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Crystallization of DsbA, an *Escherichia coli* Protein Required for Disulphide Bond Formation *in Vivo*

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DsbA is a 21 kDa protein that facilitates disulphide bond formation and is required for the correct folding and stability of a number of exported proteins in *Escherichia coli*. Crystals of oxidized DsbA have been obtained from polyethylene glycol 8000 (20 to 25%), 0·1 M-cacodylate buffer (pH 6·5) and 1% 2-methyl-2,4-pentanediol. Oxidation of the protein is critical for reproducibly obtaining high quality crystals. The resulting crystals diffract to 2 Å and belong to the monoclinic space group $C2$ with cell dimensions $a=117·5$ Å, $b=65·0$ Å, $c76·3$ Å, $\beta=126·3°$ with two molecules in the asymmetric unit.

Keywords: protein crystallization; X-ray diffraction; protein folding; oxidoreductase; protein disulphide isomerase

Mutations that render *Escherichia coli* severely defective in disulphide bond formation in exported proteins have been described in two recent papers (Bardwell *et al.*, 1991; Kamitani *et al.*, 1992). In these mutant *E. coli* strains, exported proteins lack disulphide bonds, and are often improperly folded and highly sensitive to protease degradation. The wild-type gene affected by these mutations (*dsbA*, also called *ppfA*) codes for a 21 kDa (189 residue) periplasmic protein (DsbA) exhibiting oxidoreductase activity. DsbA facilitates disulphide bond formation and the correct folding of exported proteins and may therefore be useful in the *in vitro* renaturation of improperly folded proteins.

Two DsbA homologues have also been identified; TcpG, also caller DsbA, (181 residues) from *Vibrio cholerae* (Peek & Taylor, 1992; Yu *et al.*, 1992) and Por (180 residues) from *Haemophilus influenzae* (Tomb, 1992). Complementation studies suggest that these proteins perform similar functions to *E. coli* DsbA. Although they originate from different organisms, DsbA, TcpG and Por are highly similar in sequence; DsbA and TcpG share 40% sequednce identity (Peek & Taylor, 1992); DsbA and Por shared 45% identity, (Tomb, 1992). In addition, all three proteins contain a sequence of four residues (-Cys-Pro-His-Cys-) similar to that found at the active site of proteins of the thioredoxin super-family of oxidoreductases (which includes thioredoxin and protein disulphide isomerase). Apart from this four-residue stretch, there is little sequence similarity between DsbA and the thioredoxin super-family (Bardwell *et al.*, 1991). However, on the basis of sequence homology modelling and secondary structure assignment, Ellis *et al.* (1992) predict that the structure of DsbA includes a thioredoxin structural motif.

We are interested in the structure of DsbA since it will enable a better understanding of how it facilitates a step that is crucial to the folding, stability and activity of other proteins. Structure determination will also answer the question of whether DsbA forms a new structural class of oxidoreductase enzymes, or if it is indeed a member of the thioredoxin superfamily as argued by Ellis *et al.* (1992).

To prepare DsbA for crystallization a number of modifications were made to the original purification scheme (Bardwell *et al.*, 1991). DsbA was induced by growing the overproducing strain JCB607 in

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Luria-Bertani broth (Sambrook *et al.*, 1989) at 37 °C with shaking, to an A_{600} nm of 0·2. IPTG† was added to 1 mM and incubation continued to an A_{600} nm of 2. Cells were pelleted at 4000 *g* for 30 minutes at 4 °C and then washed in 30 ml 10 mM-Hepes (pH 7·8) with or without 0·5 mM-EDTA (buffer A). The cells were then repelleted at 5000 *g* for ten minutes at 4 °C. 25 ml of buffer A containing 1 mg of Polymyxin B/ml was then added to the cell pellet. Polymyxin B permeabilizes the outer membrane without significant inner membrane permeabilization (Lehrer *et al.*, 1988) thereby releasing the periplasmic contents into the supernatant. After a 30-minute incubation at 4 °C the mixture was centrifuged at 16,500 *g* for 15 minutes at 4 °C.

The supernatant, consisting of >90% DsbA, was filtered, diluted fourfold with water and purified by ion-exchange chromatography using a Pharmacia Mono-Q HR 10/10 FPLC column equilibrated with buffer A at 4 °C. DsbA was separated with a shallow gradient into five fractions over a salt concentration range of 40 to 60 mM-NaCl. Only the second fraction ran as a single 21 kDa band on both 12% and 20% (w/v) SDS/PAGE gels. This fraction also contained over 50% of the total DsbA from this purification step and was therefore used in subsequent steps.

The purified DsbA separated into two bands, corresponding to oxidized and reduced protein, on a Pharmacia IEF 4–6·5 gel (Fig. 1). These two forms of DsbA could not be readily separated by chromatographic methods, or fully interconverted by air oxidation. However, the two bands could be fully interconverted by the addition of reducing or oxidizing agents (Fig. 1). Both the reducing and oxidizing reactions are reversible (data not shown) implying that a disulphide bond is involved in the reaction. The only two cysteines are those in the Cys-Pro-His-Cys sequence that forms the putative active site of DsbA. This evidence suggests that the FPLC-purified DsbA may be present as an equilibrium mixture of the oxidized (disulphide) and reduced (dithiol) forms of the two cysteines at the active site. For crystallization purposes, DsbA was oxidized by addition of 1·5 mM-copper (II) [1,10-phenanthroline]$_3$. The precedent for employing this particular reagent was its use in the crystallization protocol of bacterial aspartate receptor to maintain the disulphide crosslink (Jancarik *et al.*, 1991). After addition of copper (II) [1,10-phenanthroline]$_3$, the DsbA solution was left on ice for two hours and then dialysed for 24 hours at 4 °C against buffer A to remove the oxidizing agent and salt. Oxidation of the dialysed protein was confirmed by isoelectric focussing with Pharmacia IEF 4–6·5 gels, resulting in a single band at p*I* 5·4. Subsequently, DsbA was concentrated to 22 mg/ml by ultrafiltration using Centricon-10 devices (Amicon).

Triangular pyramid protein crystals were grown from this DsbA solution equilibrated against a reservoir containing the precipitant polyethylene glycol 8000 (20 to 25%, w/v), 2-methyl-2,4-pentanediol (1%) in 0·1 M-cacodylate buffer (pH 6·5) at 21 °C. The initial volume of the hanging drop was 4 μl (2 μl protein mixed with 2 μl of reservoir). Depending on the batch of protein, crystals grew within a few hours to a size large enough (0·5 mm × 0·4 mm × 0·3 mm) for data collection.

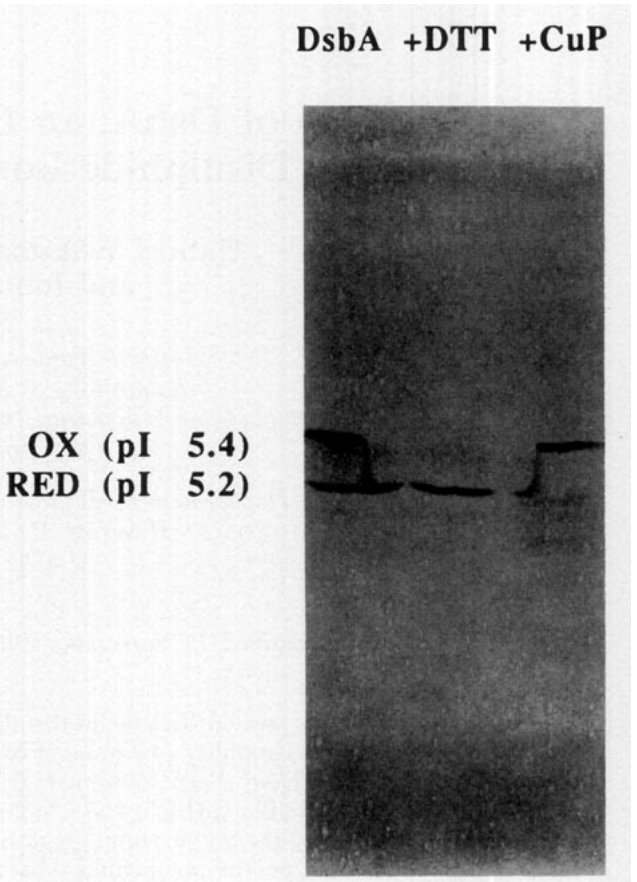

Figure 1. DsbA is a mixture of reduced and oxidized protein that can be interconverted with oxidizing and reducing agents. This Figure shows 3 lanes from a Pharmacia IEF 4–6·5 gel. The lane labelled DsbA was loaded with 4 μl of FPLC-purified DsbA. This has 2 bands corresponding to the oxidized (p*I* 5·4) and reduced (p*I* 5·2) forms of the protein. The +DTT lane was loaded with 4 μl of FPLC-purified DsbA in 10 mM-DTT. In this case there is only 1 band for the reduced protein (p*I* 5·2). In the 3rd lane (+CuP) 4 μl of FPLC-purified DsbA in 1·5 mM-Copper (II) [1,10-phenanthroline]$_3$ runs as a single oxidized band with p*I* 5·4. Note that DTT from the middle (+DTT) lane leaches out and reduces some of the oxidized DsbA in adjacent lanes. DTT, dithiothreitol.

† Abbreviations used: IPTG, isopropyl β-D-thiogalactoside; MFID, mean fractional isomorphous difference between the scaled structure factor amplitudes of the native structure, F_{NAT}, and those for the derivative structure, F_{DER}: MFID = $(\Sigma|F_{DER} - F_{NAT}|/\Sigma|F_{NAT}|) \times 100$; R_m is the merging R factor for measured intensities of symmetry related reflections; $R_m = (\Sigma_h\Sigma_i[|I_i - I|]/\Sigma_h\Sigma_i I) \times 100$ where I_i is the scaled intensity of the ith observation of reflection h, and I is the mean intensity value for this reflection; Se-Met, selenomethionine.

However, it was sometimes necessary to use a streak-seeding/microseeding technique (Stura & Wilson, 1990) to provide nucleation sites.

Two other crystal forms of oxidized DsbA grow from similar conditions. Neither is very useful for X-ray crystallographic analysis; one grows as highly twinned, thin plates and the other form is rod-shaped with a large cell edge of over 350 Å. (1 Å = 0·1 nm). Also, attempts to grow crystals of reduced DsbA by the addition of reducing agent (25 to 50 mM-dithiothreitol) to the reservoir results in the formation of extremely thin needle-shaped crystals.

Crystallographic data from the triangular-pyramid crystals have been measured on an RAXIS IIC image plate area detector connected to a Rigaku RU200 copper target rotating anode X-ray source. Crystals of oxidized DsbA diffract to beyond 2 Å and belong to the monoclinic $C2$ crystal form, with a unit cell of $a = 117{\cdot}5$ Å, $b = 65{\cdot}0$ Å, $c = 76{\cdot}3$ Å, $\beta = 126{\cdot}3°$. The unit cell and space group were confirmed both with diffractometer measurements and by precession photographs. Native data 87% complete to 2 Å and 93% complete to 2·25 Å, with an R_m of 3·5%, were measured from a single oxidized DsbA crystal (1·1 mm × 0·6 mm × 0·6 mm).

There is a striking difference between the quality of crystals grown from oxidized DsbA and those grown from an equilibrium mixture of the oxidized and reduced forms of DsbA. Most notably, the diffraction quality of the equilibrium mixture DsbA crystals is highly variable. Some crystals diffract weakly or not at all while others from the same drop diffract strongly to beyond 2 Å. In contrast, all oxidized DsbA crystals tested to date diffract strongly. Those equilibrium mixture DsbA crystals that do diffract belong to the same space group and possess the same unit cell lengths and angles as the oxidized DsbA crystals. However, structure factors measured from four different equilibrium mixture crystals merge with a mean fractional isomorphous difference (MFID) that varies between 12 and 20% (over resolution limits ranging from 2·5 Å to 3 Å), indicating that these crystals are not isomorphous. Conversely, crystallographic structure factors for three oxidized DsbA crystals merge with MFID of 5 to 7%. Clearly, the crucial oxidation step during protein purification is essential for the growth of isomorphous DsbA crystals of uniform diffraction quality.

The unit cell volume of the oxidized DsbA crystals is 469,650 Å^3. The crystal density is 1·159 g/cm^3 and the density of the mother liquor is 1·015 g/cm^3 (measured using an organic solvent density gradient with salt solution calibration similar to that described in Matthews, 1985). Substituting these values into equation (8) of Matthews (1985), gives two molecules of DsbA per asymmetric unit. The calculated cell volume per unit mass, V_m, is then 2·8 Å^3, which is within the range found for other protein crystals and indicates a solvent content of 56% (Matthews, 1968). 2-fold non-crystallographic symmetry perpendicular to b^* is indicated from self-rotation functions calculated using MERLOT (Fitzgerald, 1988).

Preliminary studies of heavy atom binding have not yielded useful heavy atom derivatives. However, the DsbA overproducing *E. coli* strain JCB607 is a methionine auxotroph (Bardwell *et al.*, 1991) and we have successfully expressed DsbA from this strain in minimal media replacing methionine with seleno-methionine (Se-Met) (details to be published elsewhere). The Se-Met DsbA produced was purified and crystallized using the same procedures described for native protein. Mass spectroscopic analysis of this modified protein (D. Fenyö & B. Chait, personal communication) indicates that all six methionines in the DsbA sequence (and therefore 12 methionines in the asymmetric unit) are substituted with Se-Met, with essentially 100% efficiency. In addition, the resulting Se-Met DsbA crystals are isomorphous with native DsbA crystals (MFID 11%) and a difference Patterson map exhibits strong peaks consistent with the presence of ordered Se atoms. It is our intention to use the Se-Met DsbA crystals in multiple wavelength anomalous scattering experiments for phase determination (Hendrickson, 1991).

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