Structural basis of the zinc- and terbium-mediated inhibition of ferroxidase activity in Dps ferritin-like proteins

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Abstract

Streptococcus suis Dpr is an iron-binding protein involved in oxidative stress resistance. It belongs to the bacterial Dps protein family whose members form dodecameric assemblies. Previous studies have shown that zinc and terbium inhibit iron incorporation in *Listeria innocua* Dps protein. In order to gain structural insights into the inhibitory effect of zinc and terbium, the crystal structures of *Streptococcus suis* Dpr complexes with these ions were determined at 1.8 Å and 2.1 Å, respectively. Both ions were found to bind at the ferroxidase center and in the same location as iron. In addition, a novel zinc-binding site formed by His40 and His44 was identified. Both His residues were found to be present within all known *Streptococcus suis* Dpr variants and in *Streptococcus pneumoniae*, *Streptococcus gordonii*, and *Streptococcus sanguinis* Dpr proteins. Amino acid sequence alignment of Dpr with other Dps family members revealed that His44 is highly conserved, in contrast to His40. The inhibitory effect of zinc and terbium on iron oxidation in Dpr was studied in vitro, and it was found that both ions at concentrations >0.2 mM almost completely abolish iron binding. These results provide a structural basis for the inhibitory effect of zinc and terbium in the Dps family of proteins, and suggest a potential role of the Dps proteins in zinc detoxification mechanisms involving the second zinc-binding site.

Keywords: ferritin; oxidative stress; ferroxidase; iron binding; zinc

The bacterial Dps (DNA-binding protein from starved cells) proteins are found in many eubacteria and in some archaeobacteria (Wiedenheft et al. 2005; Ramsay et al. 2006). Their expression is associated with starvation and oxidative stress that occur during exposure of the bacteria to hydrogen peroxide (Martinez and Kolter 1997). Dps

proteins act by removing cytoplasmic iron, thus preventing the generation of reactive oxygen species as an outcome of the Fenton reaction ($\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{OH}^- + \text{OH}^\cdot + \text{Fe}^{3+}$) (Yamamoto et al. 2002; Pulliainen et al. 2003).

Dps proteins share many structural and functional similarities with ferritins, a large family of iron-storage proteins (Hintze and Theil 2006). However, Dps proteins form a globular dodecameric assembly that is able to store up to 500 iron atoms (Grant et al. 1998) in contrast to ferritins, which are 24-meric with a storage capacity of up to 4500 iron atoms (Harrison and Arosio 1996; Bozzi et al. 1997; Zhao et al. 2002). Moreover, the active site of Dps proteins is located between neighboring monomers and not within individual monomers as in ferritins (Grant et al. 1998; Ilari et al. 2000; Kauko et al. 2004). On the

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Abbreviations: Dpr, Dps-like peroxide resistance; Dps, DNA-binding protein from starved cells or DNA protection during starvation; RMSD, root-mean-square deviation.

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other hand, the Dps active site from archaeobacteria Dps proteins is similar to the active site of bacterioferritins, although the overall structure is typical of Dps members (Gauss et al. 2006).

Dps-like proteins exhibit a diversity of roles apart from their ability to bind iron. Some Dps proteins, such as *Bacillus brevis* Dps (Ren et al. 2003), are able to protect DNA by forming a protective biocrystal structure with DNA. Moreover, *Helicobacter pylori* neutrophil-activating protein (NAP) induces neutrophil activation and acts as an adhesion molecule (Montecucco and de Bernard 2003). Finally, the induction of a Dps protein during cold-shock conditions has been observed in *Streptococcus thermophilus* (Nicodeme et al. 2004).

Streptococcus mutans Dpr has been reported to bind 11.2 zinc atoms/dodecamer (Yamamoto et al. 2002). Furthermore, the iron incorporation in *Listeria innocua* Dps has been found to be inhibited weakly by zinc but strongly by terbium (Stefanini et al. 1999) in steady-state fluorescence experiments. Terbium binding did not exclude zinc binding, leading to the suggestion that these two metals share different binding sites in *Li. innocua* Dps.

The mechanism of iron incorporation in the Dps proteins involves five main steps: iron (II) entry, binding to the ferroxidase center, oxidation, nucleation, and finally, deposition of iron as iron (III) inside the central cavity. The exact route of iron from its entry to the ferroxidase site is not known. Because of the rapid oxidation of iron (II), use of different metals could be beneficial in identifying short-lived iron-binding sites within the dodecamer. Zinc offers the advantage that it can replace iron (II) in iron-binding proteins without undergoing oxidation. Based on the inhibitory effect of zinc in iron oxidation and its potential to reveal transient iron-binding sites, we carried out crystallographic binding studies in *Streptococcus suis* Dpr, a homolog of *S. mutans* Dpr, in the presence of zinc. In addition, the binding of terbium in *S. suis* Dpr was studied crystallographically for comparison reasons and due to its previously reported inhibitory effect on ferritins (Bou-Abdallah et al. 2003).

Results

Zinc inhibition of iron oxidation

The ability of zinc to inhibit iron oxidation in Dpr was tested by an in vitro inhibition assay (Fig. 1). Different concentrations of zinc in the form of zinc chloride were used (0.0, 0.01, 0.05, 0.1, 0.2, and 0.5 mM) at constant iron and protein concentrations (0.5 mM and 0.013 mM, respectively). Significantly reduced oxidation of iron was observed for zinc concentrations >0.2 mM. Similar results

Figure 1. Effect of zinc and terbium on iron incorporation in Dpr. (A) Prussian blue staining of truncated Dpr (5 µg/lane) incubated with different concentrations of ions (as indicated) in the presence of 0.5 mM iron. (B) Coomassie blue staining of the same Prussian blue-stained gels used in A.

were obtained with 0.2 mM terbium chloride in an analogous experimental setting.

Quality of the zinc-bound Dpr structure

The structure of Dpr in the presence of zinc was determined at 1.8 Å using crystals grown in the presence of 1 mM zinc (data set ZN1). The structure was refined to a final R_{cryst} of 20.8% (last recorded $R_{\text{free}} = 24.8\%$). The RMSD in bond lengths and bond angles is 0.011 and 1.175°, respectively, and the average B-factor for all protein atoms is 31.3 Å². All subunits except chain G lack the N-terminal tail due to its flexible character that results in weak electron map densities. The Ramachandran plot shows that 96.6% of the non-proline and non-glycine residues lie at the allowed region, and no residues are found at the disallowed regions. The overall coordinate error is 0.129 Å. Details of data collection and refinement statistics are shown in Table 1.

Overall structure

The crystal structure of the Dpr–zinc complex at 1.8 Å resolution is shown in Figure 2A. It consists of 12 identical subunits (A–L), each comprising a four α-helix bundle. The 12 subunits form a spherical assembly with an external diameter of ~90 Å and an internal cavity for iron storage of ~45 Å in diameter. There are 1184 water molecules, 24 zinc ions, four HEPES ligands, three calcium ions, and two chloride ions modeled in the

Table 1. Details of data collection and refinement statistics

	ZN1	ZN2	Terbium
<i>Data collection</i>			
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Unit cell parameters (Å)	$104.5 \times 137.7 \times 142.1$	$105.1 \times 137.9 \times 142$	$104.5 \times 137.7 \times 142.4$
Resolution (Å)	1.80 (1.90–1.80) ^a	3.10 (3.29–3.10)	2.1 (2.32–2.10)
Wavelength (Å)	1.0627	1.2824	1.0627
Number of reflections	2,270,312	521,807	676,199
Unique reflections	189,126	72,378	115,334
R_{merge} (%)	9.0 (43.6)	12.4 (41.2)	8.1 (34.8)
Completeness (%)	99.9 (99.4)	99.9 (99.9)	95.6 (88.8)
I/σ (I)	18.0 (3.6)	14.7 (5.1)	13.9 (4.57)
Wilson B-factor (Å ²)	36.5	42.2	39.0
Mosaicity (°)	0.270	0.258	0.315
<i>Refinement</i>			
Resolution range (Å)	20.0–1.8		20.0–2.1
No. of reflections	189,027		115,331
No. of reflections for R_{free}	8779		5761
Protein atoms	15,821		15,214
Water molecules	1184		619
HEPES molecules	4		4
Zinc ions	24		—
Terbium ions	—		12
Calcium ions	3		4
Chloride ions	2		10
$R_{\text{cryst}}/R_{\text{free}}$ (%)	20.8/24.8		18.7/24.4
<i>RMSD from ideal geometry</i>			
Bond lengths (Å)	0.011		0.014
Bond angles (°)	1.175		1.412
<i>Average B-factors (Å²)</i>			
Main chain	30.7		33.1
Side chain	31.9		34.9
Waters	38.6		35.9
Zinc	36.0		
Terbium			33.7

^aNumbers in parentheses refer to the outermost resolution shell.

structure. The RMSD between the 12 subunits ranges from 0.135 Å to 0.191 Å for all atoms. The overall structure is similar to the native (pdb code 1UMN) and the iron-bound structure (pdb code 2BW1) of Dpr with an RMSD in C α atoms of 0.284 Å and 0.230 Å, respectively. No conformational changes were observed except those found in the active site, as discussed below.

The zinc-binding sites

Examination of the electron density map in the ZN1 data revealed clear peaks for zinc atoms. In total, 24 zinc atoms were found at the interfaces of twofold symmetry-related subunits (Fig. 2B) in two different sites (A and B). Site A corresponds to the active (ferroxidase) site of the protein that binds iron (Kauko et al. 2006), while site B is a novel zinc-binding site. The average distance between the two sites A and B is ~ 8.8 Å, and the distance between the B sites is ~ 8.7 Å. The identity of the metals has been confirmed by an anomalous scattering electron density

map that showed peaks at 24 and 16 σ levels for sites A and B, respectively. Native Dpr crystals contain no iron that could interfere with the identity of the zinc ions in these sites (Kauko et al. 2004). No other peaks for bound zinc ions were found elsewhere in the structure.

Site A

Residues Asp74, Glu78, and His47 act as zinc ligands in site A (Fig. 3A). Nearby residues His59 and Asp63 have indirect contacts with zinc through conserved water molecules (Wat2001 and Wat2002, respectively). These water molecules are also present in the native and the iron-bound structures (Kauko et al. 2006). Wat2001, in particular, is conserved within the entire Dps family. Asp74 and Glu78 belong to the same subunit; and His59, Asp63, and His47 are part of the twofold symmetry-related subunit. The average B-factor for zinc ions at site A is 27.8 Å².

A 3.1 Å data set collected near the zinc absorption edge from crystals grown in the presence of 2 mM zinc (data

Figure 2. (A) Ribbon representation of zinc-bound dodecameric Dpr. Each subunit of the dodecamer is illustrated in a different color. (B) View of the zinc-binding sites at the subunit-subunit interface. The position of sites A and B are depicted. (Orange spheres) Zinc ions. Figure was drawn with PyMOL (DeLano Scientific).

set ZN2) showed an extra density close to the zinc-binding site A in the anomalous electron density map that coincided with Wat2001. A zinc atom placed in the electron density was refined to a reasonable distance (3.3 Å) from the first zinc, suggesting that the presence of two zinc ions is structurally feasible. An attempt to refine the 1.8 Å resolution structure with two zinc ions at site A was unsuccessful. The zinc ions were settled at a distance of ~ 2.04 Å from each other after refinement, which is too close for a zinc–zinc distance.

Site B

Site B is formed by residues His40 and His44 (Fig. 3B). Both His residues belong to the same subunit, and no interactions with symmetry-related subunits of the dodecamer are observed. There are two water molecules surrounding zinc at five of the 12 B sites. Six of the remaining sites have only one water molecule as a third

zinc ligand, while no water was found at one site B next to zinc. Asp74 OD1 atom from site A interacts with His44 NE2 through a hydrogen bond (~ 2.5 Å). A second hydrogen-bond interaction involving Asp70 OD1 and His40 ND1 (distance 2.7 Å) also takes place. The B-factor for zinc ions averaged over the 12 B sites is 44.1 Å². Based on the B-factors of the zinc ions and the peak heights in the anomalous map, the occupancy for site B appears to be lower than that of site A.

Structural comparison

Superposition of the native and the iron-bound Dpr structures with the zinc-bound structure showed differences in residue positions at the active site. The site A superimposed onto the native and the iron-bound Dpr structures is shown in Figure 4. A calcium ion identified in the native Dpr structure next to the active site (Kauko

Figure 3. Close-up view of zinc binding at site A (A) and site B (B). The distances (in angstroms) between zinc, protein residues, and water molecules are given. The conserved waters, Wat2001 and Wat2002, in site A are shown.

Figure 4. Structural comparison of the active site in Dpr after superposition of the zinc-bound (ZN), iron-bound (FE), and native structure (NAT). Calcium (Ca) of the native structure, waters (Wat2001 and Wat2002) of the zinc-bound structure, zinc (Zn), and iron (Fe) ions are shown.

et al. 2004, 2006) is not present in the zinc-bound structure as a result of Asp63 side-chain movement. Only in chain A, Asp63 has a double conformation in the zinc-bound structure. No conformational changes were found for both His44 and His40 in site B. In general, the changes of the amino acid positions in the whole molecules are within the range of ~ 0.1 – 0.5 Å.

Sequence comparison

Dpr genes cloned and sequenced from 50 clinical isolates of *S. suis* exhibit an amino acid variation in 25 distinct sites (Pulliainen et al. 2005). This variation relates to almost 15% of the amino acids in Dpr, which is a rather high variation for a protein. However, none of the varying amino acids is considered to have a role in iron incorporation (Pulliainen et al. 2005). Consequently, the amino acids involved in zinc binding at site A (Asp74, Glu78, and His 47) are conserved. Furthermore, nearby amino acids (His59, Asp63, Trp160, Trp48, Met66, and Met161) were not found to be subject to any variation. Similarly, no variation was observed at site B or neighboring residues (His40, His44, Met66, and Leu73). Sequence alignment of *S. suis* Dpr against Dps family members is shown in Figure 5. Of the two His residues that form site B, His44 is highly conserved or is substituted by positively charged residues (Arg or Lys) in *Flavobacterium bacterium*, *Escherichia coli*, and *Deinococcus radiodurans*. Although Lys would be an unusual ligand for zinc, it has been reported to act as such in co-catalytic zinc-binding sites (Vallee and Auld 1993). In contrast to His44, His40 shows less conservation, although many streptococcal species have this His conserved.

Terbium binding in Dpr

The crystal structure of Dpr in the presence of terbium was determined at 2.1 Å resolution (Table 1). A total of 12 terbium ions were found bound at the ferroxidase site. In contrast to the zinc-bound Dpr, no terbium was found at site B. Terbium in site A is surrounded by a Cl-ion and three water molecules at chains A, B, C, D, E, G, I, J, and K. In the rest of the chains (F, H, and L), a fourth water molecule was found in place of the Cl-ion. The Cl-ion was placed at the active sites based on a difference electron density map that showed a peak 8 σ stronger above the average density of a water molecule. An anomalous electron density map showed no extra density close to site A, which excludes the possibility of a second terbium ion at this site.

Discussion

The results presented here provide a structural explanation for the inhibition of iron binding in Dpr and other Dps-like proteins by zinc and terbium. Both ions are able to bind at the ferroxidase center of Dpr, thus preventing iron oxidation. In addition, a second zinc-binding site (site B) was unexpectedly revealed. Both Dpr–zinc and Dpr–terbium crystal structures were determined to high resolution, and anomalous data were used to analyze and verify metal binding.

Zinc is a redox-stable alternative for iron (II) and as such can be used for characterization of iron incorporation,

	40
<i>S. suis</i>	VAHSILHQVH
<i>S. pneumoniae</i>	VAHVALHQVH
<i>S. mutans</i>	KATSIVHQVH
<i>S. mutans</i> (var)	KAASIVHQVH
<i>S. pyogenes</i>	VAAASIVHQVH
<i>S. thermophilus</i>	VAAASIVHQVH
<i>S. gordonii</i>	TAHIALHQVH
<i>S. sanguinis</i>	TAHIALHQVH
<i>S. infantarius</i>	KAASIVHQIH
<i>E. faecalis</i>	QFSVVIHQTH
<i>La. reuteri</i>	QMAMIIHQTH
<i>La. fermentum</i>	QLVMVVHQTH
<i>La. plantarum</i>	QMSMIIHQTH
<i>Li. welshimeri</i>	VFTVKIHQIH
<i>Li. innocua</i>	VFTVKIHQIH
<i>Li. monocytogenes</i>	VFTVKIHQIH
<i>B. brevis</i>	VLYVKLHNPF
<i>B. anthracis</i>	VLFTKLHNPF
<i>B. subtilis</i>	VFTVKIHQIH
<i>H. pylori</i>	VLFMKVHNPF
<i>F. bacterium</i>	LFYMNLRGPF
<i>E. coli</i>	DLSLITKQAH
<i>D. radiodurans</i>	ALQLQTKQAH
	▲ ▲ ●

Figure 5. Sequence alignment of zinc-binding site B residues in Dps family members. Residues 38–47 of *S. suis* Dpr are shown. (Triangles) His40 and His44, (oval) ferroxidase site residue His47. Sequences were obtained after a BLASTP search was carried out (<http://www.ncbi.nlm.nih.gov/blast/>), and were subsequently aligned using CLUSTALW at the European Bioinformatics Institute (<http://www.ebi.ac.uk/Tools/clustalw2/>). Figure drawn with ESPript (Gouet et al. 1999). (*Li.*) *Listeria*, (*La.*) *Lactobacillus*.

especially for identification of transient iron-binding sites or side-chain conformations in intermediates of the ferroxidase reaction. It has been suggested that the reaction mechanism of iron oxidation in the Dps proteins proceeds through a di-iron complex as in other ferroxidase centers (Hempstead et al. 1994; Stillman et al. 2001; Chiancone et al. 2004). Indeed, crystallographic studies in ferritins in the presence of zinc have provided evidence of the di-iron complex formation during the ferroxidase reaction (Stillman et al. 2001; Toussaint et al. 2007). In Dpr, the ZN1 data set showed a single zinc ion in the ferroxidase site. However, a second zinc ion in the active site was suggested by the anomalous data collected to 3.1 Å resolution. The presence of the additional zinc could be explained by the higher zinc concentration (2 mM zinc) used in co-crystallization. Refinement with two zinc ions resulted in a zinc–zinc distance of ~3.3 Å. In other proteins, as for example in GAL4, the zinc–zinc distance is 3.5 Å (Vallee et al. 1991); in yeast PPR1 it is 3.16 Å (Ball et al. 1995); in human apoferritin H-chain the distance is 3.32 Å (Ball et al. 1995); and in *E. coli* ferritin, 3.43 Å (Stillman et al. 2001). The additional zinc corresponds to the conserved Wat2001 in the Dps family members. Notably, the distance between Wat2001 and iron in the Dps family has been measured to be nearly 1 Å longer than the expected ideal distance. Thus, the position of Wat2001 could actually correspond to an iron ion (Ilari et al. 2000). In accordance with the presence of a di-iron center, two iron atoms have been modeled in *B. brevis* Dps structure (Ren et al. 2003). However, crystallographic studies have shown only a single iron ion in the ferroxidase center of Dpr and *D. radiodurans* Dps after soaking in reducing conditions (Kauko et al. 2006; Romao et al. 2006). In *H. pylori* NAP, an anion has been modeled in the place of the conserved water (Zanotti et al. 2002). Examination and refinement of the Dpr–zinc complex at 1.8 Å provided no indication of a second zinc ion at site A. Thus, Wat2001 appears to be a real water molecule and not a zinc ion in the 1.8 Å structure. The final refined distance between the water and zinc in the Dpr–zinc structure is 2.00 Å, which is close to the expected distance (2.09 Å) reported in the literature (Harding 2001). Nevertheless, further crystallographic studies may provide clear evidence for the presence of a di-iron center in the Dps family of proteins. Due to the crystallization conditions and difficulties encountered with precipitation of zinc, other metals could be more suitable for analogous studies.

Site B residue His44 is widely conserved within the Dps protein family, in contrast to His40. His or Lys residues are found in many Dps members at positions equivalent to His44, while *F. bacterium* has arginine at the same location (Fig. 5). A positively charged residue at this location has been proposed to play a role in maintaining the iron-binding site by interacting with Asp74

(Kauko et al. 2006). On the other hand, the variability of His40 might indicate a lower importance of site B amongst different bacteria. Other His40-substituting residues, such as threonine (*S. mutans*, *Li. innocua*, *Listeria monocytogenes*, *Listeria welschimeri*), serine (*Enterococcus faecalis*, *Lactobacillus plantarum*, *E. coli*) or Tyr (*B. brevis*) could act as alternative zinc-binding residues (Harding 2001). Interestingly, variants of *S. mutans* Dpr have been identified with an Ala instead of Thr. This could explain previous reports on the ability of *S. mutans* Dpr to bind 11.2 zinc atoms per dodecamer (Yamamoto et al. 2002), presumably by the ferroxidase center residues as shown in the current study. Homologs of *S. suis* Dpr possessing both His40 and His44 include *Streptococcus pneumoniae* Dpr, *Streptococcus gordinii* Dpr, and *Streptococcus sanguinis* Dpr. Further studies might bring interesting new features on the role of zinc in these pathogens.

The hydrogen bond between Asp74 and His44 suggests communication between His44 and the ferroxidase center that might have potential biological significance. This is further supported by the high conservation of His44 in Dps proteins amongst different bacteria. Hence, iron may utilize His44 on its way to the ferroxidase center. Although iron and zinc compete for site A, no iron has been found in crystallographic studies after iron (II) soaking under reduced conditions (Kauko et al. 2006). This observation, however, does not exclude the possibility that iron (II) can still use His44 as a ligand. Site-directed mutagenesis could provide more information about the role of His44 in Dpr and its effect on the ferroxidase reaction.

Zinc is toxic at high levels, and the possibility that Dpr, in addition to its role as iron scavenger, is also involved in zinc removal from the cytoplasm cannot be ruled out. Ferritins, for example, have been found to act as zinc detoxification agents and to confer resistance to zinc overload (Price and Joshi 1982; Dundon et al. 2001). Even though binding of zinc to the ferroxidase site could prevent iron oxidation, protection from high concentrations of zinc may be more important for the bacteria at these extreme conditions. Preliminary data (A. Salminen, A.T. Pulliainen, S. Haataja, A.C. Papageorgiou, and J. Finne, unpubl.) have indicated higher zinc toxicity in *S. suis* Dpr-deficient bacteria compared with the wild-type ones. In contrast, recombinant HP-NAP has been found to bestow no resistance to zinc when expressed in *E. coli* (Dundon et al. 2001). This is in agreement with the lack of the novel zinc-binding site in HP-NAP as deduced from Figure 5.

Furthermore, ferritins have been suggested to act as zinc donors to zinc-requiring enzymes (Price and Joshi 1982). Interestingly, *Bacillus subtilis* has been found to resist high concentrations of H₂O₂ more effectively in the

presence of elevated zinc concentrations (Gaballa and Helmann 2002). Transcription factors PerR and BosR related to oxidative stress and involved in regulating the expression of the *Dpr* homolog MrgA have been found to require zinc ions for effective regulation activities (Chen and Helmann 1995; Boylan et al. 2003; Traore et al. 2006). The captured zinc ions in Dpr could be transferred to zinc-binding enzymes involved in the defense mechanisms against oxidative stress in *S. suis*. Further investigations are required to verify this hypothesis and to determine the relative affinities of the participating proteins to bind and transfer zinc.

It has been suggested that zinc and terbium share different binding sites in *Li. innocua*. Based on the Dpr–zinc structure, the lack of competition between zinc and terbium can now be explained. Although *Li. innocua* Dps lacks one of the His residues acting as a zinc ligand (His40 in Dpr), the presence of a Thr residue in this position could still provide a suitable site for zinc binding. In this case, terbium and zinc could occupy the ferroxidase center and the zinc-binding site B, respectively, in competition assays.

A novel zinc-binding site has recently been identified in *D. radiodurans* (Romao et al. 2006) close to the N-terminal of the protein. It consists of two histidines, as well as an aspartate and a glutamate, and either participates in DNA interactions or acts as a metal sensor. This site, however, is unique to *D. radiodurans* and does not coincide with the zinc-binding site B of *S. suis*.

Conclusions

The use of different metals for structural studies in the Dps family of proteins can provide information regarding the reaction mechanism and new metal-binding sites. We have shown here that zinc and terbium inhibit the ferroxidase activity of Dpr by binding at the ferroxidase site of the protein. In addition, a hitherto unknown zinc-binding site in Dpr was identified. Based on amino acid sequence comparisons, this site is also present in other streptococcal Dpr proteins and in several Dps family members. A potential role of Dpr and other Dps proteins in zinc removal from the cytoplasm at elevated zinc concentrations is suggested.

Materials and Methods

Inhibition assay

Dpr (5 µg) (Haataja et al. 2002) in 5 mM Tris, pH 7.5, 0.05 M NaCl was incubated in the presence of 0.0–0.5 mM zinc chloride or 0.0–0.2 mM terbium chloride for 30 min at room temperature prior to the addition of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \times 6\text{H}_2\text{O}$ to a final concentration of 0.5 mM. The total volume of the mixture was 20 µL. The reactions were stopped after 40 min by the addition

of native gel sample buffer. The proteins were resolved with 7.5% native-PAGE and the incorporated iron was stained with Prussian blue as previously described (Kauko et al. 2004). The equal loading of the protein into each line was verified by subsequent staining of the gels with Coomassie blue. Possible interference in the assay from nonincorporated iron is unlikely because (1) the protein contains no iron (from crystallographic data), and (2) no iron staining has been detected in control protein samples where no iron was added (Kauko et al. 2004).

Protein crystallization

Crystals were grown by the vapor diffusion hanging-drop method using a well solution containing 27%–35% PEG 400, 0.2 M CaCl_2 , and 0.1 M HEPES–NaOH pH 7.4. The protein was mixed with a zinc or terbium solution prior to crystallization. The hanging drop contained 2 µL of protein–zinc/terbium solution (10 mg/mL protein solution and 1 mM or 2 mM ZnCl_2 /1 mM TbCl_3) and 2 µL of mother liquor. Data were collected from crystals grown in the presence of 1 mM and 2 mM zinc solution as well as from crystals grown in the presence of 1 mM terbium solution. Thus, the zinc samples in the text are referred to as ZN1 and ZN2, respectively. Precipitation problems were encountered at higher zinc concentrations; thus, no crystals were able to grow for crystallographic binding studies.

Data collection

The diffraction data for ZN1 and terbium crystals were collected at Max-laboratory in Lund on beamline I711. The data for ZN2 were collected at EMBL–Hamburg outstation on beamline BW7A. Most crystals grown in the presence of 2 mM zinc chloride diffracted to low resolution or showed disorder in their diffraction pattern, most likely because of protein precipitation problems; therefore, they were not useful for structural studies, and only a 3.1 Å data set was collected. A MAR CCD imaging plate detector was used in all the experiments, and XDS (Kabsch 1993) was employed for data processing. Data processing statistics for all crystals are shown in Table 1. The zinc existence in the ZN1 sample was confirmed by a fluorescence spectrum that showed a zinc absorption peak at the expected wavelength. A wavelength of 1.2824 Å near the zinc absorption edge (1.2837 Å) was used for data collection from the ZN2 sample ($\Delta f' = 3.9$). The higher redundancy and the chosen wavelength enabled the calculation of an anomalous map in the ZN2 data set to facilitate the analysis of the zinc binding and verification of the bound ions. A strong anomalous signal was detected in the terbium data at 1.0627 Å ($\Delta f'' = 8.1$).

Structure determination and refinement

Initial phases were obtained using the native Dpr structure (Kauko et al. 2004). The absence of iron ions in the native Dpr structure facilitated the structural analysis of the zinc and terbium binding. The ZN1 data set was used for structure determination and refinement due to its higher resolution. The lower-resolution ZN2 data set was partially refined and used for anomalous signal calculations. Refinement was performed with REFMAC 6.0.0 (Murshudov et al. 1997) included in the CCP4i 1.4.4 software (Potterton et al. 2003). The structures were visualized and rebuilt using COOT 0.0.33 (Emsley and Cowtan 2004). Water molecules were added using COOT during

REFMAC refinement cycles and inspected in the graphics. Water molecules with B-factors $>50 \text{ \AA}^2$ after a round of refinement were deleted. The progress of the refinement was monitored using 5% of the reflections excluded from the refinement for R_{free} calculations.

Data deposition

Atomic coordinates and structure factors have been deposited with the Protein Data Bank under the accession codes 2UX1 (Dpr-Zn) and 2V15 (Dpr-Tb).

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