The first crystal structure of manganese superoxide dismutase from the genus *Staphylococcus*

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A recombinant *Staphylococcus equorum* manganese superoxide dismutase (MnSOD) with an Asp13Arg substitution displays activity over a wide range of pH, at high temperature and in the presence of chaotropic agents, and retains 50% of its activity after irradiation with UVC for up to 45 min. Interestingly, *Bacillus subtilis* MnSOD does not have the same stability, despite having a closely similar primary structure and thus presumably also tertiary structure. Here, the crystal structure of *S. equorum* MnSOD at 1.4 Å resolution is reported that may explain these differences. The crystal belonged to space group $P3_221$, with unit-cell parameters $a = 57.36$, $b = 57.36$, $c = 105.76$ Å, and contained one molecule in the asymmetric unit. The symmetry operation indicates that the enzyme has a dimeric structure, as found in nature and in *B. subtilis* MnSOD. As expected, their overall structures are nearly identical. However, the loop connecting the helical and α/β domains of *S. equorum* MnSOD is shorter than that in *B. subtilis* MnSOD, and adopts a conformation that allows more direct water-mediated hydrogen-bond interactions between the amino-acid side chains of the first and last α -helices in the latter domain. Furthermore, *S. equorum* MnSOD has a slightly larger buried area compared with the dimer surface area than that in *B. subtilis* MnSOD, while the residues that form the interaction in the dimer-interface region are highly conserved. Thus, the stability of *S. equorum* MnSOD may not originate from the dimeric form alone. Furthermore, an additional water molecule was found in the active site. This allows an alternative geometry for the coordination of the Mn atom in the active site of the apo form. This is the first structure of MnSOD from the genus *Staphylococcus* and may provide a template for the structural study of other MnSODs from this genus.

1. Introduction

Superoxide dismutase (SOD; EC 1.15.1.1) splits superoxide (SO) into hydrogen peroxide (H₂O₂) and oxygen (O₂). The enzyme is the first line of defence against SO species in cells (Miller, 2012). The presence of SO species leads to oxidative stress in cells, to various diseases such as cancer and diabetes, and to cell death and ageing (Maritim *et al.*, 2003). SOD protects the cell from ultraviolet (UV) radiation, thereby lowering the risk of cell damage (Acton, 2013). Therefore, SOD has been used in therapeutic applications and in cosmetics (Maritim *et al.*, 2003; Lods *et al.*, 2000).

SODs are grouped into four classes based on the metal ions in the active site, which can either be a copper–zinc pair (Cu/ZnSOD, also called SodC), nickel (NiSOD, SodN), iron (FeSOD, SodA) or manganese (MnSOD, SodB) (Abreu & Cabelli, 2010). MnSOD has a nearly identical structural

architecture to FeSOD, although it has a different reaction mechanism. The metal at the active site can even be exchanged (cambialistic SODs; Abreu & Cabelli, 2010).

In initial work on *S. equorum* MnSOD only a partial nucleic acid sequence was elucidated; the full nucleotide sequence of the enzyme was reconstructed by insertion of the N- and C-termini of the gene encoding *S. saprophyticus* MnSOD (Retnoningrum *et al.*, 2016). Therefore, the enzyme has previously been referred to as a hybrid of the MnSODs from *S. equorum* and *S. saprophyticus* (Retnoningrum *et al.*, 2016). Subsequently, the amino-acid sequence was found to be almost identical to the more recently reported MnSOD sequence from *S. equorum* (GenBank WP_046465435.1) with the only substitution being at Asp13Arg. This substitution occurs in the part of the sequence of the hybrid enzyme from *S. saprophyticus* MnSOD. Nevertheless, this enzyme with the Asp13Arg substitution is designated *S. equorum* MnSOD. *S. equorum* MnSOD has amino-acid sequence similarity to other bacterial MnSODs, *i.e.* those from *Bacillus subtilis* (78.1%; PDB entry 2rcv; Liu *et al.*, 2007), *Deinococcus radiodurans* (54.5%; PDB entry 2ce4; Dennis *et al.*, 2006) and *Escherichia coli* (52.2%; PDB entry 1vew; Edwards *et al.*, 1998). Owing to their high similarity in amino-acid sequence, the structure of *B. subtilis* MnSOD has been employed to study the structure and function of *S. equorum* MnSOD. However, these homologous enzymes have significantly different characteristics. For example, *S. equorum* MnSOD is still active at 353 K (Retnoningrum *et al.*, 2016), at which temperature *B. subtilis* MnSOD is completely inactivated (Wang *et al.*, 2014). *S. equorum* MnSOD is also more resistant to inactivation by urea or guanidinium chloride (chaotropic agents), by sodium dodecyl sulfate (a detergent) or by β -mercaptoethanol (a reducing agent) than *B. subtilis* MnSOD (Retnoningrum *et al.*, 2016). These findings suggest that the *B. subtilis* MnSOD structure may be not representative of *S. equorum* MnSOD. Therefore, elucidation of the *S. equorum* MnSOD structure is of paramount importance.

Here, we report the crystal structure of *S. equorum* MnSOD, which is the first structure of an MnSOD from the genus *Staphylococcus* to be described. We discovered that the surface loop connecting the helical and α/β domains of the *S. equorum* MnSOD monomer is shorter and oriented inwards into the molecule, which might be a reason for the superior stability of *S. equorum* MnSOD over *B. subtilis* MnSOD. In addition, the active site of *S. equorum* MnSOD contains an additional water molecule that is not observed in *B. subtilis* MnSOD, but is present in the MnSOD from the eukaryote *Caenorhabditis elegans*. Thus, its presence might not be incidental and furthermore provides an alternative geometry for the Mn-atom coordination at the active site.

2. Materials and methods

All chemicals were purchased from Sigma (St Louis, Missouri, USA) or Merck AG (Darmstadt, Germany) except where otherwise indicated.

2.1. Preparation of recombinant *S. equorum* MnSOD

S. equorum MnSOD was overproduced in *E. coli* BL21 (DE3) cells as a recombinant protein containing a C-terminal His₆ tag. It was subsequently purified according to a previous report (Retnoningrum *et al.*, 2017) with minor modifications, *i.e.* the buffer used was Tris-buffered saline (TBS; 137 mM NaCl, 2.7 mM KCl, 50 mM Tris-HCl pH 7.5). In brief, the enzyme was produced in LB medium containing 100 $\mu\text{g ml}^{-1}$ ampicillin at 310 K with agitation at 150 rev min⁻¹ and induced with 1 mM IPTG. After cell disruption, the enzyme in the soluble fraction was purified using a Ni²⁺-NTA cOmplete affinity column (Roche, Singapore) on an FPLC system (NGC system, Bio-Rad). Auxiliary purification (polishing) was performed with an Enrich-70 size-exclusion column (Bio-Rad). The purified protein was concentrated using a 10 kDa cutoff Macroprep centrifugal concentrator (Merck-Millipore, Darmstadt, Germany).

2.2. Enzyme-activity assays

Colorimetric and zymographic activity assays were based on the reduction of nitro blue tetrazolium (NBT) in the presence of riboflavin (Retnoningrum *et al.*, 2016). Briefly, an enzyme solution in 16 mM phosphate buffer pH 7.8 was mixed with 85 μM NBT, 0.8 mM tetramethylethylenediamine (TEMED) and 6 μM riboflavin. The mixture was then exposed to light for 5–10 min at 310 K. The absorbance of the formazan formed was measured at a wavelength of 560 nm at room temperature (298 K). The percentage of reduction inhibition was calculated from the difference in absorbance between the blank and the sample (in the absence and the presence of the enzyme, respectively). The zymographic analysis was performed with a 12% native PAGE followed by negative staining formed by the reduction of NBT in the presence of riboflavin. The gel was immersed in 0.1 M phosphate buffer containing 0.17 M NBT for 15 min and was subsequently rinsed with water. A solution containing 0.016 mM riboflavin and 11 mM TEMED was added to the gel. Incubation was performed in the dark for 15 min at room temperature. After rinsing with water, the gel was exposed to visible light. The presence of a clear zone at the expected band after exposure in the gel is an indication of SOD activity.

2.3. Crystallization and data collection

Initial crystal screening was performed by the sitting-drop vapour-diffusion method employing a Mosquito crystallization robot system (TTP LabTech, Hertfordshire, England). Screening was performed using a protein stock solution concentration of 3.4 mg ml⁻¹ against the Index and PEG/Ion screens from Hampton Research (Alison Viejo, California, USA), in which small crystals emerged under several conditions after a few weeks. Crystals suitable for X-ray diffraction were obtained in a manual setup of the sitting-drop vapour-diffusion method in which a drop consisting of 1 μl 7.4 mg ml⁻¹ protein solution in 50 mM Tris-HCl buffer pH 8.0 mixed with 1 μl mother-liquor solution [0.2 M magnesium chloride, 0.1 M HEPES pH 7.5, 25%(w/v) PEG 3350] was

Table 1

Data-collection and refinement statistics.

Values in parentheses are for the highest resolution bin.

Data collection	
Beamline	BL-5A, PF
Temperature (K)	100
Wavelength (Å)	1.0000
Resolution range (Å)	50.00–1.40 (1.42–1.40)
Measured reflections	386764
Unique reflections	40473
Multiplicity	9.6 (9.5)
Completeness (%)	100.0 (100.0)
Mean $I/\sigma(I)$	24.4 (3.3)
R_{merge} (%)†	7.2 (47.8)
$R_{\text{p.i.m.}}$ (%)	2.5 (16.2)
$CC_{1/2}$	0.996 (0.935)
Space group	$P3_221$
Unit-cell parameters (Å)	$a = 57.36, b = 57.36, c = 105.76$
Refinement	
Resolution range (Å)	36.21–1.40 (1.44–1.40)
No. of reflections	38397 (2794)
Completeness (%)	99.9 (100)
R factor (%)	14.2 (20.2)
R_{free} (%)	18.5 (28.0)
R.m.s.d., bond lengths (Å)	0.019
R.m.s.d., bond angles (°)	1.8
Ramachandran plot	
Most favoured region (%)	91.5
Additional allowed region (%)	7.4
B factor (Å ²)	
Protein	18.4
Manganese	8.6
Water	30.7
PDB code	5x2j

† $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, in which $I_i(hkl)$ is the i th measurement and $\langle I(hkl) \rangle$ is the weighted mean of all measurements of $I(hkl)$.

placed over 50 μ l reservoir solution. The experiment was carried out in a 96-well Corning plate (Tewksbury, Massachusetts, USA) at 293 K. X-ray diffraction data were collected from an *S. equorum* MnSOD crystal mounted on a cryoloop and flash-cooled in a nitrogen vapour stream. The diffraction data were recorded with a Dectris PILATUS 2M detector upon exposure to an X-ray beam at 1.0 Å wavelength. The X-ray data collection was conducted on BL-5A at the Photon Factory (PF), High Energy Accelerator Research Organization (KEK), Tsukuba, Japan.

2.4. X-ray diffraction data processing and structure determination

The X-ray diffraction data were processed (indexed, integrated and scaled) using *HKL-2000* (Otwinowski & Minor, 1997) and the *CCP4* program suite (Winn *et al.*, 2011). The phasing was performed by molecular replacement using *MOLREP* (Vagin & Teplyakov, 2010) employing the structure of *B. subtilis* MnSOD as the homology model. Model building was then continued manually with *Coot* (Emsley *et al.*, 2010). Refinement of the model was performed with *REFMAC5* (Murshudov *et al.*, 2011) and *phenix.refine* (Afonine *et al.*, 2012). Water molecules were gradually introduced into the ($F_o - F_c$) electron-density map using *Coot* at peaks in the electron density above 3.0σ and within the distance range of a hydrogen bond. Structure validation was performed with

PROCHECK (Laskowski *et al.*, 1993). The data-collection and refinement statistics are listed in Table 1. The structure has been deposited in the Protein Data Bank with accession code 5x2j. Structural evaluation and comparison with the structures of MnSOD sequence homologues were performed manually with *Coot* (Emsley *et al.*, 2010). Analysis of the protein molecule was also performed using *PISA* (Krissinel & Henrick, 2007), *ASA-view* (Ahmad *et al.*, 2004) and *Protein-Volume* 1.3 (Chen & Makhataadze, 2015). The structural figures were prepared with *PyMOL* (<http://www.pymol.org>).

3. Results and discussion

3.1. Crystallization, X-ray diffraction analysis and structural analysis

From one crystallization drop, crystals of *S. equorum* MnSOD with two different shapes were obtained: prismatic and polygonal (Supplementary Fig. S1). During the preliminary analysis of the X-ray diffraction data, a prismatic crystal diffracted to 3.6 Å resolution (orthogonal space group $P222$) and a polygonal crystal to 1.86 Å resolution (trigonal space group $P3_221$). The latter was employed for further structural analysis because it was more reproducible and diffracted to higher resolution. However, this polygonal crystal suffered from twinning, hampering analysis and processing of the diffraction data. Fortunately, one of the polygonal crystals diffracted to 1.4 Å resolution without crystallographic problems. Diffraction data from this crystal were used in the subsequent data processing and structure determination. The data-collection and structure-refinement statistics are presented in Table 1.

Further, crystallographic analysis of the crystal suggested the presence of one protein molecule in the asymmetric unit, with a Matthews coefficient V_M of $2.2 \text{ Å}^3 \text{ Da}^{-1}$ (44.6% solvent content; Kantardjieff & Rupp, 2003; Matthews, 1968). However, the dimeric form was observed upon symmetry analysis, in agreement with the presence of the enzyme dimer in solution (Retnoningrum *et al.*, 2016). Bacterial MnSODs are mostly found in dimeric form, as shown by the structures of *B. subtilis* MnSOD and *E. coli* MnSOD, which contain four dimers (eight molecules) and two dimers (four molecules), respectively. The dimeric form of MnSOD has been proposed to be instrumental in enzyme activity and stability (Edwards *et al.*, 2001b; Whittaker & Whittaker, 1998).

3.2. Overall structure of *S. equorum* MnSOD and the active site

Bacterial MnSOD is a homodimeric enzyme, in which the monomer has the typical two-domain fold comprising of a helical domain and an α/β domain and containing one manganese ion (Liu *et al.*, 2007). Their secondary-structure elements are highly conserved, with only few deviations involving surface loops and single-turn (short) helices. The structural features of the monomers are also observed in eukaryotic MnSODs, including human MnSOD, which is a tetrameric enzyme (Borgstahl *et al.*, 1992).

Table 2
Structural comparison of *S. equorum* MnSOD with its homologues.

	<i>S. equorum</i>	<i>B. subtilis</i>	<i>E. coli</i>	<i>D. radiodurans</i>
PDB code	5x2j	2rev	1vew	2ce4
Resolution (Å)	1.4	1.8	2.1	2.2
Structural alignment				
R.m.s.d. (Å)	—	0.7859	0.9259	0.7970
No. of residues aligned	198	197	196	196
Quality normalized	—	0.1147	0.2149	0.4409
Protein volume calculation				
Molecular volume (Å ³)	26795.5	26517.1	27798.5	27212.5
Packing density	0.752	0.747	0.739	0.746
Void volume (Å ³)	6656.5	5717.9	7268.8	6914.4
van der Waals volume (Å ³)	20139.0	19799	20529.7	20298.1
PISA calculation				
Surface area (Å ²)	17590	17353	17680	17140
Buried area (Å ²)	1770	1710	1715	1810
Buried/surface area (%)	10.1	9.9	9.7	10.6

The structure of the *S. equorum* MnSOD monomer comprises an identical number of α -helices and β -sheets in an antiparallel arrangement similar to that of *B. subtilis* MnSOD (Fig. 1). The helical domain of *S. equorum* MnSOD consists of helices $\alpha 1$ – $\alpha 3$ at corresponding positions to those of *B. subtilis* MnSOD in its amino-acid sequence. Furthermore, the α/β domain starts with $\alpha 4$ and $\alpha 5$ followed by the three strands $\beta 1$ – $\beta 3$, and finally $\alpha 6$ – $\alpha 8$. All of these secondary-structure elements are nearly identical to those of *B. subtilis* MnSOD, confirming our hypothesis that the structures of the two enzymes are nearly identical. The main-chain atoms in the overall structure of *S. equorum* MnSOD are superimposable with those of *B. subtilis* MnSOD, *D. radiodurans* MnSOD and *E. coli* MnSOD (Fig. 2), with r.m.s.d. values of 0.78, 0.79 and 0.93 Å, respectively (Table 2; Krissinel & Henrick, 2004).

Furthermore, all of the previously reported interactions during dimer formation (Edwards *et al.*, 1998; Liu *et al.*, 2007) are present: for instance, the glutamate bridge between Glu164 (*S. equorum* MnSOD amino-acid sequence numbering)

of molecule *A* (Glu164-*A*) and that of molecule *B* (Glu164-*B*). Glu164-*A* is stabilized by the interaction of its O^ε atom with the N^δ atom of His165-*B* (and, *vice versa*, of Glu164-*B* with His165-*A*). An interaction of the N^ε atom of His31-*A* with the O^δ atom of Tyr168-*B* is also present in the dimer interface. This His31-*A*/Tyr168-*B* pair is highly conserved and has been proposed to guide the substrate into the active site (Whittaker & Whittaker, 1998). Other interactions in the dimer interface involve Ser126-*A* with Ser126-*B* and the water-mediated interaction of Asn145-*A* with Asn145-*B*.

His165 is one of the three histidine residues (the others are His27 and His81) that coordinate the manganese at the active site of the enzyme. The Mn atom is also coordinated by Asp161 and two water molecules, W1 and W2, which are in the axial positions opposite His27 and Asp161, respectively (Fig. 3*a*). However, while the water molecule W2 is observed in the active-site region in the structures of *B. subtilis* MnSOD (1.8 Å resolution), *E. coli* MnSOD (2.1 Å resolution) and *D. radiodurans* MnSOD (2.2 Å resolution), water molecule W1 is absent. Spectroscopic and structural study of *E. coli* MnSOD suggest a trigonal bipyramidal geometry of the manganese in the active site (Edwards *et al.*, 1998). In *S. equorum* MnSOD this coordination is achieved with the triad His27–His81–His165, Asp161 and water molecule W2 (Fig. 3*b*). Further inspection revealed that the histidine triad, the aspartate, the manganese and the water molecule W1 may form a square-pyramidal geometry (Fig. 3*c*) and, when W2 is involved, form a distorted octahedral geometry. This situation has not previously been identified owing to the absence of the water molecule W1 from the structures of *S. equorum* MnSOD bacterial homologues (MnSODs from *B. subtilis*, *E. coli* and *D. radiodurans*).

The presence of W1 in *S. equorum* MnSOD may not be artificial because its position is conserved in the structure of azide-bound *C. elegans* MnSOD (1.8 Å resolution; Hunter *et al.*, 2015), in which the W1 position is occupied by the azide.

Figure 1
Top-view (*a*) and side-view (*b*) ribbon representations of the dimeric structure of *S. equorum* MnSOD with its secondary-structure elements in red (α -helix), yellow (β -sheet) and green (coil or loop). The Mn atom is shown as a dark grey sphere and the water molecules are shown as cyan dots.

The presence of the azide completes the distorted octahedral coordination, thereby supporting the 5–6–5 manganese coordination shift during the reaction, as previously proposed for the FeSOD from *E. coli* (Lah *et al.*, 1995). In this respect, the structure of *S. equorum* MnSOD probably represents the condition in which the substrate is bound.

In addition, another water molecule (W3) that interacts with His31 (N δ) and Tyr35 (O δ) is present 3.73 Å away from water molecule W1. His31 plays a role in the aforementioned interactions in the dimer interface. A water relay involving W1 and W3 in the *S. equorum* MnSOD structure may lead to the open space outside the enzyme dimer (Fig. 4), suggesting a passage for the superoxide substrate to enter or for the peroxide product to leave the active-site cavity. This water relay has also been observed in the structure of *C. elegans* MnSOD (Hunter *et al.*, 2015).

Figure 2
Structural comparison between MnSODs from *S. equorum* (red), *B. subtilis* (blue), *E. coli* (green) and *D. radiodurans* (yellow). The structures are presented as ribbons and the Mn atom is shown as a dark grey sphere. The N- and C-termini are indicated.

Figure 3
Active site of *S. equorum* MnSOD in the presence of the Mn atom and water molecules W1 and W2 (a), with possible trigonal bipyramidal geometry involving water molecule W2 (b) and square tetrahedral geometry involving water molecule W1 (and orthogonal geometry when W2 is involved) (c). The trigonal and tetragonal squares are shown as blue lines, the interactions that form the trigonal and tetragonal pyramidal geometries are shown as red lines, and the interactions to complete the trigonal bipyramidal and orthogonal geometries are shown as green lines.

Further study of the Mn atom in the active site of MnSOD shows its relation to FeSOD because of their structural resemblance and cambialistic character, although the electron configuration of iron ([Ar] 4s² 3d⁶) differs from that of manganese ([Ar] 4s² 3d⁵). The study of MnSODs from *Saccharomyces cerevisiae* mitochondria and *Candida albicans* cytosol reveals reduced (Mn²⁺) and oxidized (Mn³⁺) states of the manganese when no reaction takes place (the so-called rest state; Sheng *et al.*, 2011). In this respect, Mn³⁺ may occur as five-coordinate Mn³⁺ or six-coordinate L-Mn³⁺ (with hydroxide as the sixth ligand). Spectroscopic study of bacterial MnSOD suggests that manganese is mostly found in its reduced state, which is supported by its structure. In addition, the activity of the MnSOD with the six-coordinate L-Mn³⁺ species is prone to reduction by ascorbate (Sheng *et al.*, 2011). This may be correlated with the observed reduced activity of *S. equorum* MnSOD in the presence of reducing agents (Indrayati *et al.*, 2014).

Finally, Gln146 in *S. equorum* MnSOD, at a position corresponding to that in *E. coli* MnSOD, plays an important role in maintaining the hydrogen-bonding network in the active site (Edwards *et al.*, 2001a). In *S. equorum* MnSOD and *B. subtilis* MnSOD, this residue resides in the surface loop (residues 151–155; *S. equorum* MnSOD amino-acid sequence numbering) that connects β 2 and β 3. This loop is five amino-acid residues shorter than the loop in *E. coli* MnSOD and *D. radiodurans* MnSOD. Consequently, the active site in the MnSODs from *S. equorum* and *B. subtilis* may be less accessible from the solvent, which may impact the reaction turnover. Solvent-accessibility analysis (Krissinel & Henrick, 2007) indicated that the Gln146 and the neighbouring Asn145 are buried in the MnSODs from *S. equorum* and *B. subtilis*, while the corresponding residues in the MnSODs from *E. coli* and *D. radiodurans* are accessible.

3.3. Structural comparison to *B. subtilis* MnSOD

Structural comparison of *S. equorum* MnSOD with *B. subtilis* MnSOD was motivated by their remarkable differences in enzyme activity and stability, despite the high

Figure 4
The water relay in the active site of *S. equorum* MnSOD to the open space involving W1–W3. His27, His81, His165 and Asp165 are the residues that coordinate the Mn atom, while His31 and Tyr35 are the residues that reside on top of the active-site cavity. His31 has been hypothesized to guide the substrate into the active site. Cyan spheres are water molecules (including W3) on the outer concave surface of the active site.

similarity in their amino-acid sequences. The only obvious difference between the structures of *S. equorum* MnSOD and *B. subtilis* MnSOD is the stretch of residues Asn91–Val99, which is the region that connects the helical and α/β domains (Fig. 5). The corresponding stretch in *B. subtilis* MnSOD is only one amino-acid residue longer (Leu89–Pro97; *B. subtilis* MnSOD amino-acid sequence numbering); thus, the loop extends slightly into the solvent. Furthermore, the shorter loop in *S. equorum* MnSOD causes the first helix of the α/β domain ($\alpha 4$) to shift inward and thereby interact better with $\alpha 8$, which is the last helix in this domain (Figs. 5a and 5b). The shift allows additional interaction between O $^{\gamma}$ of Ser92 and the carbonyl backbone of Val186, a water-mediated interaction between the amino-acid backbone of Glu93 and Trp188, and finally interaction of the O $^{\epsilon}$ atom of Glu94 and the N $^{\zeta}$ atom of Lys113. The loop itself is stabilized by side-chain interaction of the O $^{\epsilon}$ atom of Glu93 and the N $^{\zeta}$ atom of Lys95 (Fig. 5c). These interactions may increase the rigidity of the α/β domain. The active site of the enzyme is located at the interface of the helical and α/β domains, which both contribute equally to coordination of the Mn atom and the reaction. Thus, the increase in the latter domain may make the enzyme resistant to denaturation and improve its thermal stability. In addition, in the structures of other *S. equorum* MnSOD structural homologues (MnSODs from *D. radiodurans* and *E. coli*) the first helix in the α/β domain adopts an identical position to that in *B. subtilis* MnSOD.

Further evaluation of individual amino-acid residues using ASA-view suggested that the amino-acid composition of *S. equorum* MnSOD is slightly different from that of *B. subtilis* MnSOD (Supplementary Fig. S3). Overall, *S. equorum* MnSOD contains more charged residues than *B. subtilis* MnSOD. Most importantly, more charged residues in *S. equorum* MnSOD are accessible to the solvent, which may

correspond to a slight increase in the van der Waals interaction (75.2%) in *S. equorum* MnSOD compared with *B. subtilis* MnSOD (74.7%). Furthermore, residues involved in the dimer interface are mostly conserved; thus, not many differences were found during analysis using PISA (Krissinel & Henrick, 2007; Table 2).

S. equorum MnSOD has been shown to have two melting temperatures (T_m) at around 325 and 341 K, which correspond to dimer dissociation and unfolding of the monomer, respectively (Retnoningrum *et al.*, 2016). However, while the dimeric form has been associated with enzyme activity (Whittaker & Whittaker, 1998), the relationship between the dimeric form and enzyme stability has not been established. The crystal structure of *S. equorum* MnSOD suggests that enzyme stability might not be owing to the dimeric form because very little variation was observed compared with *B. subtilis* MnSOD. The *S. equorum* MnSOD structure shows additional interactions from the shorter interdomain-connecting loop in the monomer structure. Slightly higher charged residues on the surface of the enzyme molecule may improve its interaction with the aqueous environment, but this has not yet been confirmed.

4. Conclusion

We have successfully elucidated the structure of *S. equorum* MnSOD, the first example of an MnSOD from the *Staphylococcus* genus. The structure may provide an explanation on a molecular basis for the differences in the characteristics of *S. equorum* and *B. subtilis* MnSOD, two close homologues that share high homology in their amino-acid sequences. Furthermore, the structure of *S. equorum* MnSOD contains an additional water molecule that is absent from the structures of its

Figure 5

(a) Differences in the structures of MnSODs from *S. equorum* (salmon) and *B. subtilis* (green). The loop connecting $\alpha 3$ and $\alpha 4$ in *S. equorum* MnSOD (red) is shorter than that in *B. subtilis* MnSOD (blue), resulting in a structural shift of $\alpha 4$ towards $\alpha 8$. (b) Residues in the loop connecting the $\alpha 3$ and $\alpha 4$ (Glu93–Lys95), $\alpha 4$ (Lys113) and $\alpha 8$ (Trp188) helices come into proximity for interactions as a result of the structural shift. Details of interactions in the structure of *S. equorum* MnSOD (c) and their corresponding amino acids in that of *B. subtilis* MnSOD (d) as a comparison.

homologues that may shed light on the exact mechanisms of the enzymatic reaction.

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