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Structural and functional insights into the stationary-phase survival protein SurE, an important virulence factor of *Brucella abortus*

K. F. Tarique,† S. A. Abdul Rehman,§ S. Devi, Priya Tomar and S. Gourinath*

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† Present address: Public Health Research Institute Center, Rutgers New Jersey Medical School, Newark, NJ 07103, USA.

§ Present address: MRC Protein Phosphorylation and Ubiquitylation Unit, College of Life Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, Scotland.

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School of Life Sciences, Jawaharlal Nehru University, New Delhi 110 067, India. *Correspondence e-mail: sgourinath@mail.jnu.ac.in

The stationary-phase survival protein SurE from *Brucella abortus* (BaSurE) is a metal-dependent phosphatase that is essential for the survival of this bacterium in the stationary phase of its life cycle. Here, BaSurE has been biochemically characterized and its crystal structure has been determined to a resolution of 1.9 Å. BaSurE was found to be a robust enzyme, showing activity over wide ranges of temperature and pH and with various phosphoester substrates. The active biomolecule is a tetramer and each monomer was found to consist of two domains: an N-terminal domain, which forms an approximate $\alpha + \beta$ fold, and a C-terminal domain that belongs to the α/β class. The active site lies at the junction of these two domains and was identified by the presence of conserved negatively charged residues and a bound Mg^{2+} ion. Comparisons of BaSurE with its homologues have revealed both common features and differences in this class of enzymes. The number and arrangement of some of the equivalent secondary structures, which are seen to differ between BaSurE and its homologues, are responsible for a difference in the size of the active-site area and the overall oligomeric state of this enzyme in other organisms. As it is absent in mammals, it has the potential to be a drug target.

1. Introduction

Brucella abortus is a Gram-negative bacterium that infects and causes infertility and abortion in a wide variety of mammals. It represents a significant public health concern and threat to the agricultural community wherever it is endemic in nature. It is zoonotic and can be transferred to humans through infected animals or their products, causing serious acute and chronic infections (Galińska & Zagórski, 2013). *B. abortus* becomes an intracellular pathogen when taken up by macrophages, and displays a remarkable resistance to being killed by these blood cells. These bacteria are able to proliferate extensively and cause chronic infection because of their ability to maintain long-term resistance within macrophages. There is also a close relationship between the resistance of these bacteria to being killed by these phagocytes and their virulence.

As nutrients become limiting, the life cycle of *B. abortus* transitions from the exponential phase to the stationary phase, where it shows resistance to nutrient deprivation, increased acidity, exposure to reactive oxygen intermediates and other stress-induced physiological conditions. In order to survive in these harsh conditions, the metabolic processes of *B. abortus* are diverted from cell division to cell viability over prolonged periods, resulting in little or no increase in the number of cells (Roop *et al.*, 2003). Interestingly, there is a close resemblance between stationary-phase physiology and the life cycle of this

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Table 1
Macromolecule production.

Source organism	<i>B. abortus</i> strain S19
DNA source	Genomic DNA
Forward primer	5'-CTAGCTAGCTTGCCTATTCTGCTGACGAACG-ATGA-3'
Reverse primer	5'-CGGCTCGAGTGCTTCCACTCCAAGCGCCG-3'
Cloning vector	pET-21c
Expression vector	pET-21c
Expression host	<i>E. coli</i> BL21(DE3)
Complete amino-acid sequence of the construct produced	MRILLTNDGIIHAEGLAVERIARKLSDDVWVVA-PETDQSGLAHSLTLEPLRLRQIDARHFALRG-TPTDCVIMGVRHVLPGAPDLVLSGVNSGANMA-DDVTYSGTVAGAMEGTLGVRATIALSQEYEA-GDRIIPWETAEAHAPELIGRLMEAGWPEGVL-LNLNFPNCAPEEVKGVVTAQKLSHDLARLDE-RDGRGFPYFWLHFGRGKAPVADDSIAAIRS-GCISMTPLHLDLTAHKVRAELGAALGVEALEH-HHHH

pathogen inside a phagosome; in both cases the organism faces an acidic pH and nutrient deprivation (Roop *et al.*, 2003).

B. abortus (and other bacteria such as *Escherichia coli* and *Salmonella typhimurium*) possesses HF-1, an RNA-binding protein that regulates a set of genes which are essential for survival in the stationary phase of their life cycle (Robertson & Roop, 1999; Roop *et al.*, 2003). A mutation in the *hfq* gene (which codes for HF-1) in *Brucella* was shown to result in defective stationary-phase physiology (Roop *et al.*, 2003); this mutant was susceptible to acidic pH and free radicals and also lost its viability more quickly than the wild-type strain. Differential gene expression of the *hfq* mutant and the wild-type *Brucella* strain led to the identification of numerous genes that require the HF-1 protein for optimal expression during the stationary phase (Roop *et al.*, 2003). The products of these genes provide defences against various stresses in the phagosome. The stationary-phase survival protein SurE and L-isoaspartyl protein carboxymethyltransferase (Pcm) are important enzymes that have been shown to be regulated by HF-1 in the stationary phase of *E. coli* and *S. typhimurium*. The genes for SurE and Pcm overlap and exist in an operon that is essential for the survival of *E. coli* and *S. typhimurium* in stressful conditions (Roop *et al.*, 2003).

Members of the stationary-phase survival protein (SurE) family are metal-dependent nucleotidases that show phosphatase activity on nucleoside 5'-monophosphates (NMPs). This enzyme hydrolyses a 5'-ribonucleotide into a ribonucleoside and a phosphate moiety. The exact role of SurE is not known, but mutational studies have shown it to be detrimental to the growth of bacteria in the stationary phase (Zhang *et al.*, 2001). Gene duplication of SurE as an adaptation strategy at high temperatures has also been observed in *E. coli* (Riehle *et al.*, 2001). The importance of SurE can be realised by the fact that mutations in the *surE* gene increase the accumulation of L-isoaspartyl residues in an *E. coli* strain with a suppressed stress-survival phenotype (Visick *et al.*, 1998).

In silico analysis suggested a similar bicistronic arrangement of SurE and Pcm in *B. abortus*. As a step towards understanding the stationary-phase physiology of *B. abortus*, we have biochemically characterized its SurE enzyme under

various conditions and determined its crystal structure at high resolution. The biochemical and structural properties of BaSurE were compared with those of homologues of this enzyme in other organisms to gain insights into the enzymatic behaviour of this superfamily of proteins.

2. Materials and methods

2.1. Macromolecule production

Gene ID 6328006, annotated as the stationary-phase survival protein SurE from *B. abortus* (BaSurE), was amplified by polymerase chain reaction (PCR). The amplified product of BaSurE (768 bp) was digested with *NheI* and *XhoI* and ligated into a pET-21c expression vector with a C-terminal His tag (Table 1).

2.2. Expression and purification of BaSurE in *E. coli*

The pET-21c-BaSurE plasmid was transformed into a bacterial expression system in *E. coli* BL21(DE3) cells for recombinant protein production. The positive colonies were picked from LB plates containing ampicillin and inoculated into 50 ml LB medium for overnight growth. 1% of this culture was inoculated into a secondary culture containing 50 mg l⁻¹ ampicillin. The culture was incubated at 37°C until the OD₆₀₀ reached 0.6–0.8. Recombinant gene expression was initiated by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 200 μM. The temperature was decreased to 30°C after induction and the culture was allowed to grow for a further 5 h. The cells were harvested at 4°C by centrifugation at 15 940g for 5 min and the pellet was suspended in suspension buffer (1 g of pellet per 10 ml) consisting of 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.1% Triton X-100, 0.2 mM EDTA, 10 mM imidazole, 0.05 mg ml⁻¹ lysozyme, 5 mM β-mercaptoethanol (βMe). The lysed cells were sonicated and then centrifuged at 39 100g for 1 h. The cleared supernatant was applied onto an Ni²⁺ Sepharose column and the column was equilibrated with 50 ml washing buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM βMe, 20 mM imidazole). The protein was eluted in buffer consisting of 50 mM Tris pH 7.5, 150 mM NaCl, 80 mM imidazole, 5% glycerol, 5 mM βMe.

For further purification and the removal of imidazole, the concentrated protein was loaded onto a HiLoad 16/60 Superdex 200 column pre-equilibrated with running buffer consisting of 10 mM Tris pH 7.5, 150 mM NaCl, 5% glycerol, 5 mM βMe. The flow rate was maintained at 1 ml min⁻¹. Glycerol was added to increase the solubility and stability of the protein.

The purity of the desired protein and its molecular weight were checked using 12% SDS-PAGE, which showed it to be around 27 kDa, corresponding to the monomeric state of this protein (Supplementary Fig. S1). The purified fractions were pooled and concentrated using a Centricon concentrating device (Millipore) with a molecular-weight cutoff (MWCO) of 10 kDa.

Table 2
Crystallization.

Method	Vapour diffusion, hanging drop
Plate type	24-well plate
Temperature (°C)	16
Protein concentration (mg ml ⁻¹)	10
Buffer composition of protein solution	15 mM Tris pH 7.5, 150 mM NaCl, 5% glycerol, 5 mM βMe
Composition of reservoir solution	20% PEG 4000, 0.1 M Tris pH 7.0–7.5, 0.2 M MgCl ₂
Volume and ratio of drop	2 µl, 1:1
Volume of reservoir (ml)	0.5

2.3. Enzyme characterization

The phosphatase activity of BaSurE was determined using the methods described previously for homologous SurE and alkaline phosphatase enzymes using *p*-nitrophenylphosphate (PNPP) as the general substrate (Pappachan *et al.*, 2008). The rate of hydrolysis of PNPP was determined by detecting the yellow-coloured *p*-nitrophenyl (PNP) product of this hydrolysis. For the BaSurE assay, the standard 100 µl reaction mixture consisted of 200 mM bis-tris pH 6.0, 5 mM PNPP, 10 mM MgCl₂ and 20 µg purified BaSurE protein. The reaction mixture was incubated for 30 min at room temperature and then stopped by the addition of 50 µl 3 M NaOH solution. After 15 min, the absorbance of the solution was measured in a microplate reader at a wavelength of 405 nm. This absorbance is directly proportional to the amount of yellow PNP formed and hence to the activity of the enzyme.

To determine the temperature and pH at which the activity of BaSurE activity is greatest, as well as to determine which divalent metal cations can act as a cofactor for this enzyme, these factors were varied during the assay. 10 mM MgCl₂, ZnCl₂, MnCl₂, SrCl₂, NiCl₂, BaCl₂, FeCl₂ and CaCl₂ were tested as divalent metal ions.

The substrate specificity of BaSurE was measured by checking its phosphatase activity in the presence of adenosine 5'-monophosphate (5'-AMP), guanosine 5'-monophosphate (5'-GMP), cytidine 5'-monophosphate (5'-CMP), thymidine 5'-monophosphate (5'-TMP) and uridine 5'-monophosphate (5'-UMP) by the Baykov malachite green method as reported previously for other phosphatases (Baykov *et al.*, 1988; Faisal Tarique, Arif Abdul Rehman, Betzel *et al.*, 2014; Faisal Tarique, Arif Abdul Rehman & Gourinath, 2014). For this, the standard 100 µl reaction mixture included 200 mM bis-tris pH 6.0, 5 mM of each substrate, 10 mM MgCl₂ and 20 µg purified BaSurE protein. The reaction mixture was incubated for 15 min at room temperature and then stopped by the addition of 50 µl malachite green dye solution. After 20 min, the blue-coloured phosphomolybdate complex was detected at a wavelength of 650 nm in a microplate reader. The absorbance and hence the activity are directly proportional to the amount of P_i that formed along with the phosphomolybdate–malachite green complex. To determine the thermal stability of BaSurE, denaturation experiments were carried out by CD spectroscopy equipped with a Peltier-type temperature controller. The instrument was calibrated with D-10-camphorsulfuric acid. Spectra in the far-UV range (222 nm) were measured with the concentration of BaSurE kept at 0.2 mg ml⁻¹. The path length

Table 3
Data collection and processing.

Diffraction source	Beamline BM14, ESRF
Wavelength (Å)	0.978
Temperature (°C)	–173
Detector	MAR scanner 345 mm plate
Crystal-to-detector distance (mm)	183.3
Rotation range per image (°)	1
Total rotation range (°)	360
Exposure time per image (s)	1
Space group	<i>P</i> 2 ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	50.18, 121.07, 82.43
α , β , γ (°)	90, 93.58, 90
Mosaicity (°)	0.3
Resolution range (Å)	50–1.9 (1.93–1.90)
Total No. of reflections	253188
No. of unique reflections	75815
Completeness (%)	97.8 (99.5)
Multiplicity	3.3 (3.2)
$\langle I/\sigma(I) \rangle$	21.9 (2.16)
<i>R</i> _{int}	0.075 (0.651)
Overall <i>B</i> factor from Wilson plot (Å ²)	30.4

of the cell was 0.1 cm. In order to reduce the noise owing to chloride ions, sodium chloride was replaced by sodium chlorate as a salt. In each step of protein purification, *e.g.* from affinity to gel-filtration chromatography, 20 mM phosphate buffer pH 8.0 was used. The temperature was varied from 25 to 95°C and the results were interpreted in terms of the fraction of protein unfolded with increase in temperature.

2.4. Crystallization

The purified BaSurE protein was concentrated and subjected to extensive crystallization trials by varying the precipitant and its concentration, the type of salt and the pH over a wide range until good diffracting crystals were obtained (Table 2). Initially, screens from Hampton Research (Index, SaltRx and PEG/Ion) and Molecular Dimensions (PACT *premier*, Clear Strategy and Morpheus) were used to set up crystallization drops with a Mosquito robot in 96-well plates. Small crystals were found under a few conditions: Index screen condition No. 84 (0.2 M MgCl₂, 0.1 M HEPES pH 7.5, 25% PEG 3350) and Clear Strategy screen 1 condition E9 (0.2 M MgCl₂, 0.1 M Tris pH 7.5, 15% PEG 4000). The conditions were replicated and improved in 24-well plates by mixing 2 µl protein solution with 2 µl reservoir solution and equilibrating against 500 µl reservoir solution.

2.5. Data collection and processing

Crystals were carefully picked, washed and equilibrated with a cryoprotectant solution consisting of 20% PEG 4000 and 10% PEG 400 in 0.1 M Tris pH 7.0–7.5. The crystals were mounted in cryoloops and diffracted X-rays to a resolution of 1.9 Å (Table 3). The data were indexed, processed and scaled with the *HKL-2000* program suite (Otwinowski & Minor, 1997). These crystals belonged to space group *P*2₁, with a Matthews coefficient of 2.2 Å³ Da⁻¹ and a solvent content of ~45% and with four BaSurE molecules in the asymmetric unit.

2.6. Structure solution and refinement

The BaSurE structure was determined by molecular replacement using SurE from *Coxiella burnetii* (PDB entry 3ty2; 38% sequence identity; Franklin *et al.*, 2015). The structure from PDB entry 3ty2 and the aligned sequences of BaSurE and PDB entry 3ty2 were input into *CHAINS*AW (Schwarzenbacher *et al.*, 2004) to produce the specific search model used for molecular replacement, which was then carried out using *Phaser* (McCoy *et al.*, 2007) from the *CCP4* suite (Winn *et al.*, 2011). The best solution had a translation-function Z-score of 24 and a log-likelihood gain of 786.

An initial cycle of restrained refinement using this solution and *REFMAC5* (Murshudov *et al.*, 2011) resulted in a decrease in the *R* factor to 36%. The resulting model was then submitted to *ARP/wARP* (Langer *et al.*, 2008) for auto-building, which successfully built 95% of the side chains of the model into good-quality electron density. The remaining parts of the polypeptide were built manually with *Coot* (Emsley & Cowtan, 2004), and the magnesium ion and water molecules were subsequently added according to their binding geometries, coordination spheres and temperature factors and the difference Fourier electron-density map. Multiple rounds of manual adjustment of the model in *Coot* (Emsley & Cowtan, 2004) and refinement with *REFMAC5* (Murshudov *et al.*, 2011) were carried out.

Table 4

Structure solution and refinement.

Values in parentheses are for the outer shell.

Resolution range (Å)	50–1.90 (1.946–1.897)
Completeness (%)	97.7
No. of reflections, working set	71978 (5312)
No. of reflections, test set	3807 (284)
Final R_{cryst}	0.198 (0.298)
Final R_{free}	0.227 (0.336)
No. of non-H atoms	
Protein	7477
Ion	4
Ligand	0
Water	215
Total	7696
R.m.s. deviations	
Bonds (Å)	0.020
Angles (°)	2.082
Average <i>B</i> factors (Å ²)	
Protein	41.1
Ion	29.5
Ligand	0.0
Water	36.3
Ramachandran plot	
Most favoured (%)	90.9
Allowed (%)	9.1

The final model of BaSurE displays good electron density and refinement statistics (Table 4). The structure factors and coordinates of this model have been deposited in the Protein

Figure 1

Effects of pH, divalent metal ion and temperature on BaSurE activity. The phosphatase activity of BaSurE is shown as a function of (a) pH, (b) various metal ions and (c) temperature. (d) Thermal denaturation curve interpreted from circular-dichroism experiments at 222 nm, suggesting the melting temperature of BaSurE to be approximately 70°C.

Table 5
Physiochemical properties of different SurE homologues.

Temperature and pH ranges in which the enzymes show an activity greater than 70% of their maximum activity are displayed.

Organism	pH	Divalent metal ion	Temperature (°C)
<i>B. abortus</i>	5.5–6.8	$\text{Zn}^{2+} > \text{Mn}^{2+} > \text{Mg}^{2+} > \text{Sr}^{2+} > \text{Ca}^{2+} > \text{Ba}^{2+} > \text{Ni}^{2+} > \text{Fe}^{2+}$	20–80
<i>S. typhimurium</i> (Pappachan <i>et al.</i> , 2008)	7.0–8.0	$\text{Mg}^{2+} > \text{Mn}^{2+} > \text{Ca}^{2+} > \text{Zn}^{2+}$	40–50
<i>T. maritima</i> (Zhang <i>et al.</i> , 2001)	7.0–7.2	$\text{Mg}^{2+} > \text{Mn}^{2+} > \text{Ca}^{2+}$	80–85
<i>P. aerophilum</i> (Mura <i>et al.</i> , 2003)	5.7–6.0	$\text{Co}^{2+} > \text{Mg}^{2+} = \text{Mn}^{2+} > \text{Ca}^{2+}$	90
<i>E. coli</i> (Proudfoot <i>et al.</i> , 2004)	6.8–7.5	$\text{Mn}^{2+} > \text{Co}^{2+} > \text{Ni}^{2+} > \text{Mg}^{2+}$	37
<i>Xylella fastidiosa</i> (Saraiva <i>et al.</i> , 2009)	7.0	$\text{Mn}^{2+} > \text{Co}^{2+} > \text{Mg}^{2+} > \text{Ni}^{2+}$	Not available

Table 6
Substrate specificity of *B. abortus* SurE.

Substrate	<i>B. abortus</i>	<i>P. aerophilum</i> (Mura <i>et al.</i> , 2003)	<i>T. maritima</i> (Mura <i>et al.</i> , 2003)
PNPP	100	100	100
5'-AMP	69	982	49
5'-TMP	156	222	25
5'-GMP	120	800	74
5'-CMP	4	135	17
5'-UMP	139	N/A	N/A

Data Bank as entry 4zg5 and its quality was checked using PROCHECK (Laskowski *et al.*, 1993).

3. Results

3.1. Studying the activity of BaSurE under variable temperature, pH, substrate and metal-ion conditions

The physicochemical properties of BaSurE were checked by measuring its activity at different pH values and temperatures and with different metal ions using *p*-nitrophenylphosphate (PNPP) as the substrate. For BaSurE, the standard 100 µl reaction mixture volume consisted of 50 mM bis-tris pH 6.0, 5 mM PNPP, 10 mM divalent metal ion and 20 µg purified BaSurE protein. To determine which divalent metal ions can serve as a cofactor, the activity was separately checked in the presence of 10 mM MgCl₂, MnCl₂, ZnCl₂, NiCl₂, FeCl₂, SrCl₂, BaCl₂ or CaCl₂. To test the effect of pH, 200 mM each of sodium acetate pH 4.8, bis-tris in the pH range 5.5–7.0 and Tris in the pH range 7–9 were used. Activities are expressed as the percentage of the maximum observed activity. The data shown represent the average of two independent experiments. Error bars indicate the corresponding standard deviations. As measured by its ability to hydrolyze PNPP, the enzyme was found to be most active at about pH 6.0 and to display at least 40% of this activity (and hence to be considered stable) from pH 5.0 to 8.5; however, the enzyme displays relatively poor activity at higher alkalinities (Fig. 1*a*). Among the metal ions tested, BaSurE showed an almost equivalent maximal activity with Zn²⁺, Mn²⁺ and Mg²⁺; the activity then decreases in the order Sr²⁺, Ca²⁺, Ba²⁺, Ni²⁺ and Fe²⁺ (Fig. 1*b*). Magnesium, the natural cofactor of this 5'-nucleotidase, was chosen to carry out all of the enzymatic studies related to BaSurE. BaSurE showed activity over a wide range of temperatures, with a near-maximum activity maintained from 20 to 80°C; however, the activity decreased sharply at 90°C (Fig. 1*c*). All subsequent

assays were carried out at pH 6.0 at 30°C in a water bath unless stated otherwise.

The robust thermostability of BaSurE implied by the activity assay was generally confirmed from the results of circular-dichroism studies at 222 nm on this enzyme at various temperatures. The melting temperature (*T*_m) of BaSurE was calculated to be approximately 70°C from these circular-dichroism measurements (Fig. 1*d*). The phosphatase activity of BaSurE was tested on a range of phosphate esters to check its specificity. The maximum activity was found with 5'-TMP, followed by 5'-UMP, 5'-GMP, PNPP and 5'-AMP, with no or very little activity with 5'-CMP (Supplementary Fig. S2). This is difficult to explain at this stage, but it seems that the differences between the nitrogen bases are not necessarily all that minimal. It is most likely that the enzyme employs a few strategically positioned residues to create hydrogen-bonding networks that could differentiate hydrogen bonding at positions 3 and 4 in pyrimidines and eventually favour 5'-UMP and 5'-TMP over 5'-CMP.

In addition to the effects of the pH, divalent metal cation, temperature and substrate on the activity of BaSurE, similar descriptions are tabulated for homologous SurE enzymes in Tables 5 and 6. It is very clear that stationary-phase survival proteins are not specific for a single divalent metal ion but show activity with a wide variety of divalent metal ions; however, the maximum activity was most often observed with Mg²⁺ or Mn²⁺ ions. Interestingly, the temperature-dependent phosphatase activity of BaSurE was found to be similar to those of its archaeal homologues from *P. aerophilum* and *T. maritima*, where the enzyme showed reasonable activity even at high temperatures.

The activities of the *B. abortus*, *P. aerophilum* and *T. maritima* SurE enzymes with different substrates are also tabulated. Each value is shown as the percentage of the activity of the respective enzyme with *p*-nitrophenylphosphate (PNPP).

3.2. Crystal structure of BaSurE

The crystal structure of native BaSurE, determined to a resolution of 1.9 Å, was refined to *R* and *R*_{free} values of 19.8 and 22.7%, respectively. Fig. 2 shows the quality of the electron density of one of the representative regions in the structure. There are four BaSurE subunits (*i.e.* monomers) per asymmetric unit, which interact with each other about three perpendicular twofold axes to form a noncrystallo-

graphic dimer-of-dimers-type tetramer (Figs. 2*a* and 2*b*). This oligomeric state is consistent with dynamic light-scattering (DLS; data not shown) and gel-filtration experiments, in which the functional form of BaSurE was found to be a tetramer.

The BaSurE monomer (or 'subunit') is a two-domain structure consisting of six α -helices (H), 13 β -strands (B) and three short 3_{10} -helices (G) (Fig. 2*d* and Supplementary Fig. S3). Residues 1–126 in the N-terminal domain constitute a Rossmann-type fold with an $\alpha/\beta/\alpha$ structural motif that contains β -sheets B1–B8 flanked by α -helices H1 and H4 on

one side and H2, G1 and H3 on the other. Residues 127–252 constitute the C-terminal domain and form β -sheets B10–B13 and three helices: H5, G3 and H6. The N- and C-terminal domains are connected by β -strand B9 and 3_{10} -helix G2 (Fig. 2*d*).

The total surface area buried by the interactions between the four subunits is approximately 3047 Å² (as calculated by the PISA server; Krissinel & Henrick, 2007). The interactions between the subunits include hydrophobic interactions, hydrogen bonds, aromatic–aromatic interactions, aromatic–sulfur interactions, cation– π interactions and, quite notably,

Figure 2

Ribbon diagrams of different portions of the BaSurE tetramer. (a) A 'side view' of the full tetramer. Each subunit is displayed in a different colour. The rectangles labelled I and II mark two distinct intersubunit contact regions (see text). (b) A 'top view' of the tetramer, made by rotating the view in (a) by 90° about the horizontal axis. (c) A magnified view of a dimeric portion of the crystal structure. (d) Diagram of a single BaSurE subunit, showing the location of the bound Mg²⁺ ion as a green sphere. Note that unlike in (a)–(c), here the colours signify different types of secondary-structural elements, with helices in cyan and sheets in magenta. (e) 2*F*_o – *F*_c electron density at a 1 σ cutoff for a portion of the B12 strand which (as described in the text) is important for oligomerization of the protein.

ionic interactions. A total of 24 intrasubunit and 27 intersubunit ionic interactions are observed in the tetramer of the BaSurE crystal structure, as determined using the web-based *PIC* server (Supplementary Fig. S4 and Supplementary Table S1; Tina *et al.*, 2007). The ability of BaSurE to hydrolyze PNPP at high temperatures (see above) can be explained by this large network of intrasubunit/intersubunit ionic interactions or salt bridges, which are often found to be important for the

stability of these types of thermostable enzymes (Goldman, 1995; Yip *et al.*, 1995).

The oligomerization in BaSurE involves different secondary-structural elements, including two 'arms' comprising a long extended β -hairpin region (B11–B12) with a hook-like conformation and an extended α -helical 'tail' (H6). While H6 (in 'region I' of Fig. 2*a*) from any one subunit (for example the magenta subunit in Fig. 2*a*) is involved in a

Figure 3
Structural superposition. (a) Structural superpositions of the BaSurE monomeric subunit (green) on other SurE family members reveal similar core structures but prominent structural differences in terms of the numbers of secondary-structure elements and their orientations in the locations marked I, II and III. (b) Superpositions of the active site of BaSurE (green) with those of homologous SurE proteins show that the amino-acid residue coordination and the specific orientations of the residues required for the binding of the metal ions are conserved and superpose well with each other.

Figure 4
(a) Surface-charge potential of BaSurE showing the acidic region (red) in the active site, which is owing to the presence of the conserved 'DD' motif specific for metal binding. (b) Mg²⁺ bound at the dimeric interface is shown in green.

domain-swapping interaction with one other subunit (in this case the blue subunit), B11–B12 (in 'region II') interacts extensively with the two other subunits (the yellow and green subunits) to form the tetramer (Fig. 2*b*). The C-terminal domain, which includes B11, B12 and H6, is thus very important for maintaining the structural integrity of this enzyme (Figs. 2*a* and 2*b*). The length and orientation of the B11 and B12 arms vary significantly between the SurE homologues (Fig. 3*a*). As a result, the intersubunit interactions, the nature of the oligomerization state and the size of

the active-site pocket also vary between SurE proteins from different organisms.

3.3. Geometry of the active site and comparison of BaSurE with other homologous enzymes

The active site of BaSurE is acidic in nature, lies at the junction of the N- and C-terminal domains and can easily be identified by the presence of a metal ion (Mg^{2+}) in the crystal structure (Fig. 4). Amino acids interacting with the metal ions

Figure 5

Metal-ion interactions with the ligands. The interactions of the BaSurE protein with bound Mg^{2+} ions, a water molecule and docked PNPP (4NP) at the active site. The plots were generated by *LigPlot*⁺ (Wallace *et al.*, 1995). The sphere shown in green is an Mg^{2+} ion and that in cyan is a water molecule, while the PNPP is shown in stick representation. Hydrogen bonds are shown as green dotted lines, while arcs represent residues that make nonbonded contacts with the ligands.

and the substrate/product at the active site and their respective interatomic distances in the BaSurE crystal structure are listed in Fig. 5. These amino-acid residues and the associated hydrogen bonds and hydrophobic interactions at the active site are found to be largely conserved or similar in other homologous SurE structures (Mura *et al.*, 2003; Pappachan *et al.*, 2008; Antonyuk *et al.*, 2009; Iwasaki & Miki, 2007; Lee *et al.*, 2001).

Other organisms for which X-ray crystal structures of homologous SurE enzymes are available include *Pyrobaculum aerophilum* (PDB entry 1l5x; Mura *et al.*, 2003), *S. typhimurium* (PDB entry 2v4o; Pappachan *et al.*, 2008), *C. burnetii* (PDB entry 3ty2; Franklin *et al.*, 2015), *Aquifex aeolicus* (PDB entry 2wqk; Antonyuk *et al.*, 2009), *Thermus thermophilus* (PDB entry 2e6c; Iwasaki & Miki, 2007) and *Thermotoga maritima* (PDB entry 1j9k; Lee *et al.*, 2001). They all share a typical butterfly-shaped structure like that in Fig. 2(a), have a similar fold and possess the signature sequence motif NDDG for the binding of the metal ion (Supplementary Fig. S5 and Fig. 3b). Pairwise alignment and three-dimensional structure superpositions of BaSurE with these homologous proteins were carried out with the *DALI* and *MAPSCI* web-based servers (Holm & Sander, 1995) to determine the structural differences and to identify the common core secondary-structural elements. The lengths of these enzymes vary from 238 to 270 amino-acid residues, but they have a common core size of about 152 residues with an r.m.s.d. value of 0.72 Å.

Overall, the structure of BaSurE is relatively similar to the structures of PDB entries 3ty2 and 2e6c, while substantial differences can be seen with respect to the other homologous structures. These structural differences are highlighted in terms of r.m.s.d.s, numbers of C α atoms aligned, sequence identities and differences in their buried surface areas (Table 7).

The structural superposition reveals a common folding pattern among SurE homologues in the N-terminal region, while the majority of the structural differences between the homologues occur in the C-terminal region. These variations in the C-terminal regions of the homologous SurE enzymes are owing to differences in the size and the orientation of the domain-swapping helix region and a β -hairpin, which as described above lead to the dimerization and tetramerization of the enzyme and which eventually determine the active-site area of the dimeric interface (Figs. 2a, 4a and 4b, Supplementary Fig. S6 and Supplementary Table S2).

A unique feature of BaSurE, which is missing in other homologous structures, is the length of the loop connecting B6 and H4, which is longer than that in its homologues and has acquired a small β -hairpin structure made up of strands B7 and B8 (shown in green in Fig. 3a). The structure of SurE from *P. aerophilum* (PDB entry 1l5x) is different from the others in terms of the position of the swapped α -helix, which is oriented 180° away, making the dimeric interface less extensive in this enzyme.

To look into the mechanism of action of this enzyme, docking experiments were performed by placing PNPP in the active site of BaSurE. The idea for doing this was derived from

Table 7

Structural superposition and percentage sequence identity of BaSurE with homologous structures.

The PDB references for the various SurE homologues are given. Interface areas were calculated using *PISA* (Krissinel & Henrick, 2007). The values for the interface areas are rounded to the nearest tenth of the total value.

SurE homologue	Interface area (Å ²)	R.m.s.d. (Å)	No. of C α atoms aligned	Sequence identity (%)
4zg5	3050	—	—	—
1l5x	2900	2.8	231	32
2v4o	3660	2.1	243	42
3ty2	3580	1.8	245	38
2wqk	3450	3.1	244	41
2e6c	3170	1.8	240	34
1j9k	3500	3.1	227	34

interaction studies of phosphate ion with the stationary-phase survival protein from *S. typhimurium*, in which the enzyme was found to contain adenosine and phosphate ion in the active site (PDB entry 4xj7; Mathiharan *et al.*, 2015). These homologous structures were superposed and the phosphate ion from PDB entry 4xj7 was docked manually into the active site of BaSurE using *Coot* (Emsley & Cowtan, 2004); this was followed by submitting the structure of the complex to the *PatchDock* server again to give a better result (Schneidman-Duhovny *et al.*, 2005). Interestingly, the top result from these online servers showed an interaction of phosphate (of PNPP) in BaSurE that was very similar to that in the homologous SurE from *S. typhimurium*. In *S. typhimurium* SurE the phosphate molecule was found to interact with Asn96, Gly40, Gly105, Thr106, Ser104, Asn92, Ser39 and Mg²⁺ ion (Supplementary Fig. S7). The amino-acid residues in BaSurE which bind to the phosphate moiety are listed in Fig. 5 and can be compared with the equivalent residues in SurE from *S. typhimurium* (Supplementary Fig. S7).

4. Conclusion

The ability of *B. abortus* to outlast a phagosome is crucial for its survival. Stationary-phase survival protein (SurE) is an important stress-response protein and a possible virulence factor which may enable *B. abortus* to maintain its stationary-phase physiology even when in macrophages. In the present work, we report the crystal structure and functional characterization of SurE from *B. abortus*. The structural findings have been complemented by kinetic studies. BaSurE is found to be active over wide ranges of temperature and pH and with various metal ions, showing its resilient nature that could help the pathogen to withstand the harsh environment inside the phagosome. The involvement of large ion-pair networks that impart thermostability to the molecule is evident in the structure. BaSurE hydrolyses several phosphoester-containing substrates and it displays maximal hydrolytic activity with 5'-TMP. Minor differences in the nitrogen bases possibly alter the hydrogen-bond network near the active site that contributes to the precise orientation of NMPs for catalysis. This also means that the phosphate-cleaving residues have to swing

into action when the correct base is recognized, leading to the difference in substrate specificity for some of them.

The exact biological function of this enzyme in the stationary phase is not known, but we assume that this enzyme, as a nucleotide phosphatase, is possibly involved in the recycling of nucleotides to maintain a basal level of nucleosides and phosphates. These molecules can be salvaged and utilized in the synthesis of nucleic acids (DNA and RNA), energy carriers (ATP and GTP), cofactors (NAD, FAD and coenzyme A), signalling molecules (cAMP and cGMP), phospholipids and polysaccharides of the cell wall, constituents that are necessary for the survival of this bacterium.

The BaSurE protein consists of two domains: a larger N-terminal domain and a smaller C-terminal domain. While the N-terminal domain is mostly involved in the function of the molecule, the C-terminal domain is mainly important for maintaining the oligomeric state of the protein. The overall structure has a three-layer $\alpha/\beta/\alpha$ topology that is homologous to the Rossmann fold and is composed of parallel β -strands linked to a few α -helices. The structure of BaSurE bound by metal ion and docked PNPP represents the stage prior to hydrolysis. These observations support the previously proposed mechanism for the SurE enzymes, in which the metal ion seems to polarize the phosphate bond of the monophosphate substrate (PNPP) and orient the conserved Asp8 for nucleophilic attack, leading to the formation of a phosphorylated Asp8 intermediate (Pappachan *et al.*, 2008).

Owing to the close relationship between *B. abortus* stationary-phase gene expression and its virulence, BaSurE has the potential to be a drug target. What may be particularly helpful in this regard is that the structure of BaSurE displays features that are not found in other proteins. There is no similar human protein, or any protein with significant percentage identity, suggesting that a drug targeting BaSurE may cause few side effects in humans. Moreover, although the overall folds of the various available SurE homologues found in other microorganisms superficially appear to be very similar, the sizes and relative orientations of the equivalent secondary structures vary significantly, as described above, and these different detailed arrangements of the secondary structures in turn influence the volume and size of the active-site area as well as the oligomeric state of the different homologous SurE structures. It is too early to be certain, but these differences may provide a promising direction for research to design inhibitor molecules that exclusively target SurE of *B. abortus* and that not only avoid binding the protein products of human genes but also avoid targeting other bacteria as well, including those that are beneficial to humans.

5. Related literature

The following references are cited in the Supporting Information for this article: Bond (2003), Dundas *et al.* (2006), Gouet *et al.* (2003) and McWilliam *et al.* (2013).

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