corn flour-sugar-yeast medium at 25ºC. Several flies were homogenized and *Wolbachia* DNA was extracted using an STE-PK extraction method [22]. Full-length *Wolbachia α-DsbA1* (locus WD1055 GenBank accession no AE017196) was amplified from *w*Mel DNA by PCR using primers WDsbA1-Fwd1 (*Nde*I) (5'-CCATATGATTTTTAGACTATTGTTTTTATTAATT-3') and WDsbA1-Rev (*Xho*I) (5'-CCTCGAGCTTCACTATAGCTTTTCC-3'). A gene lacking the 18-residue putative signal peptide was amplified using primers WDsbA1-Fwd2 (*Nde*I) (5'-CCATATGAAGCAAGATTTATCCGATAA-3') and WDsbA1-Rev. These primers replace the two residues immediately after the signal sequence with an ATG start codon (Met) and also introduce two additional residues (Leu-Glu) at the C-terminus. PCR cycling conditions were as follows: 95ºC 3 min, (95ºC 30 s, 52ºC 30s, 72ºC 10 min) x 35 cycles, then 72ºC for 10 min. The reaction mixture contained 250 μM dNTPs, 500 nM of each primer, 1.5 mM $MgCl_2$, 10-100 ng of DNA and 1 unit of Expand DNA Polymerase (Roche) in a final volume of 20 μl. PCR products were gel-purified using a QIAquick Gel Extraction Kit (Qiagen), A-tailed and ligated into pGemT-easy (Promega) following standard protocols. The two constructs of *Wolbachia* α-DsbA1 were digested from pGemT-easy using *Nde*I and *Xho*I, and ligated into these sites in pET42a (Novagen), adding a C-terminal octa-histidine tag to the coding sequence. Positive clones were identified by colony screening and then verified by sequencing using T7 promoter and T7 terminator primers at Macrogen (Korea).

Protein Expression and Purification

E. coli BL21(DE3)pLysS (Invitrogen) was transformed with the *α-dsbA1* constructs for protein expression. Large scale (2 L) expression was carried out at 30°C for 24 h using autoinduction [23]. Cells were harvested and 100 ml lysis buffer (25 mM Tris-HCl pH 7, 150 mM NaCl, 0.5% Triton-X, EDTA-free protease inhibitor cocktail (Roche) and DNase) was added per 1L pellet. To lyse the cells, the resuspended pellet was sonicated (Sonifier 250, Branson) three times for 2 min at constant duty cycle and an output energy of 80 W. The cell lysate was spun down and then incubated for 5 min at room temperature with PrepEase Ni^{2+} affinity resin (USB) (2 g per 100 ml of lysate). The protein-bound resin was transferred to a column and washed extensively (at least 400 ml) with 50 mM Tris-HCl pH 7, 1 M NaCl, 20 mM imidazole using gravity flow, until no protein could be detected in the flow-through. Bound α-DsbA1 was eluted by adding at least 10 ml of 50 mM Tris-HCl pH 7, 150 mM NaCl, 250 mM imidazole to the column. Fractions of 1 ml were collected and tested for protein content using Protein Assay Dye (Biorad) before pooling for subsequent purification.

The protein was further purified by size exclusion chromatography (ÄKTAdesign, GE Healthcare), using a Superdex S200 or Superdex S75 column. The protein was eluted in 25 mM HEPES-NaOH pH 7, 150 mM NaCl and concentrated for further experiments using ultra centrifugal devices (Amicon) with a 10,000 Da cut off.

Protein concentration was determined spectrophotometrically by using a NanoDrop ND-1000 (NanoDrop Technologies) and calculated from absorbance at 280 nm with the calculated molar absorption coefficient $\varepsilon_{280} = 26610\ M^{-1}\ cm^{-1}$ or colorimetrically quantified by using the BCA Protein Assay Kit (Pierce). Purity was assessed by SDS-PAGE analysis and densitometry measurements using Quantity One 4.4 (Biorad). Briefly, after each purification step, aliquots were analysed by SDS-PAGE (NuPAGE novex Bis-tris gel, 4%-12% (Invitrogen)). Before loading, the samples were transferred into 2x loading dye [24] and denatured at 95˚C for 5 min. The gels were run in 1x NuPAGE MOPS SDS-running buffer

(Invitrogen, NP0001) at a constant voltage of 180 V for 50 min and visualized by staining with Coomassie Brilliant Blue (Sigma). The gels were scanned (SilverFast) and analyzed.

The amount of α-DsbA1 at each purification step was estimated using densitometry of Coomassie stained SDS gels (see Methods). Total protein was quantified using the BCA protein assay with BSA as standard and 10 μg was loaded onto each lane. The estimated yield in mg of α-DsbA1 was calculated from the estimated amount of α-DsbA1 in mg/ml and the total volume of sample at each step. Estimated yield (%) was calculated by comparison with the amount of α-DsbA1 in the lysate (=100%). Estimated purity at each step was calculated as the ratio of the estimated amount in mg/ml of α-DsbA1 and total protein.

Circular dichroism

Far-UV synchrotron radiation circular dichroism spectra (SRCD) for α-DsbA1 were recorded at 4°C at station 12.1 of the Daresbury synchrotron radiation source, UK. The protein was at a concentration of 1.1 mg/ml in 50 mM Na Phosphate buffer pH 7.5 and 150 mM NaF. Measurements were made in triplicate in a 0.005 cm path length, quartz cuvette (Hellma). Curve fitting for secondary structure analysis was performed using Selcon3, ContinLL and CDSTTR algorithms available through the CDPro suite of software (http://lamar.colostate.edu/sreeram/CDPro/). The results of each algorithm were averaged.

High Performance Liquid Chromatography/Mass Spectrometry (HPLC/MS)

Purified α-DsbA1 samples (1 mg/ml) in 50 mM HEPES pH 7, 150 mM NaCl buffer were analysed at the Mass Spectrometry Facility at the Institute for Molecular Bioscience, University of Queensland. For HPLC-ESI-MS analysis, samples were dissolved in 5% formic acid. Reverse phase HPLC was performed using a Phenomex 300A, C18 column with 0.1%

formic acid (solvent A) and 90% acetonitrile in 0.1% formic acid (solvent B). Solvent delivery and gradient formation was achieved using an Agilent 1100 binary solvent delivery system. Electrospray MS was performed using a Sciex QSTAR Pulsar/Quadropole time of flight mass spectrometer (Applied Biosystems). A full data scan was acquired at declustering and focussing potentials of 80 V and 280 V, respectively, over the mass range *m/z* 450-2000. Data collection and processing was carried out using Analyst QSi.

Sequence analysis

Sequence alignments and secondary structure predictions of *E. coli* DsbA (EcDsbA) and α-DsbA1 and *E. coli* DsbC (EcDsbC) and α-DsbA1 were performed using the 3D-PSSM server [25]. Annotations of the alignments were performed in Adobe Illustrator.

Biochemical studies

In vitro disulfide reductase activity of α-DsbA1 was measured by the ability of the protein to catalyse the reduction of insulin by DTT, as described previously [26]. The reaction mixtures were prepared fresh in 1 ml cuvettes by adding final concentrations of 0.13 mM insulin (Sigma), 0.33 mM DTT, 0.1 M sodium phosphate, 2 mM EDTA, pH 7 to various concentrations of the protein (4-10 μM). The reduction of insulin was monitored by measuring the optical density of the samples at 650 nm for 80 min at 30 s intervals.

The ability of the protein to isomerize disulfide bonds was evaluated using the scrambled RNaseA (ScRNaseA) assay. Disulfide-scrambled RNaseA (Sigma) was produced using a modified protocol of [27]. Briefly, 70 mg of native RNaseA (four disulfide bonds) was incubated overnight at room temperature in 50 mM Tris-HCl, pH 8 in the presence of 6 M GdmCl and 150 mM DTT. The reduced, unfolded protein was acidified with 100 mM acetic acid/NaOH pH 4 and purified over a PD10 column. ScRNase concentration was determined

spectrophotometrically and the eight free thiol groups were verified using Ellman's assay [28]. For random reoxidation, the reduced scRNase was diluted to a final concentration of 0.5 mg/ml in 50 mM Tris-HCl, pH 8.5 and 6 M GdmCl and incubated in the dark at room temperature for at least three days. The randomly reoxidized RNase was concentrated, acidified and purified as described above. The oxidation of disulfide bonds was confirmed by using Ellman's assay. Activity of α-DsbA1 was evaluated by measuring spectrophotometrically the renaturation of scRNaseA. Reactivated RNaseA regains native disulfide bridges and then can cleave cyclic-2',3'-cytidinemonophosphate (cCMP) into 3'-cytidinemonophosphate (3'CMP), resulting in an increase in absorption at 296 nm.

Purified α-DsbA1 (10 μM) was added to 100 mM sodium phosphate, 1 mM EDTA, 10 μM DTT pH 7 and preincubated for 5' at 25°C. To start the assay, 40 μM scRNaseA was added. At several time points, 200 μl aliquots were taken and added to 600 μl of 4 mM cCMP (in 100 mM sodium phosphate, 1 mM EDTA pH 7), so that the final assay volume was 800 μl. The rate of RNaseA cleavage in the removed aliquots was then monitored spectrophotometrically at 296 nm for 2.5 min, and the fraction of native RNase (in %) was plotted against the incubation time.

RESULTS

Expression

We attempted expression of α-DsbA1 in two ways, (1) by directing it to the cytoplasm through removal of the signal sequence and (2) by directing it to the periplasm with the native 18-residue N-terminal signal sequence. The cytoplasm-directed α-DsbA1 was successfully expressed in a soluble form (Table 1) (Figure 1A). However, the periplasm-directed α-DsbA1 did not appear to be secreted; no α-DsbA1 was detected in the periplasm but a low level of protein was detected by Western blot in the cytoplasmic pellet (not shown). We therefore concluded that α-DsbA1 containing the *Wolbachia pipientis* signal sequence is not transported to the *E coli* periplasm. Because the cytoplasmic-directed protein expressed well, we focused further work on this soluble construct.

Purification

The initial purification step of α-DsbA1 using immobilized metal affinity chromatography (IMAC) extracted 19 mg of 69 % pure protein from 1 L of culture (Table 1). High molecular mass contaminants were removed by size exclusion chromatography. This final purification step yielded 18 mg of homogeneous α-DsbA1 from 1 L of culture (Figure 1).

Purified α-DsbA1 was analysed by HPLC-ESI-MS to verify the molecular mass and identity. The analysis gave an experimental molecular mass of 26446.0 Da (Figure 2), in excellent agreement with the theoretical mass of 26446.5 or 26442.5 Da for reduced or oxidized truncated, C-terminally His-tagged α-DsbA1.

Is the protein a DsbA or a DsbC?

The sequence identity of α-DsbA1 with EcDsbA (12%) and EcDsbC (14%) is similar, and it is therefore unclear from its sequence whether the protein is a dithiol oxidase or an

isomerase. We undertook structural and functional characterizations of the protein to address this question. SRCD analysis of purified α-DsbA1 (Figure 3) indicates that native α-DsbA1 is folded and comprises 43% helix and 13% sheet. This secondary structure content compares well to the known helix and sheet content of EcDsbA (51% helix / 11% sheet) based on its crystal structure [29] but it is also close to that for EcDsbC (38% helix / 23% sheet) calculated from its crystal structure [30]. This result may reflect the fact that both DsbA and DsbC incorporate a thioredoxin fold.

The fold recognition server 3D-PSSM [25] predicts a similar helical/sheet content for α-DsbA1 to that estimated from SRCD (Figure 4A). Furthermore, this analysis indicates that α-DsbA1 has the same overall fold as *E. coli* DsbA despite some differences at the N and C-terminal segments. By comparison, 3D-PSSM comparison of α-DsbA1 and EcDsbC (Figure 4B) reveals a relatively poor alignment with several large gaps. A major difference between EcDsbA and EcDsbC, in structural terms, is that EcDsbA is a monomer whereas EcDsbC is a dimer due to an N-terminal (~60 residue) dimerization domain [30]. Notably, the predicted secondary structure at the N-terminus of α-DsbA1 is very different to that of EcDsbC, indicating that it does not include a dimerization domain. This conclusion is supported by the fact that purified α-DsbA1 is monomeric on the basis of its elution from a size exclusion chromatography column (data not shown). Overall, the secondary structure analysis, sequence comparison and oligomerization state suggests that, structurally, α-DsbA1 is more DsbA-like than DsbC-like.

Biochemical Characterization

The sequence and secondary structure of α-DsbA1 suggests that it more closely resembles the dithiol oxidase EcDsbA than the disulfide isomerase EcDsbC. On the other hand, α-DsbA1 includes residues that are commonly present in DsbC/G-type isomerases. For example, the

residue preceding the *cis*-Pro loop is generally a valine for DsbA, and a threonine for DsbC and DsbG isomerases (Figure 4B) yet α-DsbA1 also has a threonine in this position. To evaluate whether α-DsbA1 is functionally DsbA-like or DsbC-like we performed two assays that discriminate between these two activities. The insulin reduction assay is a general assay for oxidoreductase activity, and specifically probes disulfide reductase activity. As this assay relies on reducing activity, the oxidant EcDsbA has only about 10% the activity of the isomerase EcDsbC. We evaluated the activity of α-DsbA1 in this assay using EcDsbA and EcDsbC as controls. The results (Figure 5) show that EcDsbC (4 μM) rapidly reduces the disulfide bonds of insulin; disulfide reduction is detectable within 3 minutes and is complete in just over 20 min. A higher concentration of EcDsbA (10 μM) yielded detectable insulin disulfide reduction after 19 min. α-DsbA1 (10 μM) was a little less active again, with insulin reduction detectable after 22 min. Overall, the results of this assay indicate that α-DsbA1 is not a disulfide reductase, and is therefore more like the oxidant EcDsbA than the isomerase EcDsbC.

We also performed a disulfide isomerase assay by evaluating the effect of α-DsbA1 to reactivate scrambled RNaseA (scRNaseA). To restore RNaseA activity, the oxidoreductase protein must isomerize the four randomly oxidized disulfide bonds of scRNaseA. Correctly disulfide-bonded RNaseA is active and can convert cCMP to 3'CMP, detectable by an increase in absorption at 296 nm. Inactive RNase has no activity in this assay. The positive control EcDsbC (10 μM), restored ~50% RNase activity within 60 min and ~80% activity after 8 h (Figure 6). By contrast, both EcDsbA and α-DsbA1 had a much lower ability to reactivate RNase, with just ~40% of native activity restored after 8 h (Figure 6). These results show that α-DsbA1 is functionally like DsbA.

DISCUSSION

Over 50% of the *Wolbachia pipientis w*Mel genome encodes proteins with at least two cysteines, and many of these would be expected to form disulfides. This situation suggests that a disulfide pathway must be encoded in *Wolbachia* to ensure rapid and accurate disulfide bond incorporation during protein folding. *Wolbachia* does encode Dsb family members, but whereas *E. coli* encodes at least 6 Dsb proteins, *Wolbachia* encodes just three: α-DsbA1, α-DsbA2 and a DsbB-homologue. We focused on one of these three proteins, α-DsbA1, and generated soluble recombinant *Wolbachia* α-DsbA1 in milligram quantities from an *E. coli* expression system.

The primary sequence of α-DsbA1 incorporates features that are characteristic of both oxidases (DsbA) and isomerases (DsbC). EcDsbA and EcDsbC both belong to the thioredoxin fold superfamily [31] and variations in the middle two residues of the CXXC active site motif are thought to give rise to variations in activity [3]. For example, the oxidant EcDsbA has CXHC and the isomerase EcDsbC has CXYC. Furthermore, the residue preceding the highly conserved *cis*-Pro loop of the thioredoxin fold is a valine in DsbAs and a threonine in DsbCs [32]. Surprisingly, the sequence of α-DsbA1 incorporates features of both oxidases and isomerases; the active site motif is CYHC and the residue preceding the *cis*-Pro loop is a threonine rather than a valine. However, our secondary structure and sequence alignment analysis indicated that α-DsbA1 is more like EcDsbA than EcDsbC.

We then investigated the oxidoreductase activity of α-DsbA1 using the purified recombinant protein. We showed that α-DsbA1 exhibits oxidoreductase activity in the insulin reductase activity, at about the same level of activity as EcDsbA. Furthermore, α-DsbA1 has an activity similar to that of EcDsbA in the scRNase isomerase assay. In both assays, α-DsbA1 is much

less active than EcDsbC. These results indicate that α-DsbA1 is functionally more closely related to the oxidase EcDsbA than to the isomerase EcDsbC.

In conclusion, this work describes the first functional characterisation of a *Wolbachia* protein, overexpressed and purified from *E. coli*. The purified protein, α-DsbA1, is DsbA-like in secondary structure and activity, despite a low sequence homology. The methodology to produce purified and soluble α-DsbA1 now enables structural studies as well as further functional analyses to help elucidate the role of this protein in the folding and secretion of proteins involved in the unusual *Wolbachia* phenotypes. We anticipate that the strategy we used to generate this recombinant *Wolbachia* protein for functional studies can also be applied to other *Wolbachia* targets.

ACKNOWLEDGEMENTS

The work was supported by an Australian National Health and Medical Research Council (NHMRC) Senior Research Fellowship to JLM, Australian Research Council (ARC) grants to JLM and to BH, an NHMRC grant to JLM and BH, a University of Queensland Early Career Researcher Grant to BH, the ETH Zurich and the Schweizerische Nationalfond in the framework of the NCCR Structural Biology program (RG), and an International Postgraduate Research Scholarship (IPRS) to MK.

FIGURE LEGENDS

Figure 1

A. SDS-PAGE analysis of the purification of α-DsbA1. Proteins were separated on NuPage novex 4%-12% SDS-PAGE gel (Invitrogen) and visualized by Coomassie staining. Lane 1: soluble fraction of cell lysate, lane 2: peak fraction after metal (Ni^{2+}) chelate affinity chromatography, lane 3: peak fraction after size exclusion chromatography, lane 4: protein standard. 10 μg of total protein (calculated using BCA protein assay kit) was loaded. Molecular mass standards (in kDa) are indicated at the right of the gel. Expressed α-DsbA1 is indicated by a black box. **B** Lane 1: soluble fraction of cell lysate from BL21(DE3)pLysS with an α-DsbA1 expression construct, the expressed protein is indicated by a black box, lane 2: soluble fraction of untransformed BL21(DE3)pLysS cells, grown in expression media and lysed under the same conditions, lane 3: protein standard

Figure 2

HPLC-ESI-mass spectrum obtained from purified α-DsbA1 showing the major peak at 26446.0 Da (the calculated mass for reduced and oxidized is 26446.5 and 26442.5, respectively). Intensity is in counts per second (cps), and mass is in atomic mass units (amu).

Figure 3

Far-UV SRCD spectra of oxidized α-DsbA1. The spectrum was recorded at 4°C in a 0.005 cm path length quartz cuvette containing 1.1 mg/ml protein in 50 mM Na phosphate buffer, pH 7.5. The calculated secondary structure of α-DsbA1 is 43% α-helix and 13% β-sheet.

Figure 4

Sequence alignment of the mature α-DsbA1 and EcDsbA proteins (**A**) and EcDsbC (**B**) proteins. Secondary structure elements (predicted from sequence analysis for α-DsbA1 using 3D-PSSM or determined from the crystal structure for EcDsbA) are coloured in light grey (helix) and dark grey (β-sheet). Asterisks (*) denote identical residues. In the α-DsbA1 construct used herein, the two Val residues at the N-terminus are replaced with Met and additional residues are engineered at the C-terminus (Leu-Glu-His-His-His-His-His-His-His-His).

Figure 5

The oxidoreductase activity of α-DsbA1 (10 μM, ●) is compared to that for the oxidase EcDsbA (10 μM, ○), isomerase EcDsbC (4 μM, ■) and negative control (no catalyst, ▲). Reactions were performed in a final volume of 1 ml containing 0.13 mM insulin, 0.1 M sodium phosphate, 2 mM EDTA, pH 7 and purified protein. Reactions were started by adding DTT to a final concentration of 0.33 mM. Light scattering at 650 nm, caused by aggregation of reduced insulin, was recorded every 30 s for 80 min. α-DsbA1 has a similar level of activity as EcDsbA.

Figure 6

Isomerase activity of α-DsbA1 (■) was tested and compared to EcDsbA (○) and EcDsbC (□) by monitoring the catalysed reactivation of scrambled RNase (scRNase). Reactions contained 40 μM scRNaseA in 100 mM sodium phospate/NaOH, pH 7.0, 1 mM EDTA, 10 μM DTT and 10 μM protein. Folded RNase was used as a positive control (●). The cleavage of cCMP by native/ reactivated RNase was followed spectrophotometrically at 296 nm. α-DsbA1 has a similar level of activity as EcDsbA.

.

TABLE LEGEND

Table 1. Summary of the purification of recombinant α-DsbA1

REFERENCES

[1] S. Raina, D. Missiakas, Making and breaking disulfide bonds. Annu Rev Microbiol 51 (1997) 179-202.

[2] J. Messens, J.F. Collet, Pathways of disulfide bond formation in Escherichia coli. Int J Biochem Cell B 38 (2006) 1050-1062.

[3] H. Kadokura, F. Katzen, J. Beckwith, Protein disulfide bond formation in prokaryotes. Annu Rev Biochem 72 (2003) 111-135.

[4] C.W. Gruber, M. Cemazar, B. Heras, J.L. Martin, D.J. Craik, Protein disulfide isomerase: the structure of oxidative folding. Trends in biochemical sciences 31 (2006) 455-464.

[5] J.C.A. Bardwell, Building Bridges - Disulfide Bond Formation in the Cell. Mol Microbiol 14 (1994) 199-205.

[6] H. Nakamoto, J.C. Bardwell, Catalysis of disulfide bond formation and isomerization in the Escherichia coli periplasm. Biochimica et biophysica acta 1694 (2004) 111-119.

[7] A. Rietsch, J. Beckwith, The genetics of disulfide bond metabolism. Annual review of genetics 32 (1998) 163-184.

[8] J.F. Collet, J.C.A. Bardwell, Oxidative protein folding in bacteria. Mol Microbiol 44 (2002) 1-8.

[9] L. Debarbieux, J. Beckwith, Electron avenue: pathways of disulfide bond formation and isomerization. Cell 99 (1999) 117-119.

[10] M.A. Edeling, L.W. Guddat, R.A. Fabianek, L. Thony-Meyer, J.L. Martin, Structure of CcmG/DsbE at 1.14 angstrom resolution: High-fidelity reducing activity in an indiscriminately oxidizing environment. Structure 10 (2002) 973-979.

[11] J. Yu, J.S. Kroll, DsbA: a protein-folding catalyst contributing to bacterial virulence. Microbes Infect 1 (1999) 1221-1228.

[12] C.D. Ellermeier, J.M. Slauch, RtsA coordinately regulates DsbA and the Salmonella pathogenicity island 1 type III secretion system. J Bacteriol 186 (2004) 68-79.

[13] C.W. Goulding, M.I. Apostol, S. Gleiter, A. Parseghian, B. J., G. M., E. D., Gram-positive DsbE proteins function differently from Gram-negative DsbE-homologs. A structure to function analysis of DsbE from Mycobacterium tuberculosis. J Biol Chem 279 (2004) 3516-3524.

[14] A. Jeyaprakash, M.A. Hoy, Long PCR improves Wolbachia DNA amplification: wsp sequences found in 76% of sixty-three arthropod species. Insect Mol Biol 9 (2000) 393-405.

[15] J.H. Werren, S.L. O'Neill, The evolution of heritable symbionts, in: O'Neill SL, Hoffmann AA, W. JH (Eds.) Influential Passengers: Inherited Microorganisms and Arthropod Reproduction, Oxford University Press, Oxford, UK, 1997, pp. 1-4.

[16] M. Riegler, S.L. O'Neill, The Genus Wolbachia, in: M. Dworkin (Ed.) The Prokaryotes Springer-Verlag, New York, 2007, pp. 547-561.

[17] S.L. O'Neill, A.A. Hoffmann, J.H. Werren, Influential Passengers: Inherited Microorganisms and Arthropod Reproduction, Oxford University Press, Oxford, UK, 1997.

[18] M. Taylor, One in the eye for river blindness. Trends Microbiol 9 (2001) 310.

[19] M.J. Taylor, A. Hoerauf, A new approach to the treatment of filariasis. Curr Opin Infect Dis 14 (2001) 727-731.

[20] K.D. Floate, G.K. Kyei-Poku, P.C. Coghlin, Overview and relevance of Wolbachia bacteria in biocontrol research. Biocontrol Sci Techn 16 (2006) 767-788.

[21] M. Wu, L.V. Sun, J. Vamathevan, M. Riegler, R. Deboy, J.C. Brownlie, E.A. McGraw, W. Martin, C. Esser, N. Ahmadinejad, C. Wiegand, R. Madupu, M.J. Beanan, L.M. Brinkac, S.C. Daugherty, A.S. Durkin, J.F. Kolonay, W.C. Nelson, Y. Mohamoud, P. Lee, K.

Berry, M.B. Young, T. Utterback, J. Weidman, W.C. Nierman, I.T. Paulsen, K.E. Nelson, H. Tettelin, S.L. O'Neill, J.A. Eisen, Phylogenomics of the reproductive parasite Wolbachia pipientis wMel: A streamlined genome overrun by mobile genetic elements. Plos Biol 2 (2004) 327-341.

[22] S.L. O'Neill, R. Giordano, A.M. Colbert, T.L. Karr, H.M. Robertson, 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. Proc Natl Acad Sci U S A 89 (1992) 2699-2702.

[23] F.W. Studier, Protein production by auto-induction in high-density shaking cultures. Protein Expr Purif 41 (2005) 207-234.

[24] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: a Laboratory Manual, Cold Spring Harbor, New York, 1989.

[25] L.A. Kelley, R.M. MacCallum, M.J. Sternberg, Enhanced genome annotation using structural profiles in the program 3D-PSSM. J Mol Biol 299 (2000) 499-520.

[26] A. Holmgren, Thioredoxin catalyzes the reduction of insulin disulfides by dithiothreitol and dihydrolipoamide. J Biol Chem 254 (1979) 9627-9632.

[27] D.A. Hillson, N. Lambert, R.B. Freedman, Formation and isomerization of disulfide bonds in proteins: protein disulfide-isomerase. Methods in enzymology 107 (1984) 281-294.

[28] G.L. Ellman, A colorimetric method for determining low concentrations of mercaptans. Archives of biochemistry and biophysics 74 (1958) 443-450.

[29] J.L. Martin, J.C. Bardwell, J. Kuriyan, Crystal structure of the DsbA protein required for disulphide bond formation in vivo. Nature 365 (1993) 464-468.

[30] A.A. McCarthy, P.W. Haebel, A. Torronen, V. Rybin, E.N. Baker, P. Metcalf, Crystal structure of the protein disulfide bond isomerase, DsbC, from Escherichia coli. Nat Struct Biol 7 (2000) 196-199.

[31] J.L. Martin, Thioredoxin--a fold for all reasons. Structure 3 (1995) 245-250.

[32] B. Heras, M.A. Edeling, H.J. Schirra, S. Raina, J.L. Martin, Crystal structures of the DsbG disulfide isomerase reveal an unstable disulfide. Proc Natl Acad Sci U S A 101 (2004) 8876-8881.

Tables

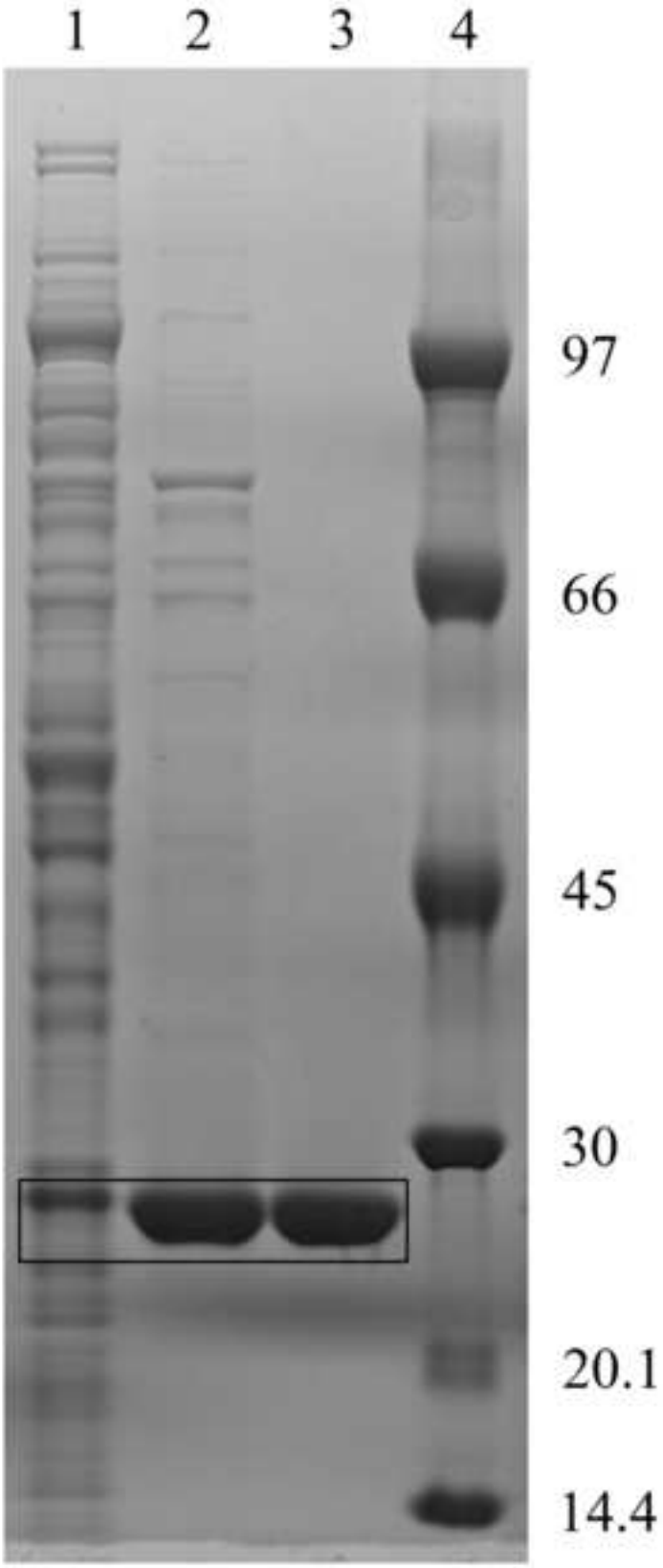
Table 1. Summary of the purification of recombinant α-DsbA1

Purification Step	Total Protein (mg/ml)	Total Volume (ml)	Estimated Amount of α-DsbA1 (mg/ml)	Estimated Yield of α-DsbA1 (mg)	Estimated Yield of α-DsbA1 (%)	Estimated Purity of α-DsbA1 (%)
Lysate	9.3	100	1.1	107	100	12
IMAC	3.5	8	2.4	19	18	69
Gel Filtration	3.8	4.8	3.8	18	17	100

Total protein was quantified using the BCA protein assay (Pierce) with BSA as standard. The amount of α-DsbA1 was estimated from densitometric analysis of Coomassie stained SDS gels (see Methods).

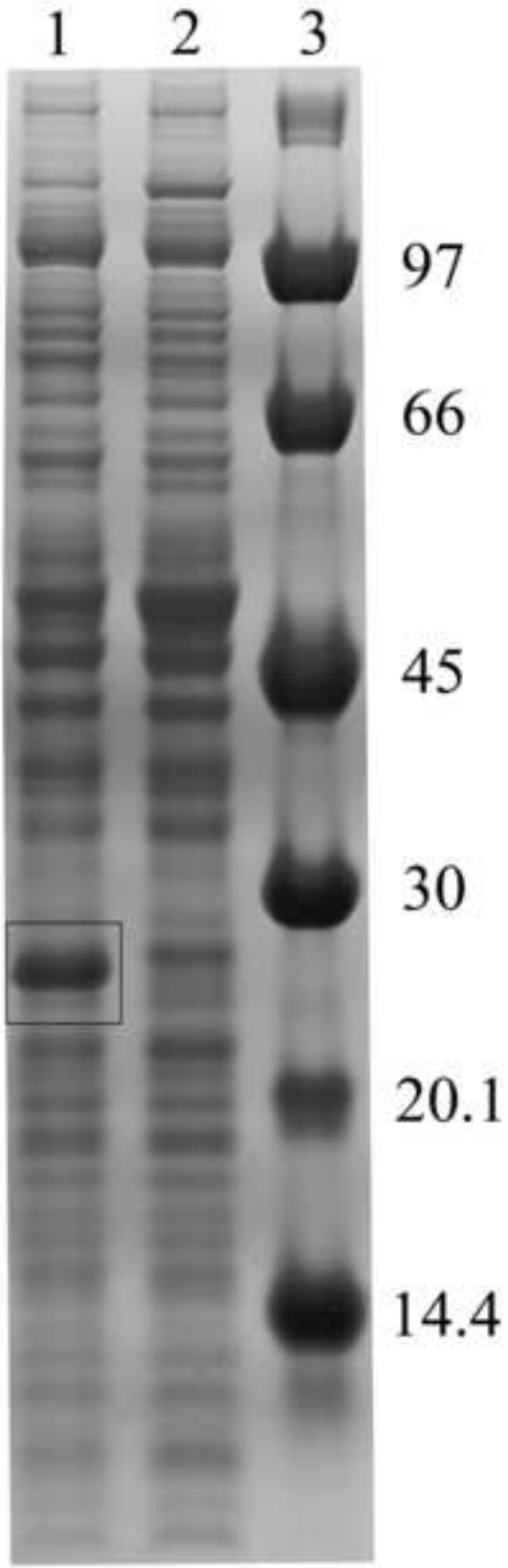
Figure

Click here to download high resolution image



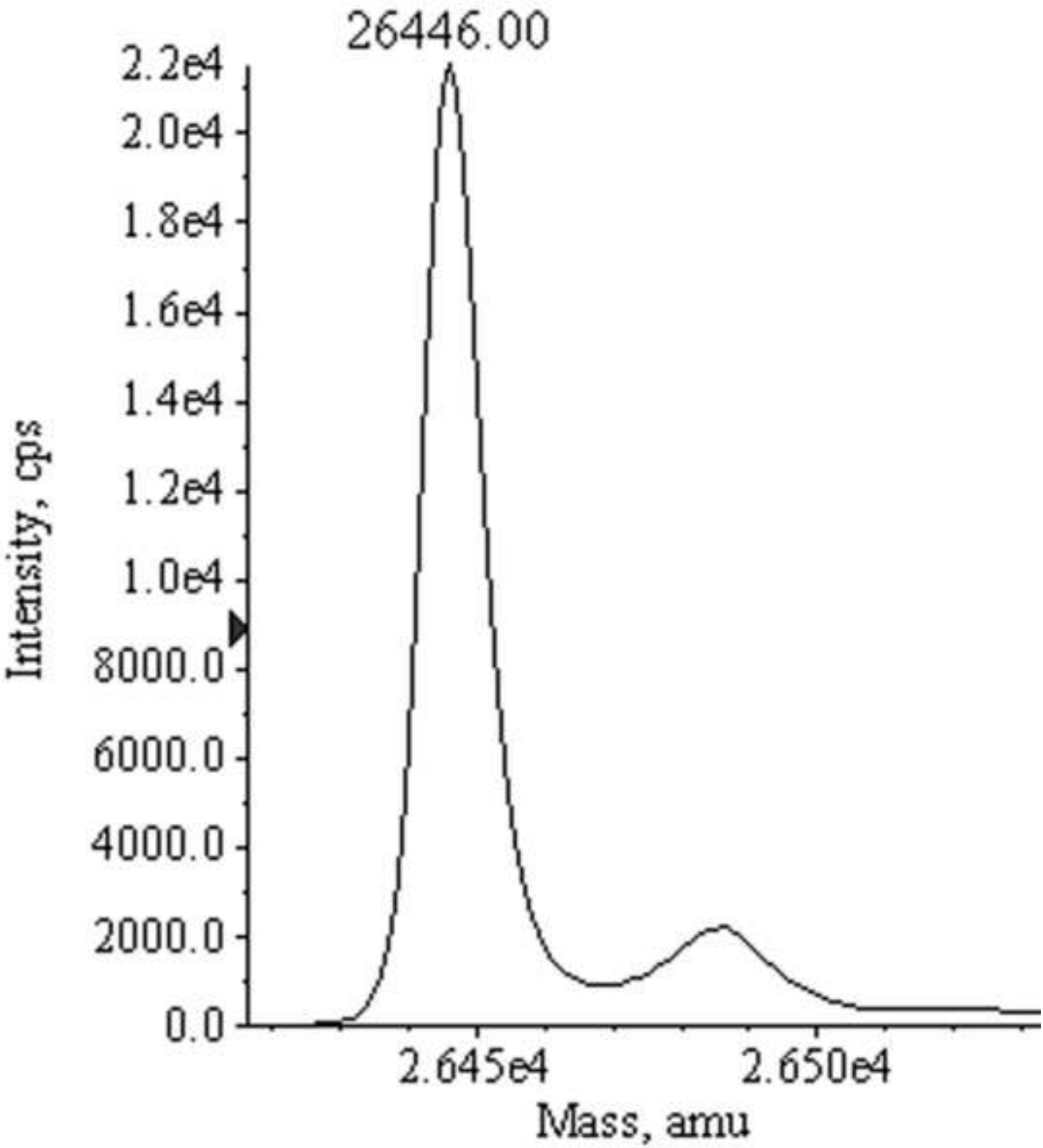
Figure

Click here to download high resolution image



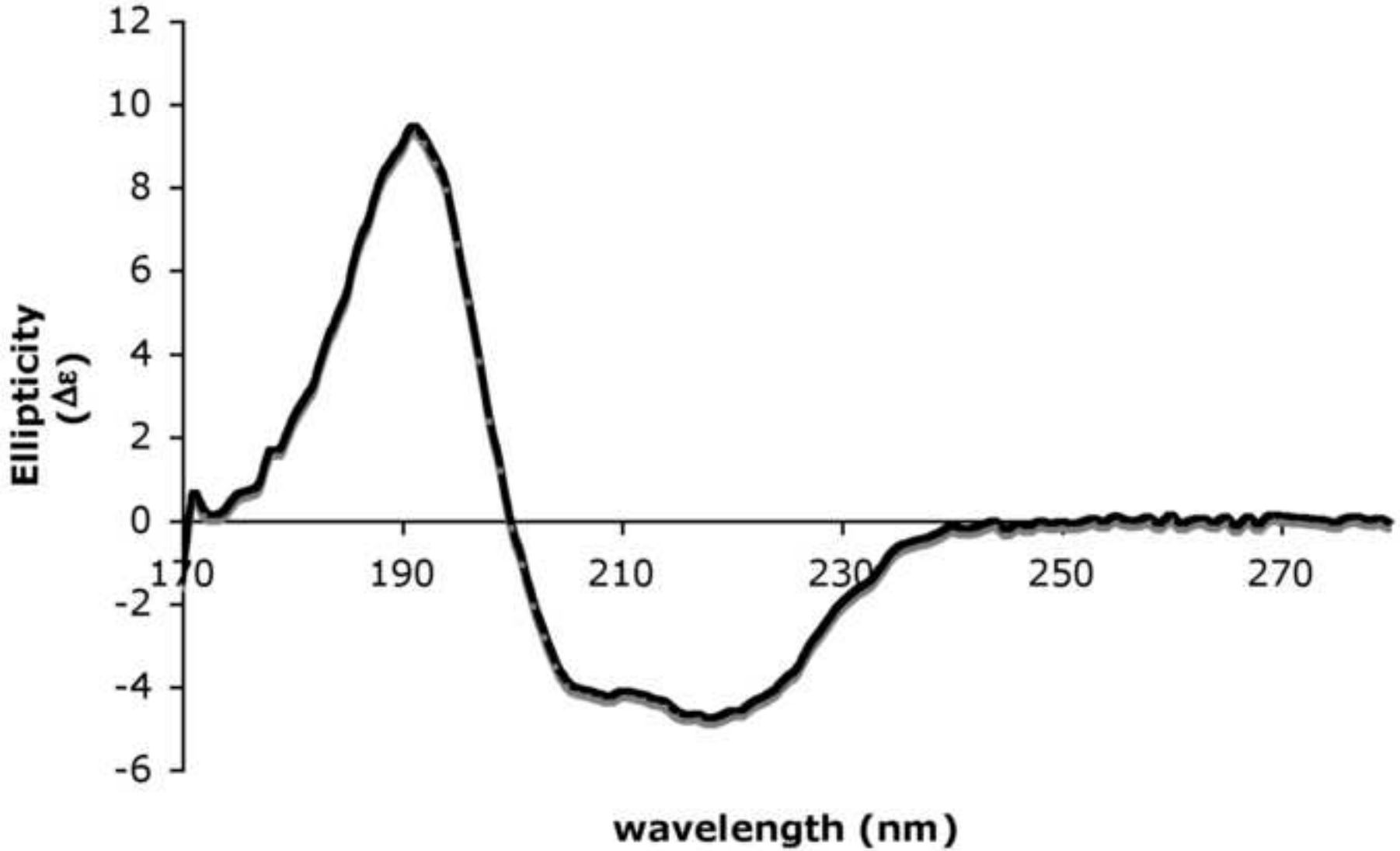
Figure

Click here to download high resolution image



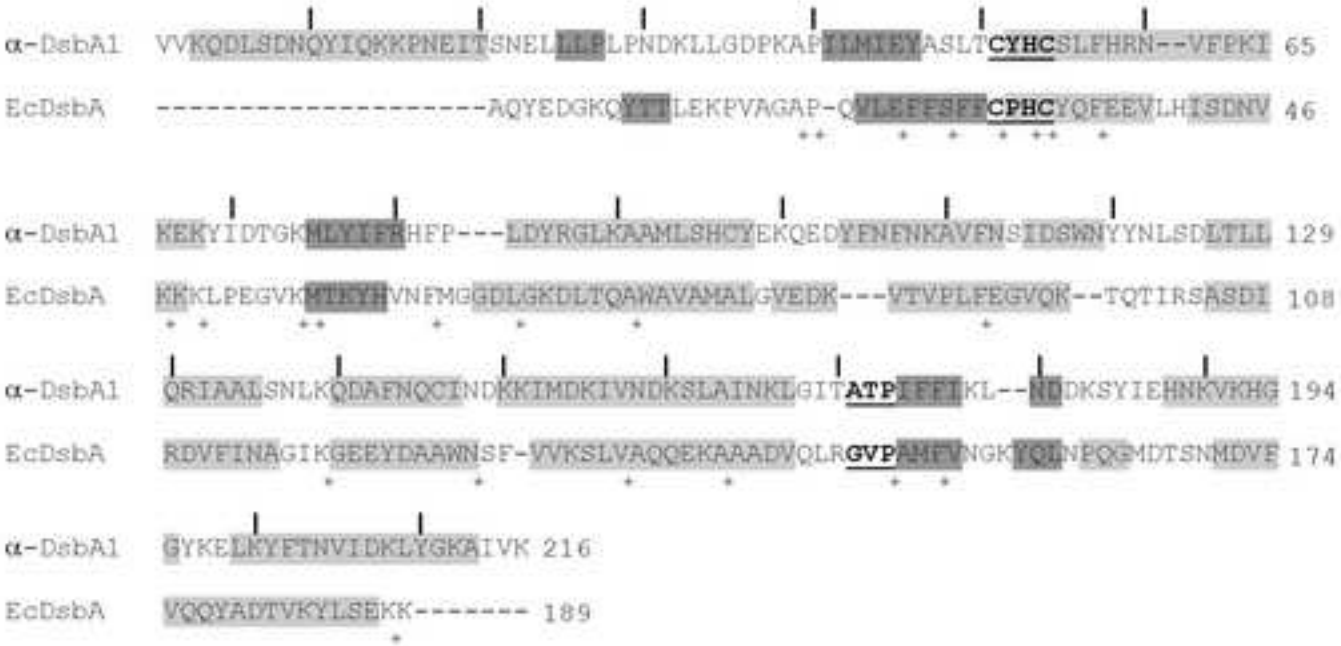
Figure

Click here to download high resolution image



Figure

Click here to download high resolution image



Figure

Click here to download high resolution image

Figure
Click here to download high resolution image

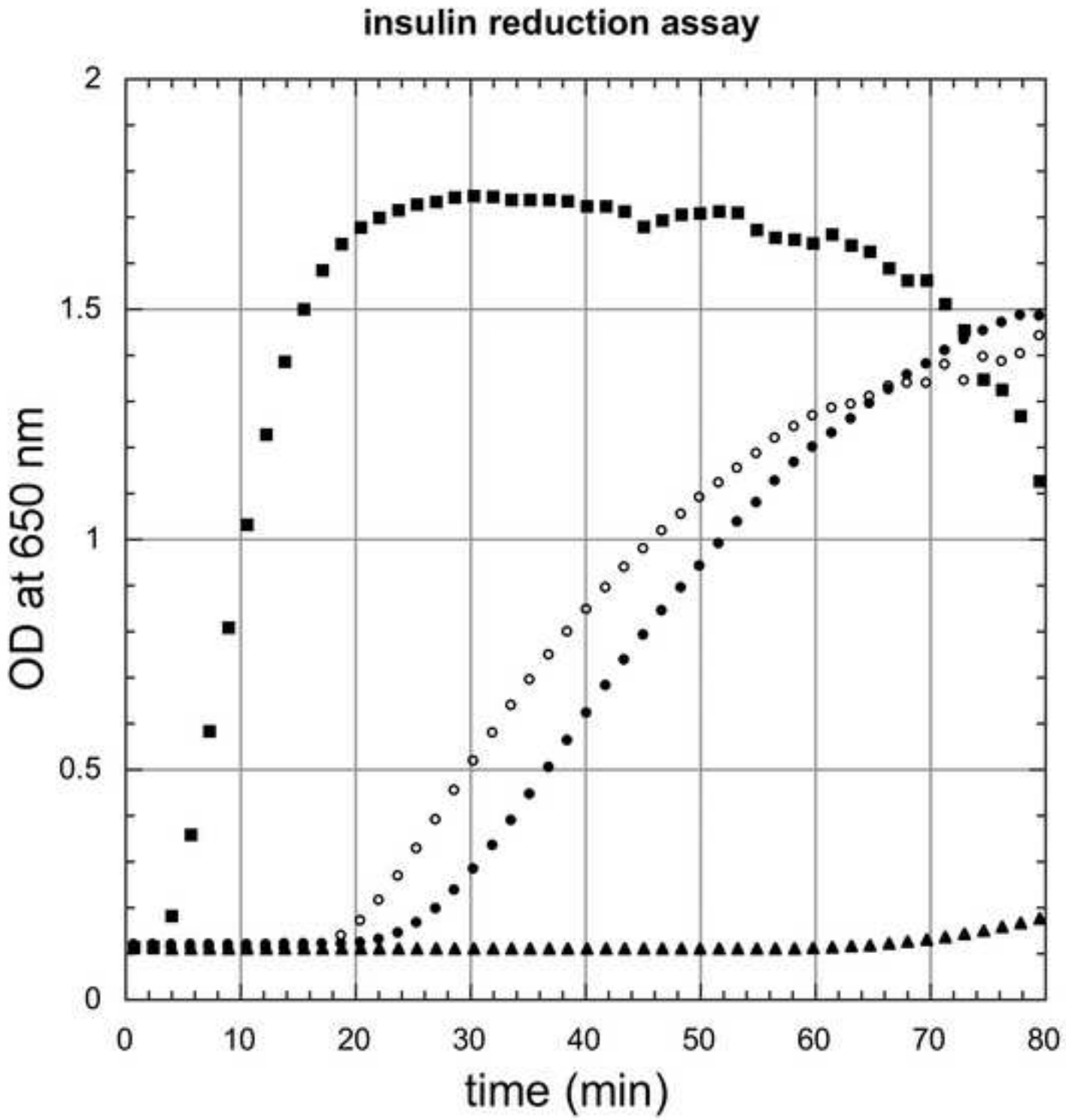


Figure
Click here to download high resolution image