Technical Note

Structure of the second Single Stranded DNA Binding protein (SSBb) from Mycobacterium smegmatis

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A B S T R A C T

All mycobacteria with sequenced genomes, except M. leprae, have a second Single Stranded DNA Binding protein (SSBb) in addition to the canonical one (SSBa). This parologue from M. smegmatis (MSSBb) has been cloned, expressed and purified. The protein, which is probably involved in stress response, has been crystalized and X-ray analyzed in the first structure elucidation of a mycobacterial SSBb. In spite of the low sequence identity between SSBas and SSBbs in mycobacteria, the tertiary and quaternary structure of the DNA binding domain of MSSBb is similar to that observed in mycobacterial SSBas. In particular, the quaternary structure is ‘clamped’ using a C-terminal stretch of the N-domain, which endows the tetrameric molecule with additional stability and its characteristic shape. Comparison involving available, rather limited, structural data on SSBBs from other sources, appears to suggest that SSBBs could exhibit higher structural variability than SSBas do.

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1. Introduction

DNA transactions such as replication, recombination and repair involve the formation of single stranded DNA (ssDNA). Single Stranded DNA Binding proteins (SSBs) protect ssDNA from chemical attacks and nuclease and prevent the formation of secondary DNA structures. The binding of DNA to SSB is independent of the sequence of DNA. SSB is one of the minimal gene products for life owing to its pivotal role in maintaining the genomic integrity (Gil et al., 2004; Mushegian and Koonin, 1996). Canonical SSBs consist of a conserved N-terminal domain and a flexible, highly disordered C-terminal tail. The N-terminal domain is composed of an oligonucleotide binding (OB) fold involved in DNA binding, while the C-terminal tail is involved in interaction with other proteins (Murzin, 1993; Shereda et al., 2008). Eubacterial SSBs are usually homo-tetrameric or contain four OB domains (Arif and Vijayan, 2012). Tetrameric SSBs have two major DNA binding modes. Experiments with the E. coli and mycobacterial SSBs showed that in the SSB35 mode, 35 nucleotides wrap around two subunits of the tetramer while in the SSB56/65 mode, 56/65 nucleotides wrap around all four subunits of the tetramer. The binding mode is dependent on concentration of the protein and salt in the solution (Acharya and Varshney, 2002; Lohman and Ferrari, 1994; Purnapatre and Varshney, 1999).

The three dimensional structures of homo-tetrameric SSBs were first characterized by X-ray studies of the proteins from E. coli (EcSSB) and human mitochondria (HMtSSB) (Raghunathan et al., 1997; Yang et al., 1997). The X-ray work involved the structural characterization of only the DNA binding N-terminal domain with the OB-fold, as the C-terminal domain was disordered in these as well as in all subsequent structure determinations. The third SSB to be characterized in detail was that from Mycobacterium tuberculosis (MtSSB) (Purnapatre and Varshney, 1999; Reddy et al., 2001). Tertiary structure determined in two crystal forms of MtSSB, as part of international and national efforts on mycobacterial proteins (Arcus et al., 2006; Arora et al., 2011; Chim et al., 2011), was very similar to that of HMtSSB and EcSSB (Saikrishnan et al., 2003). The N-domain of MtSSB, however, contains an additional β-strand (‘clamp’), which helps in stabilizing a somewhat different mode of quaternary association. Subsequent work demonstrated that the proteins from M. smegmatis and M. leprae have tertiary and quaternary structures similar to those of MtSSB, although subtle differences, especially in quaternary association, exist among the crystal structures of the three mycobacterial SSBs (Kaushal et al., 2010; Saikrishnan et al., 2005). In the meantime, structures of SSBs from several other species became available (Bernstein et al., 2004; DiDonato et al., 2006; Jedrzejczak et al., 2006; Stefanić et al., 2009). In spite of the low sequence conservation among them, all eubacterial SSBs have essentially the same tertiary structure. However, they exhibit considerable variability in quaternary association (Arif and Vijayan, 2012). The presence of an additional strand in...
mycobacterial SSBS and the related Streptomyces coelicolor SSB (ScSSB), which clamps the two subunits together, is a major determinant of the mode of quaternary association in them.

It has been discovered that several bacteria have a second paralogous SSB (SSBb), in addition to the canonical SSB (hereafter referred to as SSBa) (Kramer et al., 2007; Lindner et al., 2004). The biological role of SSBS is poorly understood. In Bacillus subtilis, SSBb (BssSSBb) has been reported to be involved in natural-competence associated recombination. It was also shown to share the load of SSBa during genetic recombination, by modulating RecA nucleation (Yadav et al., 2012). In Streptococcus pneumoniae, SSBb (SpSSBb) maintains the reservoir of naturally internalized ssDNA, thereby increasing the likelihood of multiple chromosomal transformation events in the same cell (Attaiech et al., 2011). A 3–10 fold decrease in chromosomal transformation of cells lacking SSBb, in both B. subtilis and S. pneumoniae, was seen indicating a possible redundant function (Attaiech et al., 2011; Ogura et al., 2002). S. coelicolor SSBb (ScSSBb) has been implicated in an unexpected role in chromosomal segregation during sporulation (Paradzik et al., 2013). The DNA binding properties of SSBS are often different from those of their respective SSBa counterparts. In B. subtilis, SSBb binds with lesser affinity to DNA than SSBa, while in S. pneumoniae and S. coelicolor, SSBb has a greater affinity for DNA (Attaiech et al., 2011; Yadav et al., 2012). Crystallographic studies have so far been carried out on SSBS from only two species, namely B. subtilis (BssSSBb) (Yadav et al., 2012) and S. coelicolor (ScSSBb) (Paradzik et al., 2013). The tertiary structures of BssSSBb and ScSSBb are remarkably similar to those of SSBas, with some interesting differences in the quaternary structure.

A genomic search, of 43 mycobacterial genomes, revealed the presence of SSBb in all mycobacterial species, except M. leprae (Singh et al., 2016). The genes corresponding to the two SSBS are situated far away on genomes. ssB genes are usually situated on the minus strand and are far away from the ssA gene in most mycobacterial genomes. ssB gene is encoded within an operon containing a putative ABC transporter ATP binding protein, as suggested by the ProOPDB (Taboda et al., 2012). While the SSBa sequence is highly conserved within mycobacterial genus, for example, with a sequence identity of 86% between MsSSBa and MsSSBb, SSBa proteins are much less conserved with a sequence identity of 54% between MsSSBa and MsSSBb. The sequence identity between the paralogous SSBS from same species is still lower. For instance, it is 32% in M. smegmatis and 26% in M. tuberculosis. The biological role of SSBSs in mycobacteria is still unclear. However, microarray data from M. tuberculosis H37Rv (Reddy et al., 2009) show that ssB gene is over-expressed during hypoxia and non-replicating persistence stage or dormancy (Boshoff et al., 2004; Voskuil et al., 2004). Exposure to DNA damaging agents, growth on long-fatty acids and starvation also lead to over-expression of ssBb gene and simultaneous under-expression of ssBa gene in many cases (Unpublished results: Yang Liu, et al.). These results indicate a probable role of SSBb in stress response in mycobacteria involving a hitherto unknown mechanism and provide an additional reason for pursuing further studies on mycobacterial SSBSs. Here we report the cloning, expression, purification and crystallographic studies of MsSSBb. The structure of the protein is discussed in relation to other SSBSs of known structure.

2. Materials and methods

2.1. Cloning, expression and purification

The gene encoding MsSSBb (MSMEG_4701) was amplified by PCR from the genomic DNA of M. smegmatis mc2155, using Phusion polymerase (NEB) and 100 pmol each of a forward primer (CGTACATAATACGATCTCGAGACACGGTCACTG) and a reverse primer (TGAGAACGGCTCTACCCGTCAGTCGACG). The amplified products containing Ndel and HindIII sites (underlined) were digested with respective enzymes and were cloned between the corresponding sites of the pET-14b vector (Novagen Inc.) to produce a N-terminal HIS-tagged construct. A histidine tag containing peptide stretch, MGSSHHHHHHSSGLVPRGS, was appended to the N-terminus. Cloning was confirmed by primer-based sequencing (Xcleris genomics).

The construct was transformed into E. coli BL21 (DE3) cells (Novagen Inc.) for expression. Primary and secondary cultures were grown in Luria-Bertani (LB) medium supplemented with ampicillin (100 µg ml⁻¹). The cells were harvested after 6 h of induction (0.5 mM IPTG) at 37 °C at 180 rpm to maximize the yield. The pellet was re-suspended in buffer A (30 mM Tris-HCl pH 8.0, 1 M NaCl, 10 mM imidazole and 10% (v/v) glycerol) and lysed by sonication. The lysate was then centrifuged at 15,000 g for 60 min. The supernatant was loaded onto an Ni-NTA column (GE Healthcare) equilibrated with buffer A, washed with the same buffer supplemented with 20 mM imidazole and eluted with a linear imidazole gradient from 30 to 500 mM. The histidine tag containing peptide stretch was retained in subsequent procedures. The protein was further purified by size-exclusion chromatography using High load 16/600 Superdex 200 column (GE Healthcare) in buffer B (30 mM Tris-HCl pH 8.0, 750 mM NaCl and 10% (v/v) glycerol). Two separate peaks were obtained on subjecting the protein to gel filtration. No detectable DNA contamination was observed in the second peak. The fractions containing pure protein were concentrated to 13 mg ml⁻¹ for crystallization trials. The purity was established by SDS-PAGE. The quaternary state and molar mass of purified MsSSBb were established by size-exclusion chromatography with multi–angle light scattering. A Superdex 200 10/300 column (GE healthcare) was connected in-line to a mini-DAWN detector (Wyatt Technology, California, USA). Pure MsSSBb (0.5 mg ml⁻¹) was injected in the column pre-equilibrated with buffer containing 30 mM Tris-HCl pH 8.0, 250 mM NaCl. The signals were collected at a 90° angle from the laser and were analyzed using the Astra software (Wyatt Technology). Proteins were also dialyzed against buffer C (30 mM Tris-HCl pH 8.0, 200 mM NaCl and 5% (v/v) glycerol) and stored for further use in biochemical characterization.

2.2. Crystallization and structure solution

Commercially available screens from Hampton research, Molecular dimensions and Jena biosciences were employed for crystallization trials. Microbatch under-oil method was used in all crystallization experiments. 1 µl protein solution (13 mg ml⁻¹) in buffer B was mixed with 1 µl precipitant solution in a 1:1 mixture of paraffin oil and silicon oil. Poorly diffraction crystal were obtained with Hampton Crystal Screen lite condition No. 14, consisting of 0.2 M calcium chloride dehydrate, 0.1 M HEPES pH 7.5, 7.5% (v/v) PEG 400. Addition of 3% (w/v) 1.5-diaminopentane dihydrochloride to the precipitant solution resulted in better crystals that could be used for diffraction studies. Crystals were soaked in a mother liquor containing 5% (v/v) glycerol as cryo-protectant, for about 1 min. Diffraction data were collected at a home source using a MAR345 detector mounted on a Bruker MICROSTAR ULTRA II Cu Kα rotating-anode X-ray generator, at 100 K. The data were processed using iMosflm (Barty et al., 2011) and were scaled using SCALA from the CCP4 program suite (Winn et al., 2011). A Matthews coefficient of 2.22 Å³Da⁻¹ correspond to the presence of two subunits in the asymmetric unit, with 40% solvent content (Matthews, 1968). The structure of SSBa from S. coelicolor (PDB:3EIV), which exhibits the maximum sequence similarity with
MsSSBb among SSBs of known three dimensional structure, was used as the search model in the molecular replacement solution of MsSSBb. Using Phaser (McCoy et al., 2007), the best solution was obtained in space group P6₅22, with a log-likelihood gain of 342 and a Z score of 22.7. Model building was done using COOT (Emsley et al., 2010) and refinement was carried out using REFMAC (Vagin et al., 2004). During the final cycles of refinement, water O atoms were added successively using peaks with heights of greater than 3.0 in Fo – CₐFc maps and 1.0 in 2Fo – CₐFc maps. The C-terminal residues and a few residues in loops are not defined in both the subunits. Statistics pertaining to data collection, refinement and model are given in Table 1.

### Table 1

<table>
<thead>
<tr>
<th>Space group</th>
<th>P6₅22</th>
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<tr>
<td>Unit cell dimensions</td>
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<tr>
<td>a (Å)</td>
<td>73.61</td>
</tr>
<tr>
<td>b (Å)</td>
<td>73.61</td>
</tr>
<tr>
<td>c (Å)</td>
<td>216.21</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
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<tr>
<td>Vₐ (Å³ Da⁻¹)</td>
<td>2.22</td>
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<tr>
<td>Solvent content (%)</td>
<td>39.69</td>
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<tr>
<td>Unique reflections</td>
<td>12,839 (1821)</td>
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<tr>
<td>Multiplicity</td>
<td>9.9 (9.6)</td>
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<tr>
<td>Completeness (%)</td>
<td>100.0 (100.0)</td>
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<tr>
<td>&lt;I/σ(I)&gt;</td>
<td>16.4 (2.3)</td>
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<tr>
<td>CC(1/2)</td>
<td>0.999 (0.671)</td>
</tr>
<tr>
<td>Rmerge (%)</td>
<td>9.3 (100.0)</td>
</tr>
</tbody>
</table>

#### Refinement and model statistics

| R-factor (%) | 21.1 |
| Rfree (%) | 25.6 |
| R.M.S. deviation from ideal bond lengths (Å) | 0.016 |
| Bond angles (°) | 1.78 |
| Ramachandran plot statistics (Residues in %) | |
| Favored region | 92.5 |
| Allowed region | 7.5 |
| Disallowed region | 0.0 |

#### a Rmerge = Σᵢ[|Iᵢ(hkl)| - <I(hkl)>]/Σᵢ|Iᵢ(hkl)|, where Iᵢ(hkl) is the ith observation of reflection hkl and <I(hkl)> is the weighted average intensity for all i observations of reflection hkl.

### 2.3. Analysis of the structures

ALIGN was used to superpose three dimensional structures (Cohen, 1997). Surface area calculations and interface analysis were carried out using PISA and NACCESS (Hubbard and Thornton, 1993; Krissinel and Henrick, 2007). PyMOL was used for visual analysis of structures and generating figures (DeLano, 2002).

### 3. Results and discussion

#### 3.1. Purification and characterization

The ssbB gene from M. smegmatis was successfully cloned and expressed using E. coli expression system. The His-tagged proteins obtained after Ni-NTA chromatography were partially pure and had DNA contamination, as established by OD₂₆₀/₂₈₀ and running the protein samples on an agarose gel. Protein was further purified to homogeneity on a Superdex 200 gel filtration column and samples with no DNA contamination were pooled together. SDS-PAGE indicated that the purified MsSSBb has a subunit molecular weight of approximately 20 kDa, along with the tag (Fig. 1A). A single peak at a molecular mass of 75.4 (±2.9) kDa corresponding to a stable tetramer was seen in SEC-MALS (Fig. 1B).

#### 3.2. Overall structural features

The tetrameric molecule of MsSSBb (Fig. 2A) has 222 symmetry. One of the molecular dyads coincides with a crystallographic two-fold axis. This relates subunits A and C to subunits B and D. The two subunits in the asymmetric unit have the same structure with a r.m.s.d. of 0.46 Å on superposition of Cα positions. In both subunits, only the N-domain (residues 1–121) is ordered (Fig. 2B). Even in the N-domain, residues in the loops are disordered to different extents. The core of the OB-fold made of a β-barrel capped by an α-helix, the elaboration of which constitute the subunit (Fig. 2C), has the same geometry in all SSBs and SSBbs of known structure (Fig. 2D). The main difference between the tertiary structures of SSBa from mycobacteria and S. coelicolor on one hand and the rest

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**Fig. 1.** Purification of MsSSBb. (A) SDS-PAGE analysis and (B) SEC-MALS profile.
Fig. 2. Structure of MsSSBb. (A) Tetramer with three dyads (P, Q and R) labelled. Axis P coincides with the crystallographic symmetry axis. The interfaces illustrated in Fig. 3 are encircled in dashed lines. (B) Secondary structure elements of MsSSBb marked over the amino acid sequence. Disordered residues in subunit A are indicated by dots. (C) Tertiary structure, with secondary structure elements marked. (D) Superposition of the DNA binding domains of two representative SSBa structures along with those of the three known SSBb structures.
of the SSBs of known structure on the other hand, is the presence of an additional strand that ‘clamps’ two neighbouring subunits together in the tetramer. That strand (β6 in Fig. 2C) is present in MsSSBb as well.

3.3. Quaternary association in relation to other SSBs

The structure of MsSSBb is best discussed in terms of its relationships with the known structures of other SSBs and SSBs. Structural data available on SSBa are extensive. It has been established that the structural diversity among them is generated primarily through variation in the quaternary structure. The mutual orientation of the subunits A and B are nearly the same in all SSBs and, for convenience, these two subunits together will be referred as the AB dimer. Thus, an AB dimer and the equivalent CD dimer together can be considered to constitute the functional tetramer. In all mycobacterial SSBs and in ScSSBa, the two dimers are clamped together at the two ends to give the tetramer an ellipsoidal shape. In the absence of the ‘clamp’ formed by strand β6, as in EcSSBa, the mutual orientation of AB and CD dimers is variable, often giving a different shape to the molecule. The probable biological and energetic implication of the two quaternary arrangements have been discussed earlier (Saikrishnan et al., 2003).

Tetramerization in MsSSBb involves clamping through β6 and is similar to that in mycobacterial SSBs and ScSSBa. The tetramer has three interfaces, namely AB (CD), AC (BD) and AD (BC). Among them, the first two deserve special consideration. Both the interfaces involved burial of surface area and hydrogen bonded interactions. The area buried at the AB interface in MsSSBb is

<table>
<thead>
<tr>
<th>PDB ID</th>
<th>Distance between AC (Å)</th>
<th>Distance between AB (Å)</th>
<th>Surface area buried (non polar component) (Å²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MsSSBb</td>
<td>5GQO</td>
<td>20.6</td>
<td>8117 (5444) 2575 (123)</td>
</tr>
<tr>
<td>BsSSBb</td>
<td>3VDY</td>
<td>26.9</td>
<td>6883 (4168) 555 (1255)</td>
</tr>
<tr>
<td>ScSSBb</td>
<td>4DAM</td>
<td>22.2</td>
<td>7577 (5005) 1493 (94)</td>
</tr>
<tr>
<td>MsSSBa</td>
<td>1X3E</td>
<td>22.7</td>
<td>8932 (5446) 2770 (35)</td>
</tr>
<tr>
<td>ScSSBa</td>
<td>3EIV</td>
<td>22.8</td>
<td>9312 (5661) 2838 (17)</td>
</tr>
<tr>
<td>EcSSBa</td>
<td>1KAW</td>
<td>30.0</td>
<td>3690 (1179) 957 (897)</td>
</tr>
</tbody>
</table>

Fig. 3. Secondary structure elements at the subunit interfaces of MsSSBb. (A) AB and (B) AC interfaces. Subunits A, B and C are in green, maroon and orange, respectively. Water molecules are shown as spheres.
1152 Å² (757 Å² non-polar), while it is 2575 Å² (1751 Å²) at AC the interface. These values are comparable to those, for instance, in the clamped tetramer of MsSSBa [Table 2]. In contrast, the surface area buried at the AB and AC interfaces in the typical unclamped EcSSBa tetramer are 2528 Å² (1179 Å²) and 449 Å² (389 Å²) respectively. The total surface areas buried on tetramerization of MsSSBb and MsSSBa is 8117 Å² (5444 Å²) and 8932 Å² (5446 Å²) respectively. The corresponding value in EcSSBa is much lower at 6542 Å² (3553 Å²). It was also noticed that the distance between centres of mass of subunits A and B is higher in clamped tetramers (24.3–28.1 Å) than in unclamped tetramers (around 20.3 Å). In contrast, the distance between the centres of mass of subunits A and C is lower in the former (20.6–22.8 Å) than in the latter (26.9–30.0 Å).

An interesting feature at the AB interface of MsSSBa is water mediated interactions between the N-terminal segments of the A and B subunits, which mimics the formation of an anti-parallel ribbon involving the two strands (Fig. 3A). This feature, with variations, exists in all relevant SSB structures as well. In tetrameric SSBs without clamps, the two strands are directly connected by hydrogen bonds as in canonical anti-parallel β sheets. When the tetramers are clamped, as in mycobacterial SSBs and ScSSBa, both the arrangements, one with direct hydrogen bonds and other involving water-mediated interactions have been observed (Arif and Vijayan, 2012). In MsSSBa, as indeed in SSBs with clamped tetramers, clamping involves formation of a short anti-parallel β ribbon between β6 strands of subunits A and C (Fig. 3B). Obviously, this feature cannot occur in the unclamped SSBs. Furthermore, inter-subunit hydrogen bonds between A and C subunits are much less numerous in them than in clamped tetramers.

The above observations lead to the conclusion that clamped tetramers, including that of MsSSBa, are more stable than unclamped ones in terms of surface area buried and hydrogen bonds. Indeed, the clamped tetramer of MsSSBa has been demonstrated to be more stable than the homologous unclamped EcSSBa tetramer (Saikrishnan et al., 2003; Handa et al., 2000). If SSB tetramers are to be treated as dimer of dimers, subunits A and B constitute the dimer in unclamped tetramers, while it is A and C in clamped tetramers. This distinction is perhaps of no biological consequence in the dT56/95 mode of binding, as the DNA wraps around the whole tetramer. However, in the dT35 binding mode, the DNA wraps around a dimer, which may be affected by the dimerization effects. In any case, clamping of A and C subunits results not only in strengthening the interactions between the two, but also weakening those between subunits A and B.

Structural studies on other SSBs have been limited to those on BsSSBa and ScSSBa. The tetrameric association of the DNA binding domain of BsSSBa is similar to the that in unclamped tetramers. The mutual orientation of AB and CD dimers in it is very similar to that seen in EcSSBa. It may be recalled that ScSSBa has a clamped tetramer as in mycobacterial tetramers. However, no clamp exists in ScSSBa. Instead, subunits A (B) and C (D) are linked by a disulfide bridge involving Cys7 in the N-terminal region. This bridge and its symmetry equivalent which occur at the centre of the tetramer, do not have the same effect as the clamps, which occur at the periphery of the tetramer, on the strength of the AB and AC interactions. Therefore, the tetrameric organization in ScSSBa is intermediate in nature between those of clamped and unclamped tetramers. The surface area buried in ScSSBa at the AB interface is 2327 Å² (1414 Å²), which is close to those found in the unclamped tetramers. However, the surface area buried at the AC interface [1493 Å² (1073 Å²)] is intermediate between the values for the two types of tetramers. In ScSSBa, the distances between the centres of mass of subunits A and B (21.3 Å) and between A and C (22.2 Å) are both short, suggesting similarity to both types of tetramers.

4. Conclusion

Mycobacteria are organisms among eubacteria which have a paralogous SSBb, in addition to the canonical, well characterized SSBa. The first structure determination of mycobacterial SSBb, from *M. smegmatis* (MsSSBa), suggests that the two paralogues have essentially the same tertiary and quaternary structure for the DNA binding domain, in spite of the low sequence identity between SSBa and SSBb in each species. In particular, the DNA binding domains of MsSSBa possesses the additional peptide stretch which clamps the subunits together as in all mycobacterial SSBs of known structure. This clamping mechanism leads to a stable tetramer with a characteristic geometry. The structures of the SSBbs of only two other organisms, namely *S. coelicolor* and *B. subtilis*, are now available. ScSSBa has a structure somewhat different from those observed so far in the SSBs of both types, in that pairs of OB domains in the molecule are linked together by disulfide bridges.

Accession numbers

The atomic coordinates and structure factors of MsSSBa have been deposited in the Protein Data Bank with accession code 5GQO.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsb.2016.09.012.

References


