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Crystallization of DsbC, the disulfide bond isomerase of *Escherichia coli*

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Abstract

DsbC is a 2×23 kDa soluble dimeric protein molecule involved in protein disulfide bond formation in the *E. coli* periplasm, primarily catalyzing disulfide bond rearrangements. Crystals of both the native and selenomethione protein suitable for structure determination were obtained using the hanging-drop vapour-diffusion method. The best crystals were obtained using 18–22%(*v*/*v*) polyethylene glycol 550 monomethyl ether in 100 m*M* Tris–HCl (pH 8.9). Seeding methods were used to produce large crystals diffracting to 2 Å resolution, and the detergent *n*-octyl-β-glucoside was used to improve crystal quality. Significant variation in cell dimensions and crystal order was observed. Cell dimensions obtained for frozen crystals were in the range $a = 58.8(0.3)$, $b = 78.9(0.5)$, $c = 95.2(5.0)$ Å. The lattice is orthorhombic and systematic absences indicate that the space group is $P2_12_12_1$.

1. Introduction

The formation of disulfide bonds in secreted proteins is known to be catalyzed *in vivo* (Bardwell, 1994). In the eukaryotic cell, disulfide formation occurs primarily in the endoplasmic reticulum, where oxidized glutathione is thought to be the source of oxidizing power (Hwang, Sinskey & Lodish, 1992). Protein disulfide isomerase (PDI) (Freedman, Hirst & Tuite, 1994) catalyzes both net formation of disulfide bonds and intramolecular disulfide rearrangement, even in species with stable conformations (Creighton *et al.*, 1993). In Gram-negative bacteria such as *Escherichia coli*, disulfide bonds are formed rapidly in the periplasm, in a process catalyzed by several proteins: DsbA, DsbB and DsbC.

DsbA is a soluble, monomeric protein of 21 kDa (Bardwell, McGovern & Beckwith, 1991; Kamitani, Akiyama & Ito, 1992) that is believed to be the direct oxidant that transfers its disulfide bond to newly secreted proteins (Bardwell *et al.*, 1993), but it is a very poor disulfide isomerase (Zapun & Creighton, 1994). The membrane-embedded protein DsbB is believed to recycle DsbA from its dithiol to the disulfide form (Bardwell *et al.*, 1993; Missiakas, Georgopoulos & Raina, 1993). How DsbB is itself reoxidized is not known. The absence of DsbA and/or DsbB results in pleiotropic defects that are consequences of the non-functionality of extracellular proteins lacking their disulfide bonds.

The absence of a third protein, DsbC, also causes defects that are associated with deficient formation of disulfide bonds (Shevchik, Condemine & Robert-Baudouy, 1994; Massiakas, Georgopoulos & Raina, 1994). It is a soluble protein of 23 kDa that is dimeric in solution and is believed to function primarily to catalyze intramolecular disulfide rearrangements (Zapun *et al.*, 1995). The sequence of DsbC (Lovett & Kolodner, 1991; Zapun *et al.*, 1995, SwissProt database code DSBC_ECOLI) indicates a p*I* of 6.2, consistent with the result obtained by experiment (unpublished results).

DsbA and PDI belong to the thioredoxin family and share this common structural motif (Martin, 1995). Two segments of the PDI sequence are homologous to thioredoxin (Edman, Ellis, Blacher, Roth & Rutter, 1985), and the structure of one has been shown recently by NMR spectroscopy to be very similar to that of thioredoxin (Kemmink, Darby, Dijkstra, Scheek & Creighton, 1995). The crystal structure of DsbA revealed the thioredoxin structural motif, with insertion of an α-helical domain (Martin, Bardwell & Kuriyan, 1993). On the basis of its biochemical properties, DsbC is likely also to contain the thioredoxin motif, even though this is not apparent in its primary structure. DsbC is, however, double the size of thioredoxin, so there must also be a substantial amount of other structure. The position of the active-site residues in the sequence indicates that the extra residues of DsbC are not in the same position as in DsbA.

All these proteins have in their active site two cysteine residues, separated by only two other residues, that are involved in the thiol–disulfide exchange reactions responsible for their respective activities. A disulfide bond can be formed reversibly between the S atoms of these residues, but only the more N-terminal of the two cysteine residues is exposed and reacts with other thiol-containing molecules. Apart from these common features, the active-site thiol–disulfide chemistry varies greatly between the different members of the family. The disulfide bonds of PDI, DsbA and DsbC are very unstable (Lundström & Holmgren, 1993; Zapun, Missiakas, Raina & Creighton, 1995) and destabilize the folded conformation of the protein (Zapun, Bardwell & Creighton, 1993). The reduced form of these proteins is more resistant to denaturation than the form with the intact active-site disulfide bond. In contrast, the disulfide bond of thioredoxin is much more stable, about 57 kJ mol^{-1} (7 kcal mol^{-1}) more than that of DsbA, and stabilizes the folded structure of the protein (Holmgren, 1995). The active-site disulfide bond of DsbC is almost as unstable as that of DsbA, but the reactivities of the cysteine residues of the two proteins are significantly different (Zapun, Missiakas, Raina & Creighton, 1995). Understanding how active sites with widely different properties are accommodated in the same overall thioredoxin fold requires the structures of more such proteins to be solved at high

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Table 1. *Crystallographic data-collection statistics*

DsbC	X-ray source*	Temperature (K)	Unit cell (Å)	Resolution (Å)	Completeness (%)	R_{merge}†
Native	BL19	100	58.2 79.2 94.8	2.1	85.6	6.0
Sel-met	X11	100	59.3 80.4 96.4	2.4	96.8	8.3
Sel-met	RA	278	59.5 81.2 106.5	3.5	94.6	12

*BL19 ESRF synchrotron beam, X11 synchrotron beam EMBL/DESY, RA rotating-anode laboratory X-ray source. †$R_{merge} = \sum_{hkl} \sum_i |I_{hkl,i} - \langle I \rangle_{hkl}| / \sum |\langle I \rangle_{hkl}|$, where $I_{hkl,i}$ are the intensity measurements for a reflection hkl and $\langle I \rangle_{hkl}$ is the mean value for this reflection.

resolution. The structure of DsbC should provide valuable clues as to how these proteins function.

2. Experimental

DsbC was isolated from an overproducing strain of *E. coli* [BL21 (DE3) harboring the plasmid pDM801] as previously described (Missiakas, Georgopoulos & Raina, 1994; Zapun, Missiakas, Raina & Creighton, 1995). DsbC with all ten methionine residues replaced by selenomethionine was isolated in the same way from the methionine auxotrophic strain B834 (DE3) carrying the expression plasmid pDM801, but the cells were grown in a minimal medium derived from that of Hoffman & Spicer (1991) containing per litre: 40 mg of each of the five nucleotides, 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, 2 mmol of $MgSO_4$, 0.1 mmol of $CaCl_2$, 0.1 mmol of $MnCl_2$, 1 µmol of $FeCl_3$, 5 mg of thiamine, 1 mg each of pyridoxal, niacinamide, biotin, folic acid, choline chloride and pantothenate, 0.1 mg of riboflavine, 4 g of glucose, 1 g of NH_4Cl, 40 mg of seleno-L-methionine (Sigma) and 100 mg of carbenicillin. Nucleotides were added to the minimal media to increase the protein yield and the bacterial growth rate (Hoffman & Spicer, 1991). Mass spectroscopic analysis of the DsbC-Se-Met indicated that all ten methionines in DsbC were substituted by Se-Met with essentially 100% efficiency.

Crystallization experiments were performed by the hanging-drop vapour-diffusion method at 291 K using Linbro 24-well tissue-culture plates. Initial trials were carried out using an established crystallization screen (Jancarik & Kim, 1991) which, while it failed to produce useful crystals, did produce granular precipitates in drops containing PEG 400, PEG 2000, PEG 550 MME (polyethylene glycol 550 monomethyl ether) and Jeffamine 500. Further trials were carried out with different molecular weight PEG's in combination with a range of salts [$MgCl_2$, $ZnCl_2$, $NiCl_2$, $FeCl_3$, $CdCl_2$, $Pb(NO_2)_3$] and additives (*n*-octyl-β-glucoside, glycerol and isopropanol) at pH 4–9. Large crystals were obtained with 25% PEG 4000 and 50% isopropanol in pH 5.0 100 m*M* Na-citrate and a protein concentration of 30 mg ml^{-1}. These 'isopropanol' crystals proved to be very unstable and difficult to freeze, and no useful diffraction data were obtained from them. Further trials varying only the PEG 550 MME concentration and pH produced small needle crystals. Less elongated crystals were obtained using the additive *n*-octyl-β-glycoside [1%(*v*/*v*)] and 18–22%(*v*/*v*) PEG 550 MME, 100 m*M* Tris–HCl (pH 8.9) and a protein concentration of 15–20 mg ml^{-1}. Microseeding techniques (Stura & Wilson, 1991) were employed to obtain crystals more reproducibly that were large enough for data collection (0.15 × 0.15 × 0.4 mm) and diffracted to 2.0 Å (Fig. 1).

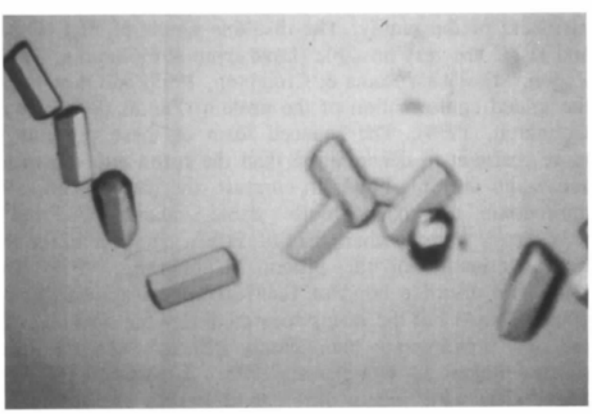

Fig. 1. Crystals of DsbC-Sel-Met protein. Crystals grow up to dimensions of 0.15 × 0.15 × 0.4 mm.

Crystals to be used for cryogenic data collection were mounted in thin nylon loops and frozen by plunging into liquid nitrogen. Before freezing, the crystals were gradually equilibrated with cryobuffer (33% PEG 550 MME, 1% *n*-octyl-β-glucoside, 100 m*M* Tris–HCl, pH 8.9) using a five-step transfer procedure. Frozen crystals were stored in ethane blocks and maintained at 100 K during data collection using an Oxford Cryostream cryogenic device.

Monochromatic diffraction data were collected using a rotating-anode X-ray equipped with a graphite monochromator and collimator or using the synchroton beamline X11 at EMBL/DESY (States, 1995). MAD data (multi-wavelength anomalous dispersion; Hendrickson, Horton & LeMaster, 1990) from a frozen selenomethione crystal were collected at beamline BL19 at the ESRF Grenoble (Thompson, 1994). MAR area detectors (Hendrix, 1995) were used, and the diffraction images were processed using the program *DENZO/SCALEPACK* (Otwinowski & Minor, 1996).

3. Results and discussion

DsbC crystals were very radiation sensitive at room temperature, and significant decay at 278 K (50% during a 40 h exposure) limited the resolution of single-crystal rotating-anode source data sets to 3.5 Å. Radiation decay was eliminated by cryocooling, and data sets to 2.1 Å resolution could be collected from single frozen crystals at 100 K (Table 1). The ability to collect several accurate data sets from a single frozen DsbC crystal without significant radiation decay suggested the possibility that the structure could be obtained by MAD methods, assuming the data were good enough to allow the positions of the ten Se atoms per monomer to be determined.

Processing statistics for data collected from wet and frozen native and selenomethionine DsbC crystals are listed in Table 1. Systematic absences from several DsbC native and selenomethionine data sets were consistent with the

orthorhombic space group $P2_12_12_1$. Significant variation in cell dimensions and differences in crystal mosaicity were encountered, however, even between crystals harvested from the same drop and frozen by similar methods. Typical cell dimensions were $a = 58.8(0.3)$, $b = 78.9(0.5)$, $c = 95.2(5.0)$ Å for frozen crystals, with the variation being most evident in the c-axis length. The small unit-cell differences observed between the native and selenomethionine crystals (Table 1) are not significant and do not indicate a conformational change between the native and substituted molecules. Assuming that there are two monomers in the asymmetric unit, the V_M value is approximately 2.3 Å^3 Da^{-1} (Matthews, 1968), corresponding to a typical protein crystal solvent content of 43%. The probable occurrence of two DsbC monomers per asymmetric unit implies that the positions of 20 Se atoms would need to be determined to exploit MAD data fully, and that additional phase information or high-resolution accurate anomalous data would be necessary for the challenging task of locating them.

The irreproducible quality of the frozen crystals has hampered both the MAD data collection and the search for heavy-metal derivatives. The most likely explanation for the variability of the crystals may be that the freezing procedure introduces uncontrolled changes to the crystal structure. Crystal freezing can increase mosaicity, reduce resolution and alter unit-cell parameters (Rodgers, 1994; Mitchell & Garman, 1994). DsbC crystals were sensitive and difficult to transfer to cryobuffer without cracking. Small variations in the cryobuffer PEG 550 MME concentration markedly reduced resolution. Crystal damage may occur both during transfer from the hanging drop to the cryo-buffer or by drying or pre-cooling of the crystal in the loop immediately before freezing. We have been addressing these possibilities by testing different transfer and freezing protocols.

The structure determination by a combination of MIR (multiple isomorphous replacement) and MAD methods is in progress.

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