A novel bacterial class V dye-decolourizing peroxidase from the extremophile *Deinococcus radiodurans*: cloning, expression optimization, purification, crystallization, initial characterization and X-ray diffraction analysis

Kelly Stefany Tuna Frade,^a Andreia Cecília Pimenta Fernandes,^a Celia Marisa Silveira,^b Carlos Frazão^a and Elin Moe^{a*}

Received 1 March 2018

Accepted 8 June 2018

Edited by N. Sträter, University of Leipzig, Germany

Keywords: peroxidases; oxidative stress; radiation resistance; dye-decolourizing peroxidases; *Deinococcus radiodurans*.

^aMacromolecular Crystallography Unit, Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa (ITQB-NOVA), Avenida da República – EAN, 2780-157 Oeiras, Portugal, and ^bRaman BioSpectroscopy Laboratory, Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa (ITQB-NOVA), Avenida da República – EAN, 2780-157 Oeiras, Portugal. *Correspondence e-mail: elinmoe@itqb.unl.pt

Deinococcus radiodurans is a bacterium with extreme resistance to desiccation and radiation. The resistance mechanism is unknown, but an efficient reactive oxygen species (ROS) scavenging system and DNA-repair and DNA-protection mechanisms are believed to play important roles. Here, the cloning and small- and medium-scale expression tests of a novel dye-decolourizing peroxidase from *D. radiodurans* (*DrDyP*) using three different *Escherichia coli* strains and three different temperatures in order to identify the optimum conditions for the expression of recombinant *DrDyP* are presented. The best expression conditions were used for large-scale expression and yielded ~10 mg recombinant *DrDyP* per litre of culture after purification. Initial characterization experiments demonstrated unusual features with regard to the haem spin state, which motivated the crystallization experiment. The obtained crystals were used for data collection and diffracted to 2.2 Å resolution. The crystals belonged to the trigonal space group $P3_1$ or $P3_2$, with unit-cell parameters $a = b = 64.13$, $c = 111.32$ Å, and are predicted to contain one *DrDyP* molecule per asymmetric unit. Structure determination by molecular replacement using previously determined structures of dye-decolourizing peroxidases with ~30% sequence identity at ~2 Å resolution as templates are ongoing.

1. Introduction

Deinococcus radiodurans is a bacterium that has a remarkable capacity to overcome oxidative stress (Battista, 1997). Oxidative stress is caused by reactive oxygen species (ROS), which can damage cellular macromolecules (Slade & Radman, 2011). When oxidative modifications occur in humans, they can induce cancer, neurodegenerative diseases and premature aging (Birben *et al.*, 2012; Cui *et al.*, 2012). Given that *D. radiodurans* displays an outstanding resistance to oxidative stress and that reactive oxygen species are associated with these disorders, a comprehensive study of the strategies used by *D. radiodurans* to combat oxidative stress may open novel opportunities for anti-aging and anticancer treatments and for the treatment of degenerative disorders (Gerber *et al.*, 2015; Slade & Radman, 2011).

To date, the mechanism behind the resistance of *D. radiodurans* to oxidative stress remains unclear, but it is believed to be caused by the combination of an efficient DNA-repair process and an antioxidant defence system, which enables proteins to retain catalytic activity and provides a swift

Table 1

Macromolecule production.

In the primers, the nucleotides encoding the His tag are underlined, the TEV site nucleotides are in italics and the *attB1* and *attB2* sites (for recombination into the cloning vector) are in bold. The lowercase letters indicate the gene-encoding nucleotides.

Source organism	<i>D. radiodurans</i>
DNA source	<i>D. radiodurans</i>
Forward primer 1 (5'–3')	CATCACCATCACCATCACG <u>AAAACTGTATTCCA</u> <i>GGGAGCAatgacgctgttcaaaaagctg</i>
Reverse primer (5'–3')	GGGGACCACCTTTGTACAAGAAAGCTGGGTC <i>ttact</i> <i>tcagttcactcagcca</i>
Forward primer 2 (5'–3')	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAG GAGATAGAACCATG <u>CATCACCATCACCATCAC</u>
Cloning vector	pDONR221
Expression vector	pDEST14
Expression host	<i>E. coli</i> BL21(DE3)*, BL21(DE3)pLysS and BL21(DE3)*pRARE
Complete amino-acid sequence of the construct produced after TEV cleavage	GAMTLFKKLRELHVHNDKIDLDLDDIQTALVLRER EPYYGTHAMVRFDTAEGGRELLKLLPHIASAEK WWDVKYAWTAAAIISYGLKKGVPQDSDLSDFPE SPKVMAGRAEHLFDVGENDPKHWEKPFPGTGQV HLALTFAENEENWQKALVIAEHELEATKGVTL LMREDFGAQPDNRSLGYKDMISNPAIEGSGIK PFPQGGPAIKPGEFVLGYPGAGVPLGMPKPEV LGKNGTFVALRKYHTNAGSFNRYLKENAETGG DAELLAALKLVGRWSGAPLTLPKEDDEPGLHD PNRNNDFTYKNDPEGLEVPLGSHIRRMNPRDTK LELLTVDNIHRIIRATAYGPAYDPKADSLAED KVERGLYFIFISAKAMDTTEFLQKEWINKANFI GGSERDPIVGLQDEDLTFLPKEPVRQRLRGM DTFNVLRGGEYLFMPSLSALKWLSLK
Theoretical molecular mass (kDa)	51.2

response under stress conditions (Slade & Radman, 2011). The genome sequence of *D. radiodurans* shows that it possesses an expanded repertoire of genes encoding cellular antioxidant protection proteins (Makarova *et al.*, 2001). It has three catalases (the *katE* catalases DR1998 and DRA0259, and the eukaryotic-type catalase DRA0146), four superoxide dismutases (the manganese-dependent superoxide dismutase DR1279, and three copper/zinc-dependent superoxide dismutases DR1546, DRA0202 and DR0644) and two peroxidases (the cytochrome *c* peroxidase DRA0301 and an iron-dependent peroxidase, the putative dye-decolourizing peroxidase DRA0145). It also possesses two DNA-binding proteins from starved cells (Dps) proteins Dps1 (DR2263) and Dps2 (DRB0092) (Romão *et al.*, 2006; Cuypers *et al.*, 2007).

Peroxidases are oxidoreductases that catalyze the oxidation of substrate molecules using hydrogen peroxide as an electron acceptor (Smulevich *et al.*, 2010). The vast majority of peroxidases, although phylogenetically unrelated, contain a haem cofactor. They are present in both the eukaryotic and prokaryotic kingdoms and are classified into two superfamilies: the animal peroxidase and plant peroxidase superfamilies (Rahmanpour & Bugg, 2015). The DyP-type peroxidases are a newly identified superfamily of peroxidases which are unrelated to general peroxidases (Sugano *et al.*, 2007), showing structural divergence and low sequence similarity with disparity in the haem pocket (which possesses a distal aspartic acid instead of histidine). DyP-type peroxidases also show different reaction characteristics compared with

other peroxidases, such as low optimal pH values for activity and high affinity for structurally different substrates, including synthetic dyes (for example anthraquinone and azo dyes), β -carotene, aromatic sulfides, phenolic or nonphenolic lignin-compound units and metal ions (for example Mn^{2+}) (Salva-chúa *et al.*, 2013; Sezer *et al.*, 2013).

Initially, the DyP-type peroxidases were classified into four subfamilies, A, B, C and D, based on primary sequence. The bacterial enzymes were grouped into subfamilies A, B and C, and the fungal enzymes into subfamily D (Rahmanpour & Bugg, 2015). Subsequently, a reclassification into the classes P (primitive), I (intermediate) and V (advanced) was proposed based on tertiary structure (Yoshida & Sugano, 2015). Here, class P consists of the former class B, class I of the former class A and class V of the previous classes C and D.

Here, we present the expression optimization, purification, crystallization and initial characterization and X-ray diffraction analysis of a putative dye-decolourizing peroxidase (DRA0145) from *D. radiodurans* (*DrDyP*). Based on the initial classification and the PeroxiBase (Fawal *et al.*, 2013), *DrDyP* belongs to the largely unexplored class C DyPs, which are organized within class V of the new categorization. Here, we will use the latter nomenclature when describing *DrDyP*. Our initial characterization experiments indicate unusual features of *DrDyP* arising from the presence of a low-spin haem state, suggesting that it lacks peroxidase activity at pH 7.5, which has not been observed previously for other DyPs. The structure determination of *DrDyP* could provide valuable information to explain these properties and provide further insight into this class of DyPs and their potential role in the mechanism of oxidative-stress resistance by *D. radiodurans*.

2. Materials and methods

2.1. Macromolecule production

The gene encoding a three-amino-acid N-terminally truncated version ($\Delta 3$) of DR_A0145 (accession code Q9RZ08), hereafter called *DrDyP*, from *D. radiodurans* was cloned into the pDEST14 expression vector from the Gateway system (GE Healthcare), as previously performed for endonuclease III (Sarre *et al.*, 2014), using the primers described in Table 1. The primers contain nucleotides encoding an N-terminal His tag followed by a TEV cleavage site upstream of the gene encoding the protein. Insertion of the gene into the correct reading frame of the pDEST14 vector was confirmed using the GATC Biotech Sanger sequencing service (GATC Biotech).

The expression plasmid containing the gene encoding *DrDyP* was transformed into three different *Escherichia coli* expression strains, BL21(DE3)*, BL21(DE3)pLysS and BL21(DE3)*pRARE, by heat shock. One transformant of each strain was used to inoculate 5 ml LB medium with ampicillin (200 $\mu\text{g ml}^{-1}$) and, in the case of pLysS, chloramphenicol (34 $\mu\text{g ml}^{-1}$), and was grown at 37°C until an OD₆₀₀ of ~0.6 was attained. At this point gene expression was induced by the addition of 0.5 mM IPTG. After 3 h of induction, the cells were harvested and dissolved in 200 μl extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM

Table 2
Crystallization conditions.

Method	Robot	Manual (hanging drop)
Plate type	Sitting-drop MDL3 plates	XRL hanging-drop 24-well MD3-11 plates
Temperature (K)	293.15	293.15
Protein concentration (mg ml ⁻¹)	9.10	9.10
Buffer composition of protein solution	50 mM Tris-HCl pH 7.5, 150 mM NaCl	50 mM Tris-HCl pH 7.5, 150 mM NaCl
Composition of reservoir solution	0.1 M CHES pH 9.5, 20% PEG 8000	0.1 M CHES pH 8.5, 18% PEG 8000
Volume and ratio of drop	0.2 µl, 1:1	2 µl, 1:1
Volume of reservoir (µl)	40	500

MgCl₂, 0.1 mg ml⁻¹ lysozyme, 1 µg ml⁻¹ DNase), followed by three freeze–thaw cycles in liquid nitrogen and a water bath at 22°C. The resulting extract was centrifuged at 13 200 rev min⁻¹ in a tabletop centrifuge at 4°C and the supernatant (soluble fraction) was collected for SDS–PAGE analysis. The resulting pellet was dissolved in 50 µl 1% SDS and heated at 95°C for 5 min followed by centrifugation (insoluble fraction). The supernatant (15 µl) and the dissolved pellet (5 µl) were then loaded onto an SDS–PAGE gel for analysis after adding 5 µl SDS–PAGE sample-loading buffer [0.5 M Tris–HCl pH 6.8, 0.5% (w/v) bromophenol blue, 10% (w/v) SDS, 50% glycerol, 50 mM β-mercaptoethanol] to each and were heated to 95°C for 5 min. A non-induced expression culture of *E. coli* BL21(DE3)* cells was also included as a negative control, and samples from these cultures were treated the same as the cultures with induced expression.

One of the strains that overexpressed DrDyP was BL21(DE3)*, and this strain was used in a temperature-optimization expression test in order to determine the temperature at which the strain expressed the most protein. Three 1 ml pre-cultures of the strain were used to inoculate three 50 ml cultures of LB medium with 200 µg ml⁻¹ ampicillin at 18, 25 and 37°C. The cells were grown to an OD₆₀₀ of ~0.6 and expression was induced by the addition of 0.5 mM IPTG as above. The length of induction varied depending on the growth temperature and lasted for 18, 6 and 3 h at 18, 25 and 37°C, respectively. After induction, the cells were harvested and the pellet was dissolved in 1 ml extraction buffer. Next, the samples were subjected to three freeze–thaw cycles as described for the small-scale expression test. The resulting extract was centrifuged at 13 200 rev min⁻¹ in a tabletop centrifuge at 4°C and the supernatant (soluble fraction) was collected for SDS–PAGE analysis. The resulting pellet was dissolved in 250 µl 1% SDS and heated at 95°C for 5 min followed by centrifugation (insoluble fraction). The supernatant (15 µl) and the dissolved pellet (5 µl) were then loaded onto an SDS–PAGE gel for analysis after adding 5 µl SDS–PAGE sample-loading buffer each and heated to 95°C for 5 min. A fourth culture with non-induced expression was also grown at 37°C and acted as a negative control.

Finally, DrDyP was expressed on a large scale in 1 l LB medium with 200 µg ml⁻¹ ampicillin in *E. coli* BL21(DE3)* cells at 37°C. The cells were harvested after 3 h of induction by the addition of 0.5 mM IPTG. The pellet was dissolved in 20 ml extraction buffer followed by four freeze–thaw cycles in

liquid nitrogen and a water bath at 22°C. The resulting extract was centrifuged at 18 000 rev min⁻¹ at 4°C and the soluble fractions were applied onto a 1 ml HisTrap column (GE Healthcare) equilibrated with buffer A (50 mM Tris–HCl pH 7.5, 150 mM NaCl). The resin was washed with ten column volumes (CV) of buffer A and 10 CV of 5% buffer B (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 500 mM imidazole). The protein was eluted with a gradient from 5 to 100% buffer B over 20 CV. The red/brown-coloured fractions containing DrDyP were pooled and dialyzed overnight (4°C) into buffer A to remove imidazole. His-tagged TEV protease (Kapust *et al.*, 2001) was added to the sample in a 1:20 ratio before dialysis in order to facilitate cleavage of the His tag. After dialysis, the protein solution was applied onto a second 1 ml HisTrap column in order to recover the protein without the His tag from the flowthrough. The pure red-coloured protein, with a molecular mass of 51.2 kDa after removal of the His tag, was concentrated to 9.1 mg ml⁻¹ and stored at 4°C. The purity was analysed by SDS–PAGE and was estimated to be greater than 95%.

The iron content in the sample was determined by inductively coupled plasma atomic emission spectrometry (ICP–AES) using an Ultima spectrometer from Horiba Jobin-Yvon.

Electronic absorption measurements were recorded at 18°C using a Shimadzu UV-1800 spectrophotometer. The protein concentration was ~4 µM in 40 mM Britton–Robinson buffer solutions pH 3.0, 4.0, 5.0 and 7.5.

2.2. Crystallization

The purified protein (9.1 mg ml⁻¹) in 50 mM Tris–HCl pH 7.5, 150 mM NaCl was used in sitting-drop crystallization experiments on a Honeybee Cartesian (Genomic Systems) crystallization robot with MDL3 plates (100 nl protein solution and 40 µl reservoir solution) and the JCSG+ crystallization screen (Molecular Dimensions). Small and fragile red crystals appeared in condition A7 with a reservoir solution consisting of 0.1 M CHES pH 9.5, 20% PEG 8000. With the purpose of improving the crystallization condition, hanging-drop optimization experiments were performed manually by varying the concentration of PEG 8000 from 18 to 23% (w/v) and the pH between 8.5 and 10. The best-looking crystals from the optimization experiment appeared in 18% PEG 8000, 0.1 M CHES pH 8.5. The crystallization conditions for the crystals used for diffraction data collection are described in Table 2.

2.3. Data collection and processing

The crystals from the JSCG+ screen were briefly soaked in 20% PEG 8000, 0.1 M CHES pH 9.5, 25% glycerol and the optimized crystals were soaked in 18% PEG 8000, 0.1 M CHES pH 8.5, 25% glycerol before being flash-cooled in liquid nitrogen and used for data collection on beamline ID30B at the European Synchrotron Facility (ESRF) for the crystals from the JCSG+ screen and beamline I03 at Diamond Light Source (DLS) for the optimized crystals. A crystal from the screen diffracted to 2.8 Å resolution, while an optimized crystal diffracted to 2.2 Å resolution. Here, we present the results for the best diffracting crystal. A total of 1420 images were collected using an oscillation width of 0.1° and the data were processed with *XDS* (Kabsch, 2010). The data-collection parameters and processing statistics for the optimized crystal are listed in Table 3.

3. Results and discussion

The gene encoding *DrDyP* was inserted into the pDEST14 expression vector containing nucleotides encoding an N-terminal His tag and TEV cleavage site and used for small-scale test expression (5 ml cultures) in three different *E. coli* strains [BL21(DE3)*, BL21(DE3)pLysS and BL21(DE3)*pRARE] at 37°C. *DrDyP* was overexpressed in soluble form in *E. coli* BL21(DE3)* and BL21(DE3)pLysS cells, and migrated as a single band at ~50 kDa on a 12% SDS–PAGE gel, as expected from theoretical calculations (the theoretical mass is 53.2 kDa with the His and TEV tags); it could not be identified in the non-induced control cells [BL21(DE3)*] or in BL21(DE3)*pRARE (Fig. 1*a*). BL21(DE3)* was selected for medium-scale test expressions at three different temperatures (18, 25 and 37°C) and three different induction times (18, 6 and 3 h, respectively). A high yield of overexpressed protein

Table 3
Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	I03, DLS
Wavelength (Å)	0.97625
Temperature (K)	100
Detector	PILATUS3 6M
Crystal-to-detector distance (mm)	384.54
Rotation range per image (°)	0.10
Total rotation range (°)	142
Exposure time per image (s)	0.102
Space group	<i>P</i> ₃ ₁ or <i>P</i> ₃ ₂
<i>a</i> , <i>b</i> , <i>c</i> (Å)	64.13, 64.13, 111.32
Mosaicity (°)	0.117
Resolution range (Å)	55.54–2.20 (2.33–2.20)
Total No. of reflections	104559 (16905)
No. of unique reflections	25843 (4160)
Completeness (%)	99.5 (99.1)
Multiplicity	4.0 (4.1)
<i>I</i> / <i>σ</i> (<i>I</i>)†	11.0 (1.0)
<i>R</i> _{meas} (%)	7.6 (124.4)
Overall <i>B</i> factor from Wilson plot (Å ²)	62

† Diffraction images were processed to 2.20 Å resolution, where CC_{1/2} reaches 50.1% and they still contain significant data according to the authors of the data-processing program (Karplus & Diederichs, 2012). *I*/*σ*(*I*) falls below 2.0 at 2.4 Å resolution.

at ~50 kDa was identified in all conditions and expression at 37°C for 3 h was chosen for the large-scale experiment (Fig. 1*b*).

After large-scale expression, the protein was purified using two HisTrap steps with a dialysis and TEV cleavage step between them. The protein solution had a red colouration, as expected owing to the presence of the haem group. Above pH 4.0 the UV–Vis spectra of *DrDyP* displayed Soret bands around 416 nm and Q-bands in the range 500–580 nm, which are consistent with a low-spin haem iron (Fig. 2). This is very unusual for DyPs (and other peroxidases), which typically display heterogeneous spin populations mainly comprising

Figure 1
Result of small- and medium-scale expression testing and purification. Protein bands showing overexpressed *DrDyP* are circled. (a) 12% SDS–PAGE gels from small-scale expression in *E. coli* BL21(DE3)*, BL21(DE3)pLysS and BL21(DE3)*pRARE. S and I indicate soluble and insoluble fractions, respectively. (b) 12% SDS–PAGE gels from medium-scale expression in *E. coli* BL21(DE3)* at 18, 25 and 37°C. (c) Purified *DrDyP* after concentration.

high-spin haems, with the sixth coordination site either vacant for substrate binding or weakly bound to water (Smulevich *et al.*, 2010). The high abundance of low-spin haem iron may indicate poor peroxidase activity, as previously observed for other DyPs (Sezer *et al.*, 2013). Upon lowering the solution pH below 4.0, additional features were observed in the spectrum. The appearance of a 639 nm charge-transfer band in particular suggested the presence of a mixture of high-spin and low-spin states and could indicate that the protein is only activated at low pH.

Large-scale expression of *DrDyP* was also performed in the presence of haemin, as this has been used as a supplement to growth media for the recombinant expression of some DyPs to obtain 100% incorporation of haem (Fernández-Fueyo *et al.*, 2015; Santos *et al.*, 2014). Here, the addition of haemin had a negative effect on the growth of the cells and resulted in a low yield of recombinant protein, and thus it was not used as a supplement to the growth medium for the expression of

DrDyP. Quantification of the iron content in recombinant *DrDyP* also confirmed that the addition of haemin was not needed since it is mostly produced in the holoprotein form (0.99 ± 0.07 moles of iron per mole of protein) in LB medium. The Reinheitszahl value (A_{416}/A_{280} ratio) was greater than 1.0, indicating good purity.

The purified *DrDyP* was used to screen crystallization conditions using the JCSG+ crystallization suite and a Cartesian Mini-Bee crystallization robot. Red-coloured crystals appeared in condition A7 [20% (w/v) PEG 8000, 0.1 M CHES pH 9.5] after two months (Fig. 3a). The crystals were further optimized by hanging-drop vapour diffusion (manually), and after two weeks crystals that were suitable for data collection were obtained in 18% (w/v) PEG 8000, 0.1 M CHES pH 8.5 (Fig. 3b). The crystals were flash-cooled in crystallization buffer with 25% glycerol and were used for data collection on beamline ID30B at the ESRF to a resolution of 2.8 Å for the crystals from JCSG+ screen condition A7 and on beamline I03 at DLS to a resolution of 2.2 Å for the optimized crystals. The data-collection statistics for the optimized crystal are shown in Table 3.

Preliminary X-ray diffraction analysis showed that the *DrDyP* crystals belonged to space group $P3_1$ or $P3_2$, with unit-cell parameters $a = b = 64.46$, $c = 111.82$ Å. Unit-cell volume considerations (Matthews, 1968; Kantardjieff & Rupp, 2003) indicated the presence of one *DrDyP* monomer in the asymmetric unit, with a calculated V_M of $2.61 \text{ Å}^3 \text{ Da}^{-1}$ and an estimated solvent content of 52%.

The crystal structures of DyPs from many organisms are already known (Rahmanpour & Bugg, 2015), but those with the highest sequence identity to *DrDyP* (~30%) are from an *Anabaena* sp. (Yoshida *et al.*, 2016), an *Amycolatopsis* sp. (Brown *et al.*, 2012), *Bjerkandera adusta* (Yoshida *et al.*, 2011) and *Auricularia auricula-judae* (Strittmatter *et al.*, 2013). Attempts to solve the structure of *DrDyP* using these structures as templates for molecular replacement is ongoing. The crystal structure of *DrDyP* from *D. radiodurans* will provide crucial information regarding the observed unusual features of the spin state of the haem and the possible lack of activity at high pH, and thus provide novel information about the members of the class V DyPs.

Figure 2
UV-Vis spectra of *DrDyP* at different pH values. From top to bottom: pH 3.0 (red), 4.0 (green), 5.0 (blue) and 7.5 (black). The inset shows the magnified 450–670 nm region. The protein concentration was $\sim 4 \mu\text{M}$.

Figure 3
Crystals obtained in (a) condition D7 of the JCSG+ crystallization screen (20% PEG 8000, 0.1 M CHES pH 9.5) and (b) a hanging-drop vapour-diffusion optimization experiment in 18% PEG 8000, 0.1 M CHES pH 8.5 (full size and enlarged view). (c) The crystal ($\sim 100 \mu\text{m}$) used for data collection on beamline I03 at DLS.

Acknowledgements

Beamtime at ID30B at the ESRF and I03 at DLS and assistance from the beamline staff is gratefully acknowledged.

Funding information

This work was financially supported by Project LISBOA-01-0145-FEDER-007660 (Microbiologia Molecular, Estrutural e Celular) funded by FEDER funds through COMPETE2020 – Programa Operacional Competitividade e Internacionalização (POCI), the European Community's Seventh Framework Program (FP7/2007-2013) under Grant Agreement 283570 (BioStruct-X) and by national funds (PTDC/BBB-BEP/0561/2014) and postdoctoral fellowships SFRH/BPD/94050/2013 and SFRH/BPD/79566/2011 to EM and CMS through FCT – Fundação para a Ciência e a Tecnologia.

References

- Battista, J. R. (1997). *Annu. Rev. Microbiol.* **51**, 203–224.
- Birben, E., Sahiner, U. M., Sackesen, C., Erzurum, S. & Kalayci, O. (2012). *World Allergy Organ. J.* **5**, 9–19.
- Brown, M. E., Barros, T. & Chang, M. C. Y. (2012). *ACS Chem. Biol.* **7**, 2074–2081.
- Cui, H., Kong, Y. & Zhang, H. (2012). *J. Signal. Transduct.* **2012**, 1–13.
- Cuyper, M. G., Mitchell, E. P., Romão, C. V. & McSweeney, S. M. (2007). *J. Mol. Biol.* **371**, 787–799.
- Fawal, N., Li, Q., Savelli, B., Brette, M., Passaia, G., Fabre, M., Mathé, C. & Dunand, C. (2013). *Nucleic Acids Res.* **41**, D441–D444.
- Fernández-Fueyo, E., Linde, D., Almendral, D., López-Lucendo, M. F., Ruiz-Dueñas, F. J. & Martínez, A. T. (2015). *Appl. Microbiol. Biotechnol.* **99**, 8927–8942.
- Gerber, E., Bernard, R., Castang, S., Chabot, N., Coze, F., Dreux-Zigha, A., Hauser, E., Hivin, P., Joseph, P., Lazarelli, C., Letellier, G., Olive, J. & Leonetti, J.-P. (2015). *J. Appl. Microbiol.* **119**, 1–10.
- Kabsch, W. (2010). *Acta Cryst.* **D66**, 125–132.
- Kantardjieff, K. A. & Rupp, B. (2003). *Protein Sci.* **12**, 1865–1871.
- Kapust, R. B., Tözsér, J., Fox, J. D., Anderson, D. E., Cherry, S., Copeland, T. D. & Waugh, D. S. (2001). *Protein Eng.* **14**, 993–1000.
- Karplus, P. A. & Diederichs, K. (2012). *Science*, **336**, 1030–1033.
- Makarova, K. S., Aravind, L., Wolf, Y. I., Tatusov, R. L., Minton, K. W., Koonin, E. V. & Daly, M. J. (2001). *Microbiol. Mol. Biol. Rev.* **65**, 44–79.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Rahmanpour, R. & Bugg, T. D. H. (2015). *Heme Peroxidases*, edited by E. Raven & B. Dunford, pp. 334–357. Cambridge: Royal Society of Chemistry.
- Romão, C. V., Mitchell, E. P. & McSweeney, S. (2006). *J. Biol. Inorg. Chem.* **11**, 891–902.
- Salvachúa, D., Prieto, A., Martínez, Á. T. & Martínez, M. J. (2013). *Appl. Environ. Microbiol.* **79**, 4316–4324.
- Santos, A., Mendes, S., Brissos, V. & Martins, L. O. (2014). *Appl. Microbiol. Biotechnol.* **98**, 2053–2065.
- Sarre, A., Ökvist, M., Klar, T., Moe, E. & Timmins, J. (2014). *Acta Cryst.* **F70**, 1688–1692.
- Sezer, M., Santos, A., Kielb, P., Pinto, T., Martins, L. O. & Todorovic, S. (2013). *Biochemistry*, **52**, 3074–3084.
- Slade, D. & Radman, M. (2011). *Microbiol. Mol. Biol. Rev.* **75**, 133–191.
- Smulevich, G., Feis, A., Howes, B. D. & Ivancich, A. (2010). *Handbook of Porphyrin Science*, Vol. 6, edited by K. M. Kadish, K. M. Smith & R. Guillard, pp. 367–453. Singapore: World Scientific.
- Strittmatter, E., Liers, C., Ullrich, R., Wachter, S., Hofrichter, M., Plattner, D. A. & Piontek, K. (2013). *J. Biol. Chem.* **288**, 4095–4102.
- Sugano, Y., Muramatsu, R., Ichianagi, A., Sato, T. & Shoda, M. (2007). *J. Biol. Chem.* **282**, 36652–36658.
- Yoshida, T., Ogola, H. J. O., Amano, Y., Hisabori, T., Ashida, H., Sawa, Y., Tsuge, H. & Sugano, Y. (2016). *Proteins*, **84**, 31–42.
- Yoshida, T. & Sugano, Y. (2015). *Arch. Biochem. Biophys.* **574**, 49–55.
- Yoshida, T., Tsuge, H., Konno, H., Hisabori, T. & Sugano, Y. (2011). *FEBS J.* **278**, 2387–2394.