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Crystallization and preliminary diffraction studies of native and selenomethionine CcmG (CycY, DsbE)

Melissa A. Edeling,[a]† Luke W. Guddat,[b]† Renata A. Fabianek,[c]‡ Judy A. Halliday,[a]§ Alun Jones,[a] Linda Thöny-Meyer[c] and Jennifer L. Martin[a]*

[a]Centre for Drug Design and Development and Special Research Centre for Functional and Applied Genomics, Institute for Molecular Bioscience, University of Queensland, Brisbane, QLD 4072, Australia, [b]Department of Biochemistry and Molecular Biology, School of Molecular and Microbial Science, The University of Queensland, Brisbane, QLD 4072, Australia, and [c]Institute of Microbiology, ETH Zürich, Schmelzbergstrasse 7, CH-8092 Zürich, Switzerland

† These two authors contributed equally to this work.
‡ Present address: RAF, Stratagene Europe, Amsterdam, The Netherlands.
§ Present address: Alchemia Pty Ltd, Upper Mt Gravatt, QLD 4122, Australia.

Correspondence e-mail: j.martin@imb.uq.edu.au

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Disulfide-bond (Dsb) proteins are a family of redox proteins containing a Cys-*X*-*X*-Cys motif. They are essential for disulfide-bond exchange in the bacterial periplasm and are necessary for the correct folding and function of many secreted proteins. CcmG (DsbE) is a reducing Dsb protein required for cytochrome *c* maturation. Crystals of *Bradyrhizobium japonicum* CcmG have been obtained that diffract X-rays to 1.14 Å resolution. The crystals are orthorhombic, space group $P2_12_12_1$, with unit-cell parameters $a = 35.1$, $b = 48.2$, $c = 90.2$ Å. Selenomethionine CcmG was expressed without using a methionine auxotroph or methionine-pathway inhibition and was purified without reducing agents.

1. Introduction

Secreted proteins face a harsh environment that requires additional structural stabilization to prevent unfolding and protease degradation. For most secreted proteins, this is achieved through bracing the polypeptide fold by forming disulfide bridges between cysteines in the sequence. The formation of disulfide bonds is a catalysed event that takes place in the oxidative environment of the endoplasmic reticulum in eukaryotes. In bacteria, disulfide-bond formation occurs in the periplasmic space and is catalysed by the Dsb (disulfide-bond) family of proteins (Bardwell, 1994), which are characterized by a conserved Cys-*X*-*X*-Cys motif. The Dsb proteins include oxidizing proteins that catalyse disulfide-bond formation quickly and efficiently (DsbA; Bardwell *et al.*, 1991), disulfide-shuffling proteins that isomerize incorrectly bridged disulfide bonds (DsbC; Zapun *et al.*, 1995; DsbG; Bessette *et al.*, 1999) and membrane proteins that convert Dsb proteins into their respective catalytically competent redox form (DsbB; Bardwell *et al.*, 1993; DsbD; Katzen & Beckwith, 2000).

CcmG was originally named DsbE because it was identified through a genetic screen for Dsb proteins (Missiakas & Raina, 1997). It is now known that this protein has a very specific disulfide-reducing role in the maturation of the haem-binding *c*-type cytochromes (Fabianek *et al.*, 1998), which transfer electrons in respiratory and/or photosynthetic electron-transport chains. The biogenesis of *c*-type cytochromes in bacteria is quite complex: the Gram-negative bacterium *Escherichia coli* expresses eight specific genes (*ccmA–H*; Thöny-Meyer *et al.*, 1995) for the task. CcmG is one of the eight proteins encoded by these genes. The homologous protein in *B. japonicum* (formerly known as CycY) is a 20 kDa periplasmic protein attached to the cytoplasmic membrane by an N-terminal membrane anchor (Fabianek *et al.*, 1997). The redox potential of CcmG is −0.217 V (Fabianek *et al.*, 1997), indicating that it is a reducing protein, and its role is proposed to be in the reduction of the apocytochrome before haem attachment (Fabianek *et al.*, 2000). Here, we describe the production of *B. japonicum* CcmG crystals that are suitable for high-resolution structural studies.

2. Materials and methods

2.1. Protein expression and purification

A soluble form of *B. japonicum* CcmG without the N-terminal membrane anchor (corresponding to residues 39–194) was expressed and purified as described previously (Fabianek *et al.*, 1997), except that a gel-filtration step was added to remove low molecular weight contaminants. For expression of selenomethionine CcmG (SeMet CcmG), the pET22b (Novagen) expression vector containing CcmG with a cleavable signal sequence (Fabianek *et al.*, 1997) was used to transform BL21 (DE3) cells. The cells were cultured in minimal medium containing SeMet (L/D mixture) at a concentration of 50 μg ml^{-1}. Native CcmG was only expressed successfully in the absence of induction by isopropyl β-D-thiogalactopyranoside (IPTG): inclusion bodies containing CcmG formed if IPTG was added. However, expression of SeMet CcmG in minimal medium required the addition of 0.1 m*M* IPTG. The IPTG was added at the exponential phase of growth ($OD_{600\,nm} = 0.6$) and the cells were harvested during the stationary phase ($OD_{600\,nm} = 1.4$). Protein

concentration was estimated by measuring the absorption at 280 nm in a Shimadzu UV-1201 spectrophotometer and homogeneity was assessed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis. SeMet incorporation was assessed by mass spectrometry using a PE SCIEX API III triple-quadrupole mass spectrometer.

2.2. Crystallization and cryoprotection

CcmG samples for crystallization were prepared in Milli-Q water without any additives such as buffers or salts. The hanging-drop vapour-diffusion method was used with a sparse-matrix screening approach (Jancarik & Kim, 1991) to identify promising conditions. Grid screens were prepared to investigate the effect of temperature (277 and 293 K), pH (varied over pH 6–8 in 0.5 pH unit steps), precipitant concentration [ammonium sulfate varied from 1.4–2.4 *M* in 0.2 *M* steps, polyethylene glycol (PEG) 400 varied from 2–10% in 2% steps] and protein concentration (two concentrations used, 20 and 40 mg ml^{-1}). Crystallization trials were set up using a 2 µl hanging drop (1 µl of protein solution and 1 µl of reservoir solution) suspended over a reservoir volume of 1 ml. Microseeding (Stura & Wilson, 1990) was performed by preparing a crystal seed stock from a crushed crystal diluted tenfold in stabilizing solution [0.1 *M* *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) pH 6.5, 2%(*v*/*v*) PEG 400, 2.2 *M* ammonium sulfate]. Serial tenfold dilutions were made from the seed stock in stabilizing solution. A cat's whisker was used to streak seed crystallization drops (Stura & Wilson, 1990) to identify the optimal concentration for microseeding.

2.3. Cryocooling and X-ray diffraction data measurement

Crystals of native and SeMet CcmG were cryoprotected in a stepwise manner. Crystals were soaked using a sequential soaking technique (Garman, 1999), with 2 min in stabilizing solution (see §2.2) containing 5% glycerol then 2 min each in 10, 15 and finally 20% glycerol in stabilizing solution. Crystals were then flash-cooled in the gaseous nitrogen stream at 100 K. In-house X-ray diffraction data were measured using a Rigaku RU-200 Cu $K\alpha$ rotating-anode X-ray generator operating at 46 kV and 60 mA and equipped with Yale focusing mirror optics (MSC) and a Rigaku R-AXIS IIC imaging-plate area detector. Synchrotron X-ray diffraction data were measured at a wavelength of 1.08 Å on beamline BL 7-1 using a MAR 300 imaging-plate detector at the Stanford Synchrotron Radiation Laboratory (SSRL), USA. All diffraction data were integrated and scaled using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

3.1. Native CcmG

Crystals of native oxidized CcmG grew at 293 K from microseeded hanging drops containing 1 µl of 40 mg ml^{-1} CcmG and 1 µl of a reservoir solution containing 0.1 *M* HEPES pH 6.5, 2%(*v*/*v*) PEG 400 and 2.0 *M* ammonium sulfate. CcmG crystals grown in this way can reach dimensions of up to 0.8 mm in 7 d. However, these larger crystals were not amenable to cryocooling (diffraction resolution worsened and mosaicity increased), presumably because their size hindered flash-cooling through to the core of the crystal. Smaller crystals of around 0.4 mm in length were cryocooled and diffract to 1.35 Å on the in-house rotating-anode/image-plate X-ray diffraction equipment (Table 1). To measure a higher resolution data set, the largest crystals were cooled to 277 K rather than cryocooled and then exposed to synchrotron radiation at the SSRL. This gave a high-quality 1.14 Å resolution native data set from a single CcmG crystal (Table 1). Crystals that were cryocooled at the SSRL did not diffract beyond 1.3 Å resolution. The crystals belong to the space group $P2_12_12_1$ and have unit-cell parameters $a = 35.1$, $b = 48.2$, $c = 90.2$ Å. The solvent content based on one molecule per asymmetric unit is 45%, which is within the usual range for proteins (27–65%; Matthews, 1968).

3.2. SeMet CcmG

Expression of SeMet-substituted proteins is now commonplace for X-ray structure determination, since it enables rapid phase determination from a single crystal using multiwavelength anomalous diffraction. However, the production of SeMet CcmG is unusual for two reasons. Firstly, it did not use either of the two common methods of producing SeMet proteins: (i) use of a methionine auxotrophic *E. coli* strain of bacteria or (ii) inhibition of the methionine pathway by addition of high concentrations of specific amino acids at the mid-log phase (Doublie, 1997). SeMet CcmG was simply expressed from the usual BL21 (DE3) strain in minimal medium supplemented with selenomethionine. Secondly, since crystallization of native CcmG requires the active site to be in the oxidized disulfide form, SeMet CcmG was expressed, purified and crystallized in the absence of reducing agents. This is unusual for SeMet protein preparations. Reducing agents are usually added to prevent oxidation of the selenium (Smith & Thompson, 1998).

Table 1
Data-collection statistics for native CcmG crystals.

Values in parentheses refer to the highest resolution shell.

Temperature (K)	100	277
Resolution range (Å)	50–1.35	20–1.14
Mosaicity (°)	0.55	0.2
No. of observations [$I > 0\sigma(I)$]	165149	174559
No. of unique reflections [$I > 0\sigma(I)$]	30573	50056
Highest resolution shell (Å)	1.40–1.35	1.18–1.14
R_{sym}† (%)	6.7 (16.4)	6.2 (29.1)
Completeness (%)	92.1 (52.2)	88.4 (80.3)
$I/\sigma(I)$	18.4 (4.1)	12.0 (2.6)

† $R_{sym} = \sum|I - \langle I\rangle|/\sum\langle I\rangle$, where I is the intensity of each individual reflection.

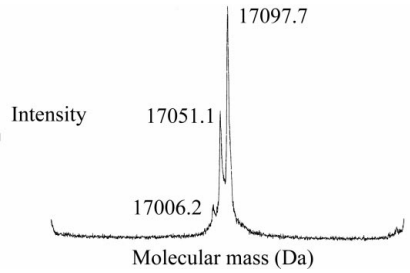

Figure 1
Electrospray mass spectrum of SeMet CcmG. Masses for the three peaks are shown. The largest peak (mass 17 097.9 Da) corresponds to CcmG with two SeMet substitutions and the shoulder peak (mass 17 051.1 Da) corresponds to CcmG with only one of the two methionines substituted with SeMet. The smallest peak (mass 17 006.2 Da) corresponds to native CcmG.

The soluble periplasmic domain of CcmG incorporates two methionines, one of which is at the N-terminus. The calculated molecular mass of this soluble form of CcmG is 17 005.4 Da. If both methionines are replaced by SeMet, the mass would increase to 17 099.2 Da. If only one methionine is substituted by SeMet, the mass would be 17 052.3 Da. The mass spectrum of the purified SeMet CcmG protein (Fig. 1) shows that the dominant peak corresponds to two SeMet substitutions. A second peak (at much lower intensity) corresponds to a single SeMet replacement. Very little of the sample appears to have no SeMet replacement and there is no indication of SeMet oxidation.

Crystals of SeMet CcmG grow under similar conditions to those used to produce native CcmG crystals, except that a lower protein concentration is required (32 mg ml^{-1}) and the final drop size is 4 µl (2 µl of protein and 2 µl of reservoir). We plan to solve the structure of CcmG using these SeMet CcmG crystals in multi-wavelength anomalous diffraction experiments.

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References

Bardwell, J. C. (1994). *Mol. Microbiol.* **14**, 199–205.
Bardwell, J. C., Lee, J. O., Jander, G., Martin, N., Belin, D. & Beckwith, J. (1993). *Proc. Natl Acad. Sci. USA*, **90**, 1038–1042.
Bardwell, J. C., McGovern, K. & Beckwith, J. (1991). *Cell*, **67**, 581–589.
Bessette, P. H., Cotto, J. J., Gilbert, H. F. & Georgiou, G. (1999). *J. Biol. Chem.* **274**, 7784–7792.
Doublie, S. (1997). *Methods Enzymol.* **276**, 523–530.
Fabianek, R. A., Hennecke, H. & Thöny-Meyer, L. (1998). *J. Bacteriol.* **180**, 1947–1950.
Fabianek, R. A., Hennecke, H. & Thöny-Meyer, L. (2000). *FEMS Microbiol. Rev.* **24**, 303–316.
Fabianek, R. A., Huber-Wunderlich, M., Glockshuber, R., Künzler, P., Hennecke, H. & Thöny-Meyer, L. (1997). *J. Biol. Chem.* **272**, 4467–4473.
Garman, E. (1999). *Acta Cryst.* D**55**, 1641–1653.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Katzen, F. & Beckwith, J. (2000). *Cell*, **103**, 769–779.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Missiakas, D. & Raina, S. (1997). *J. Bacteriol.* **179**, 2465–2471.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Smith, J. L. & Thompson, A. (1998). *Structure*, **6**, 815–819.
Stura, E. A. & Wilson, I. A. (1990). *Methods*, **1**, 38–49.
Thöny-Meyer, L., Fischer, F., Künzler, P., Ritz, D. & Hennecke, H. (1995). *J. Bacteriol.* **177**, 4321–4326.
Zapun, A., Missiakas, D., Raina, S. & Creighton, T. E. (1995). *Biochemistry*, **34**, 5075–5089.