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Purification, crystallization and preliminary X-ray analysis of a Sco1-like protein from *Bacillus subtilis*, a copper-binding protein involved in the assembly of cytochrome *c* oxidase

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The putative copper-delivery protein BsSco from *Bacillus subtilis* is a member of the Sco family of cytochrome *c* oxidase assembly proteins. BsSco is a membrane protein and the soluble domain has been cloned and expressed in *Escherichia coli* as a fusion with glutathione-*S*-transferase. The fusion protein was isolated from the cell lysate using a glutathione-affinity column and the soluble domain of BsSco was released by treatment with thrombin. Sufficient amounts of the soluble domain have been obtained for crystallization. Crystals obtained by hanging-drop vapour diffusion diffract to a resolution of 2.3 Å at a synchrotron source. The space group is *P*6 and the unit-cell parameters are $a = 67.74$, $b = 67.74$, $c = 189.58$ Å.

1. Introduction

Aerobic respiratory chains usually terminate with an integral membrane protein that is a member of the heme-copper family of oxidoreductases (Pereira *et al.*, 2002). It is becoming increasingly apparent that the assembly of these multimeric enzymes involves several accessory proteins for the coordinated insertion of their redox centers (*e.g.* Brown *et al.*, 2002; Nittis *et al.*, 2001). Proteins belonging to the Sco family are proposed to be involved in the delivery of copper to cytochrome *c* oxidase (Glerum *et al.*, 1996). Sco was initially identified as a requirement in the assembly of functional cytochrome *c* oxidase within yeast mitochondria (Schulze & Rodel, 1988). Genetic manipulations of the gene encoding Sco1p within yeast cells demonstrate that its disruption results in a phenotype that is cytochrome *c* oxidase deficient and is incapable of growth on non-fermentable substrates. A second highly similar gene, *Sco2*, has also been identified in yeast, but Sco2p does not complement the *Sco1* knockout. The role of Sco2p in cytochrome *c* oxidase assembly is not known. A human homolog of yeast Sco1 has been identified (*i.e.* hSco2) and three lethal point mutations have been reported (Papadopoulou *et al.*, 1999).

The aerobic bacterium *Bacillus subtilis* expresses two distinct members of the heme-copper based cytochrome oxidase family (Hill *et al.*, 1993). One of these is a cytochrome *c* oxidase and has the same redox-active metal sites as found in the well studied mitochondrial cytochrome *c* oxidase, including the two copper centers known as Cu_A and Cu_B. The second member of the cytochrome oxidase family expressed in aerobically grown *B. subtilis* is a menaquinol oxidase, but menaquinol oxidase has only three of the four redox-active metal centers found in cytochrome *c* oxidases. The menaquinol oxidase does not have the Cu_A center found in cytochrome *c* oxidases. In *B. subtilis*, there are therefore two members of the same enzyme family but with different copper requirements. We reasoned that this would be a useful system to explore the role of metal-delivery proteins such as the Sco-type protein found in yeast. A sequence-based search of the *B. subtilis* genome reveals a candidate gene that has the features of a potential Sco homolog (Mattatall *et al.*, 2000). The gene is designated *ypmQ* and when this gene is deleted *via* homologous recombination the bacterium is deficient in cytochrome *c* oxidase, but the level of menaquinol oxidase is unaffected. The level of cytochrome *c* oxidase recovers when the growth medium is supplemented with copper or when *ypmQ* is expressed from a plasmid. The *B. subtilis* gene *ypmQ* expresses a homolog of Sco and we hereafter refer to this protein as BsSco, for *B. subtilis* Sco. The metal-binding activity has been reported for the Sco homolog from *Rhodobacter sphaeroides* (McEwan *et al.*, 2002). In addition, it has been recognized that the Sco proteins may have a thiol–disulfide oxidoreductase activity in addition to their putative role in copper acquisition (Chinenov, 2000). In order to better understand the functional role of the Sco protein, it is of interest to determine the structure of a member of this protein family.

Both the yeast and *B. subtilis* versions of Sco are integral membrane proteins. BsSco is expressed as an approximately 21 000 Da lipoprotein with a diacylglyceride moiety attached to a cysteine residue that is at the N-terminus following processing by signal peptidase II (Tjalsma *et al.*, 1999). Over-

expression of native BsSco in *B. subtilis* does not produce sufficient protein for structural studies. Furthermore, expression of the entire gene sequence from *B. subtilis* is lethal in *Escherichia coli* (Mattatall & Hill, unpublished observation). Therefore, we developed an expression and purification system for the soluble domain of BsSco that involves fusing this sequence with glutathione-*S*-transferase and expressing the fusion protein in *E. coli*. Here, we describe the crystallization and preliminary X-ray data of the soluble domain of BsSco.

2. Gene construction

The polymerase chain reaction was used to amplify the soluble portion (*i.e.* amino acids 22–193) of *ypmQ* from pNM137 (Mattatall *et al.*, 2000). The forward (ypmQGST-F; 5′-GGA ATT CC**G GAT CC**C AGC AGA TTA AAG ATC CGC TCA ATT AC-3′) and reverse (ypmQGST-R; 5′ GG**G GAT CC**T CGA GTT ACT TGA GTG TAC TGG CTG ACT T-3′) primers contained *Bam*HI (in bold) and *Xho*I (underlined) restriction sites. The 516 bp PCR product was cloned into the pDrive cloning vector using a Qiagen PCR cloning kit. The insert was then subcloned into pGEX-4T3 (Amersham Pharmacia Biotech Inc., Baie d'Urfe, Quebec) using *Bam*HI/*Xho*I restriction sites to give DH5α (Gibco BRL)/pJR395.

3. Expression and purification of BsSco

E. coli DH5α cells harboring pJR395 were grown in 10 ml of Luria broth (LB medium) containing 0.1%(*w*/*v*) glucose and 100 µg ml^{-1} ampicillin. At an A_{600} of 0.6–0.8, the culture was added to a 2.8 l Fernbach flask with 700 ml of the above medium and incubated with shaking at 310 K. When the A_{600} reached 0.5, the temperature was decreased to 303 K. At A_{600} 0.6–0.8, isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 1.0 m*M* and the culture incubated for another 3 h. Cells were harvested by centrifugation at 3260*g* at 277 K for 30 min in a Beckman J-6M/E centrifuge using the JS-4.2 rotor. The cell pellet (approximately 5 g) was resuspended in 40 ml PBS/EDTA/PMSF buffer [1× phosphate-buffered saline (1× PBS; 10 m*M* Na_2HPO_4, 2.7 m*M* KCl, 137 m*M* NaCl, 2 m*M* KH_2PO_4 pH 7.4), 1 m*M* EDTA, 0.5 m*M* phenylmethylsulfonyl fluoride (PMSF)] and frozen at 203 K until use. The cells were thawed and lysed by addition of lysozyme (0.04 g per 5 g of cells and 40 ml of added PBS/EDTA/PMSF buffer). To improve the binding of GST-BsSco to the affinity resin, Tris(2-carboxyethyl)phosphine hydrochloride (TCEP; Sigma) was added to a final concentration of 5 m*M* before cell lysis. After 30 min lysis at 277 K, 20 ml of 0.2% Triton X-100 and 300 µl of 1 mg ml^{-1} DNase and RNase were added. After rocking for 10 min at 277 K, the sample was centrifuged at 3260*g* at 277 K for 30 min. The pH of the supernatant was adjusted to 6.4 prior to being loaded onto a Glutathione Sepharose 4 Fast Flow (Amersham Pharmacia Biotech AB) column (4 ml bed volume) equilibrated with 1× PBS pH 7.4. All purification steps were performed at room temperature. The column was washed with 1× PBS pH 7.7 until the A_{280} returned to the background level. After 16 h on-column thrombin cleavage (100 units per 100 mg of protein loaded), BsSco, including two residues (glycine-serine) from the thrombin cleavage site, was eluted from the column in 1× PBS pH 7.7. The purity and identity of BsSco were assessed using SDS–PAGE and Western blotting, respectively.

The molecular mass of the BsSco, as determined by mass spectroscopy (Micromass Q-Tof Ultima) is 19 721.30 ± 0.18 Da, in comparison with a theoretical value of 19 652.1 Da. One possible explanation of this discrepancy is that our preparations contain bound metal ions, although the protein isolation was performed under metal-limited conditions. We are in the process of pursuing both the metal-binding capacity and redox status of BsSco. The yield of highly purified BsSco was approximately 13 mg per litre of *E. coli* culture. In the last stage of purification, BsSco was dialyzed into 50 m*M* Tris–HCl pH 7.4 and concentrated to a concentration of 10–28 mg ml^{-1} using 5K NMWL membranes (Millipore).

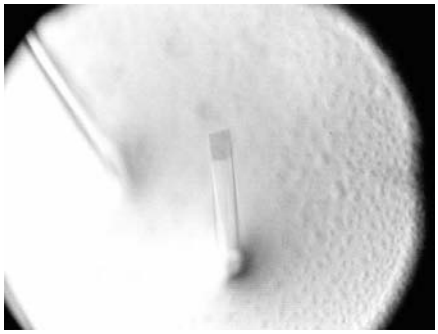

Figure 1
Typical crystal of BsSco.

4. Crystallization

The protein concentration was adjusted to 8–10 mg ml^{-1} in 10 m*M* sodium acetate pH 4.5 for crystallization trials. Initial crystal screening by hanging-drop vapour diffusion was carried out using a sparse-matrix screen (Jancarik & Kim, 1991) with commercial Crystal Screen kits (Hampton Research) at room temperature. Expansion and fine-tuning of the initial crystallization conditions led to the final condition containing 15% PEG MME 5000, 0.2 *M* $CaCl_2$, 0.1 *M* cacodylate pH 6.5 and 5 m*M* $NiCl_2$. Crystals usually appeared in 1–2 d, reaching a maximum size of 0.6 × 0.2 × 0.2 mm in ∼7 d (Fig. 1).

Table 1
Statistics of X-ray diffraction data.

Values in parentheses are for the highest resolution shell.

No. measured reflections	51796
No. independent reflections	19918
Resolution range (Å)	30.0–2.7 (2.38–2.30)
R_{sym} (%)	8.6 (28.8)
Completeness (%)	91.2 (91.8)
Average $I/\sigma(I)$	15.3 (4.7)

5. X-ray diffraction analysis

Data collection was carried out at the F2 beamline of the Cornell High Energy Synchrotron Source using a Quantum IV CCD detector at a wavelength of 0.979 Å. Prior to data collection at 100 K, crystals were soaked in crystallization buffer containing 20% PEG 400 for 2–3 min followed by flash-cooling in a liquid-nitrogen gas stream. All data were processed with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The crystals belong to the *P*6 space group, with unit-cell parameters a = 67.74, b = 67.74, c = 189.58 Å (Table 1). If we assume there to be three molecules per asymmetric unit, then the solvent content would be ∼40%. For structure determination, we have expressed and crystallized SeMet-labeled protein and also obtained a lead co-crystal that will be used for phase solution.

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