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***Mycobacterium tuberculosis* copper-regulated protein SocB is an intrinsically disordered protein that folds upon interaction with a synthetic phospholipid bilayer**

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Abstract

Multiple genes in *Mycobacterium tuberculosis* (*Mtb*) are regulated by copper including *socAB* (small orf induced by copper A and B), which is induced by copper and repressed by RicR (regulated in copper repressor). *socA* and *socB* encode hypothetical proteins of 61 and 54 amino acids, respectively. Here, we use biophysical and computational methods to evaluate the SocB structure. We find that SocB lacks evidence for secondary structure, with no thermal cooperative unfolding event, according to circular dichroism measurements. 2D NMR spectra similarly exhibit hallmarks of a disordered structural state, which is also supported by analyzing SocB diffusion. Altogether, these findings suggest that by itself SocB is intrinsically disordered. Interestingly, SocB interacts with a synthetic phospholipid bilayer and becomes helical, which suggests that it may be membrane associated.

Keywords

SocB; *Mycobacterium tuberculosis* (*Mtb*); intrinsically disordered proteins; synthetic phospholipid bilayer; membrane protein

INTRODUCTION

Tuberculosis is caused by the bacterium *Mycobacterium tuberculosis* (*Mtb*) and is one of the most lethal communicable diseases worldwide. Statistics for this disease are staggering; in

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2013, it was estimated that about 9.0 million people were newly infected with *Mtb*. Tuberculosis kills about 1.5 million people annually, many of whom were HIV-positive.¹ In 2013, it was estimated that 480,000 people acquired multidrug-resistant tuberculosis, presenting a major challenge for the possibility of its global elimination.¹

Mtb has evolved defense mechanisms to survive in host macrophages, including a proteasome, which counteracts reactive nitrogen intermediates.² The *Mtb* proteasome is also associated with normal copper homeostasis that must be maintained for robust virulence in mice.³ Although it is unknown how the proteasome directly affects copper homeostasis in *Mtb*, it appears that an unusual copper responsive regulon is repressed in *Mtb* strains defective for proteasomal degradation.⁴ Under copper depleted conditions, the transcriptional repressor RicR binds to the operator of five different promoters to prevent their expression. Under copper sufficient conditions RicR dissociates from DNA, enabling expression of the regulon. The RicR regulon includes *ricR* itself, a gene encoding a copper methallothionein required for copper resistance, a multi-copper oxidase gene, and several genes of unknown function.^{4–6} Although disruption of individual RicR-regulated genes does not result in measurable effects on *Mtb* pathogenesis in mice, constitutive repression of the RicR regulon in its entirety leads to a significant copper sensitivity *in vitro* and attenuation of bacterial growth in mice.⁶

Most of the genes of the RicR regulon are only found in pathogenic mycobacteria, and in some cases, only in strains associated with human infections⁴. In particular, the *socAB* operon, which encodes the hypothetical proteins SocA (61 amino acids) and SocB (54 amino acids), is only found in the *Mtb* complex.⁴ Interestingly, SocA and SocB share no similarity with any proteins in any database, according to searches performed with the blastp algorithm.⁷ Here, we characterize SocB by using biophysical approaches, including nuclear magnetic resonance (NMR) and circular dichroism (CD) spectroscopy, and test for its interaction with a synthetic phospholipid bilayer.

METHODS

Protein production and purification

socB was amplified using primers 5'-gaccatatgtggacgctgaaagcccg-3' and 5'-gactcgagactaccgctggcaccagcgtaacgccctgagcgccacagcgatc-3' by PCR and cloned into the NdeI and XhoI sites of plasmid pET24b(+) (Novagen), positioning it N-terminal to a thrombin cleavage site and histidine tag. The *socB* protein was expressed in *E. coli* BL21(DE3) cells with induction by 0.4 mM IPTG at an optical density at 600 nanometers (nm) of 0.5–0.6. Expression was performed at 37°C in LB media or M9 minimal media supplemented with a mixture of vitamins and trace elements as well as ¹⁵N-labeled ammonium chloride as the sole nitrogen source.⁸ SocB was purified from *E. coli* by affinity chromatography with TALON (Clontech) resin followed by size-exclusion chromatography on an ÄKTA FPLC system (GE Healthcare) with a HiLoad 16/600 Superdex 75pg column (GE Healthcare). Ubiquitin was purchased from Sigma, and Pup obtained as previously described.⁹ The Superdex 75pg column elution profiles were done in 20 mM NaPO₄, 50 mM NaCl, pH 6.5. The final concentration of ubiquitin loaded onto the Superdex 75pg column was 100 µM.

Electrospray ionization mass spectrometry

Mass spectrometry experiments were performed on a 6130 Quadrupole LC/MS mass spectrometer with 5–10 μM SocB in 20 mM NaPO_4 , 50 mM NaCl, 2 mM DTT, 1% acetonitrile, pH 6.5. Analysis was performed with Open Lab CDS Chem Station Edition software (Agilent Technologies).

NMR spectroscopy

HSQC (heteronuclear single quantum coherence) experiments were performed at 800 MHz on a Bruker spectrometer equipped with a cryogenically cooled probe. Spectra were collected at 25°C unless otherwise indicated, and on ^{15}N labeled protein samples. SocB was at 50 μM concentration and dissolved in Buffer 1 (20 mM NaPO_4 , 50 mM NaCl, 2 mM DTT, pH 6.5, 5% D_2O , and 1% NaN_3). Pup was at 700 μM concentration and in Buffer 2 (20 mM HEPES, 50 mM NaCl, 10% glycerol, and pH 6.5) whereas ubiquitin was at 300 μM concentration and in Buffer 1 but without DTT. The details on Pup and ubiquitin sample preparation and data collection were reported elsewhere.⁹ Thermal denaturation and NOESY experiments were acquired on 500 μM SocB in Buffer 1. Data processing and visualization was performed by NMRPipe and Sparky, respectively.^{10,11}

MSP1D1:DPPC nanodisc preparation

MSP1D1:DPPC (1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine) nanodiscs were prepared by using a previously published protocol.^{12,13} Briefly, the purified membrane scaffold protein MSP1D1 (Addgene plasmid # 20061) was mixed from a 5 mg/mL stock with cholate-solubilized 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine (DPPC, Avanti Polar Lipids, # 850355P) lipid at a 1:90 molar ratio and incubated for 2 hours at 37°C. The mixture was then added to Bio-Beads SM-2 (Bio-Rad), incubated on a rotating platform shaker overnight at 4°C, and spun down to extract nanodiscs from the beads. The collected nanodiscs were fractionated by FPLC with a Superdex 200 10/300 GL column (GE Healthcare Life Sciences), using a flow rate of 0.5 mL/min and fraction volumes of 1 mL. Homogeneity of the fractions was analyzed by dynamic light scattering to find an average radius and molecular weight for the nanodiscs of 5 nm and 125 kDa, respectively. The polydispersity value varied depending on the fraction and was in the range of 10–15%.

Circular dichroism

SocB and ubiquitin in 20 mM NaPO_4 , 50 mM NaCl, pH 6.5 were diluted 6-fold into water to a final concentration of 8.6 μM and 26 μM , respectively, to accurately detect low far UV wavelengths. CD spectra of SocB, ubiquitin and buffer (as a control) were collected on an Aviv Biomedical Int. 420SF circular dichroism spectrometer with QS quartz cuvettes of 1.0 mm cell path from 25 to 80°C (5°C steps with 5 minute temperature stability time). Ubiquitin CD spectra were collected additionally at 85°C, 90°C, 95°C and 99°C. The wavelength step was 0.5 nm, averaging time 5 seconds, and the bandwidth was 1.0 nm. The Aviv Biomedical Int. software was used to analyze and smooth the data with a second-degree polynomial. Pup data were collected at 17 μM concentration in Buffer 2. Parameters for data collection and analysis were described elsewhere.⁹ Measurements of the SocB:nanodisc sample were performed at a final concentration of 3 μM in Buffer 3 (20 mM

NaPO₄, 50 mM NaCl 0.5 mM EDTA, pH 6.5); the MSP1D1 concentration in this sample was 30 μ M. Data were collected with above-mentioned parameters in a 0.2 mm quartz cells at 25°C.

RESULTS

SocB is predicted to be largely disordered

We initially tried to produce recombinant SocA in *E. coli*, but were unsuccessful unless SocA was fused to maltose binding protein (data not shown). Unlike SocA, SocB was predicted to be a stable protein by ProtParam.¹⁴ Consistent with this prediction, we were able to produce SocB in *E. coli* with a carboxyl (C) terminal 14-amino acid histidine tag for NMR structural studies [Fig. 1(A)]. With the tag included, our recombinant protein is 7.6-kDa. By using affinity and size exclusion chromatography for purification, we were able to produce samples with no contamination and at the correct molecular weight, as assessed by mass spectrometry [Fig. 1(B)]. Attempts to remove the tag resulted in the protein not eluting from Talon resin and GST-tagged constructs exhibited poorer protein yields (data not show). Therefore, all studies were performed in the presence of the C-terminal histidine tag.

We used the programs PrDOS¹⁵ to predict degree of disorder and Jpred4¹⁶ to predict secondary structure for SocB and compared the results to ubiquitin and Pup^{9,17,18}, as examples of ordered and disordered proteins, respectively. PrDOS correctly identified ubiquitin as an ordered protein, as indicated by disorder probability values of less than 0.5 [Fig. 1(C), top panel, red] and summarized by black and red sequence coloring for order and disorder, respectively [Fig. 1(C), bottom third panel]. An over-estimation of disorder was found for ubiquitin at the N-terminal end [Fig. 1(C), red]. Jpred4 correctly identified all regions with secondary structure in ubiquitin, with the exception of a short β -strand and 1-turn 3_{10} -helix [Fig. 1(C)].

As published previously^{9,17,18}, Pup is largely disordered with anticipated secondary structure but increases order in a region that adopts a helix after binding to the *Mycobacterium* proteasomal ATPase Mpa.¹⁹ Like Pup, SocB was predicted to be largely disordered, with order only in a region spanning S37 - P45 [Fig. 1(C)]. Moreover, only Q35-F41 was predicted in SocB to adopt secondary structure; these residues are anticipated to form a helix.

SocB shows hallmarks of an intrinsically disordered protein by NMR and diffusion

We subjected ¹⁵N labeled SocB to a 2D NMR HSQC experiment, in which structured proteins exhibit well-dispersed signals, as demonstrated by ubiquitin [Fig. 2(A), right panel]. Intrinsically disorder proteins, such as Pup, yield amide signals that lack dispersion [Fig. 2(A), center].^{9,17,18} SocB resembled Pup, with congested amide signals [Fig. 2(A), left panel]. SocB contains one tryptophan and two glutamines, with side chain groups observable at their expected chemical shift values, and seven glycines that appear in a cluster at a position characteristic of random coil [Fig. 2(A)]. The peak dispersion and relative peak position did not change significantly upon changing the temperature, offering further support that SocB is intrinsically disordered (Supporting Information Fig. S1).

Additionally, a 2D homonuclear NOESY experiment lacked any hallmarks of protein secondary structure (Supporting Information Fig. S2).

Like Pup⁹, SocB elutes earlier than expected for its molecular weight during size-exclusion chromatography, and before 8.6-kDa ubiquitin [Fig. 2(B)]. By using four proteins (myoglobin, ovalbumin, albumin, and ferritin) of known molecular weight and Stokes radius (R_s), we estimated the R_s for SocB from its elution volume to be 21.7 Å, as done previously.^{9,20–22} This result was compared to expected R_s values as a function of molecular weight for five known protein folding states, including native, molten globule, pre-molten globule, natively unfolded coil-like, and natively unfolded pre-molten globule like (PMG-like).²³ The elution profile of SocB fits best to that of natively unfolded PMG-like proteins [Fig. 2(C, D)].

CD analysis indicates SocB to behave as an intrinsically disordered protein

Circular dichroism (CD) spectroscopy is sensitive to protein secondary structure. SocB exhibits a spectrum characteristic of a disordered peptide, with negative ellipticity below 200 nm and little ellipticity at 190 and 222 nm [Fig. 3(A)]. The overall CD profile of SocB resembles that of intrinsically disordered Pup and differs from that of ubiquitin [Fig. 3(A)].^{9,24}

We next monitored the CD spectrum of SocB, Pup, and ubiquitin as a function of temperature, starting at 25°C and extending until 80°C for SocB and Pup [Fig. 3(B–C)] and 99°C for ubiquitin [Fig. 3(D)]. We plotted ellipticity at 198.5, 197.7 and 198 nm as a function of temperature for SocB, Pup, and ubiquitin, respectively, to find a somewhat linear profile with no transition point for SocB and as reported previously, Pup [Fig. 3(E)].⁹ By contrast, ubiquitin exhibits a sharp transition beginning at 80°C and retains characteristics of secondary structure even up to 99°C [Fig. 3E, right panel], as previously observed.^{25,26} This comparison clearly demonstrates that the SocB melting curve does not present the profile expected for a structured protein, which typically transitions from a folded state to an unfolded ensemble.

SocB interacts with a synthetic phospholipid bilayer and appears to become helical

Small bacterial proteins have recently been appreciated as having critical roles in bacterial physiology, and it has been noted that they are generally membrane associated (reviewed in ²⁷). Consistent with these reports, the high positive charge of SocB (pI 11) may indicate that it interacts with the negatively charged membrane of *Mtb*. To determine whether or not SocB interacts with membranes, we used a commercially available model system for phospholipid bilayers composed of the membrane scaffold protein MSP1D1, genetically engineered from Apolipoprotein A-1 (ApoA-1), and DPPC (1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine). We prepared a mixture of 3 μM SocB and 30 μM MSP1D1:DPPC nanodiscs for CD measurements. The resulting spectrum was compared to 3 μM SocB alone and 30 μM MSP1D1:DPPC nanodiscs [Fig. 4]. As described above, SocB by itself presents a spectrum characteristic of a disordered protein, with little negative ellipticity below 200 nm [Fig. 3(A) and Fig. 4, black]. Addition of the synthetic phospholipid bilayer yielded significant changes, indicating SocB interaction with these nanodiscs [Fig. 4, red]. To rule

out the possibility that the observed effects were from SocB interaction with MSP1D1, we also acquired a spectrum on this mixture, and found no change (Supporting Information Fig. S3). Moreover, a difference spectrum in which the signal recorded for the MSP1D1:DPPC nanodiscs alone is subtracted from that of the SocB:nanodisc complex shows hallmarks of helicity [Fig. 4, purple]. In particular, positive ellipticity is found between 190–200 nm and negative bands at 208 and 222 nm. We evaluated these results further with DichroWeb, a software package that converts CD data to predicted secondary structural content.^{28,29} This program found free SocB to have 9% helicity. This value increased significantly when SocB was in the synthetic lipid bilayer, yielding a value of 81% helicity.

DISCUSSION

In summary, we find that *Mtb* protein SocB lacks signatures of order and of structural elements when by itself in vitro. It appears to be an intrinsically disordered protein, based on CD, NMR, diffusion, and thermal melting experiments. However, in the presence of a synthetic phospholipid bilayer, SocB appears to become helical. This finding is suggested by CD data collected with SocB at 10-fold less than MSP1D1:DPPC. SocB is a highly basic protein (pI 11) and this finding suggests that it forms interactions with phosphate groups of lipid bilayers.

SocB, so far, appears to exist only in the *Mtb* complex, which suggests that it is only relevant to infections in higher mammals, such as humans; this may explain why virulence phenotypes have not yet been associated with the *socAB* locus in mice. Among the copper-regulated RicR regulon genes, several are putative and confirmed membrane proteins.³⁰ Because SocB is highly basic in charge and interacts with a synthetic phospholipid bilayer, one possibility is that SocB regulates the localization or function of one or more of the RicR-regulated membrane proteins. Alternatively, SocB may affect the function of RicR-independent proteins, including protein secretion systems. We are currently testing these hypotheses. Importantly, SocB appears to be a new member of a fascinating and growing family of small proteins that may affect physiology at a bacterial membrane.²⁷

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

SocB is purified from *E. coli* and predicted to be a disordered protein. **A:** SocB produced with a histidine tag in *E. coli*. –, uninduced, +, induced with IPTG, P, after purification. Molecular weight markers are indicated to the left in kilodaltons (kDa). **B:** ESI-MS spectrum of purified SocB protein demonstrates it at the expected molecular weight. **C:** Results from the disorder prediction program PrDOS for SocB (in black), Pup (in purple), and ubiquitin (in red) are plotted; scores above 0.5 (grey dashed line) indicate predicted disorder. Below the plot, the SocB (top), Pup (middle) and ubiquitin (bottom) sequence is displayed with amino acids predicted to be disordered or ordered in red or black, respectively. Predicted secondary structures are indicated below each sequence with helical regions annotated with an “H” and extended regions with an “E.” Established secondary structure elements of ubiquitin are also indicated for comparison (II° structure).

Figure 2.

SocB lacks signal dispersion in a 2D NMR experiment and diffuses like a natively disordered protein. **A:** ^1H - ^{15}N HSQC spectra from ^{15}N labeled SocB (left), Pup (center), or ubiquitin (right). **B:** Size-exclusion chromatography by FPLC for SocB (black) and ubiquitin (red) with molecular weight indicated above. At the bottom of the chromatogram, the elution volume for ferritin and IgG, albumin, ovalbumin, and myoglobin is indicated with arrows labeled a, b, c, and d, respectively. **C:** Log plots of molecular weight (MW) versus R_s for indicated protein conformations with the value for SocB displayed (black star), as generated by using the elution time in (B). **D:** The theoretical Stokes radius (R_s) for SocB assuming different folding states. Stokes radius, R_s . Molecular Weight, MW.

Figure 3.

SocB does not unfold cooperatively. **A:** CD spectra of SocB, Pup, and ubiquitin in the far UV region spanning from 190 to 240 nm at 25°C. **B–D:** CD spectra of SocB, Pup and ubiquitin collected in the far UV region at temperatures collected in 5° increments from 25 – 80°C (SocB), 20 – 80°C (Pup) and 25 – 99°C (ubiquitin). **E:** The change in ellipticity of SocB at 198.5 nm, ubiquitin at 198 nm, and Pup at 197.7 nm from the data presented in (B–D) to visualize the SocB thermal unfolding transition curve in comparison to Pup and ubiquitin.

Figure 4. SocB interacts with a synthetic phospholipid bilayer and becomes helical
CD raw spectra of SocB (black) and with the synthetic phospholipid bilayer MSP1D1:DCCP (red). A difference spectrum is included in which the signal from MSP1D1:DPPC nanodisks is subtracted from that of the SocB: nanodisc complex (purple). The spectrum of the MSP1D1:DCCP nanodisks is shown in blue for comparison.