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The 2.2 Å resolution structure of the catalase-peroxidase KatG from Synechococcus elongatus PCC7942

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The crystal structure of catalase-peroxidase from Synechococcus elongatus PCC7942 (SeKatG) was solved by molecular replacement and refined to an Rwork of 16.8% and an Rfree of 20.6% at 2.2 Å resolution. The asymmetric unit consisted of only one subunit of the catalase-peroxidase molecule, including a protoporphyrin IX haem moiety and two sodium ions. A typical KatG covalent adduct was formed, Met248–Tyr222–Trp94, which is a key structural element for catalase activity. The crystallographic equivalent subunit was created by a twofold symmetry operation to form the functional dimer. The overall structure of the dimer was quite similar to other KatGs. One sodium ion was located close to the proximal Trp314. The location and configuration of the proximal cation site were very similar to those of typical peroxidases such as ascorbate peroxidase. These features may provide a structural basis for the behaviour of the radical localization/delocalization during the course of the enzymatic reaction.

1. Introduction

Animals, plants and most microorganisms contain hydrogen peroxide-scavenging enzymes that act as independent catalases and peroxidases, whereas some bacteria and fungi contain bifunctional catalase-peroxidases (KatGs). Catalase-peroxidases are haem-containing enzymes which decompose hydrogen peroxide via two distinct mechanistic pathways, catalatic (2H2O2→2H2O+O2) and peroxidic (2AHred+H2O2→2Aox+2H2O) reactions, achieved within a single active site. The enzymes belong to the peroxidase superfamily class I, a group that includes ascorbate peroxidases and cytochrome c peroxidases (Welinder, 1992). Despite striking sequence homologies among class I enzymes, KatGs exhibit a dominant catalatic activity that can be compared with typical monofunctional catalases, while also demonstrating substantial peroxidatic activity.

The crystal structures of KatGs from Haloarcula marismortui (HmKatG; Yamada et al., 2002), Burkholderia pseudomallei (BpKatG; Carpena et al., 2003), Mycobacterium tuberculosis (MtKatG; Bertrand et al., 2004) and Magnaporthe grisea (MagKatG; Zámoky et al., 2012) have been reported. The X-ray structures revealed that KatGs possess unique covalent bonds formed among the side chains of three distal residues, Met–Tyr–Trp, which are located on the distal side of the haem active site. Mutagenesis studies confirmed that the Met–Tyr–Trp cross-link is required for catalatic activity (Regelsberger et al., 2000; Jakopitsch et al., 2004; Ghiladi et al., 2005; Kapetanaki et al., 2007; Zhao et al., 2010, 2013). These structures have provided many insights into the structure and function of KatGs. However, the details of the reaction mechanism are still uncertain. Although the peroxidatic activity of KatG may participate in the formation of specific products, the endogenous peroxidase substrate has not been identified and the binding site remains unknown. KatGs have attracted considerable attention by virtue of their role in the activation of isoniazid (INH), a prodrug used to treat tuberculosis (Cade et al., 2010; Zhao et al., 2006). INH also quenches amino-acid-based radical intermediates, thereby raising the need to better understand the mechanism and function of catalase-peroxidase (Ghiladi et al., 2005; Wiseman et al., 2010).

Both catalatic and peroxidatic reactions involve the oxidation of the haem iron to form compound I, accompanied by the formation...
of a porphyrin π-cation radical or a protein-based radical species (Dunford, 1999). Recently, three distinct sites for the formation of radical intermediates, Trp330, Trp139 and Trp153, were identified as the unique site for protein-based radical species (Jakopitsch et al., 2006). The stabilities of the tryptophyl radicals in KatGs may be influenced by their surroundings.

We have previously reported the crystallization of KatG from *Synechococcus elongatus* PCC7942 (SeKatG; Wada et al., 2002; PDB entry 1ub2). The enzyme shares 76.2% amino-acid sequence identity with *Synechocystis* PCC6803 KatG. A detailed study of the three-dimensional structure of SeKatG, including a comparison with *Bacillus* KatG and typical peroxidases, should provide some information regarding the KatG stabilization/delocalization mechanism. Here, we report the refined structure of SeKatG at 2.2 Å resolution, highlighting the structural differences and similarities among SeKatG, *Bacillus* KatG and typical peroxidases.

### 2. Materials and methods

#### 2.1. Overexpression, purification, crystallization and data collection

Although crystallization and data collection have previously been described (Wada et al., 2002), we noticed that the SeKatG previously used included a substantial sequence error (a A97T mutation). Therefore, in this study, an expression plasmid encoding SeKatG with the correct sequence based on the UniProt database (accession No. Q31MN3) was newly constructed by using site-directed mutagenesis according to the manufacturer’s protocol. Two oligonucleotides (5'-ACTTGGCAACCGGCGGCGACCTACC3' and 5'-GTTAAGTGTCGCGCCGGGCGGCTGGAAT3') were used to produce the error-free pET3a-SekatG using the previously generated pET3a-SekatG as the template (Wada et al., 2002). Protein overexpression and purification were performed as reported previously. We successfully obtained crystals of purified SeKatG under the same conditions as used previously (4.0-4.3 M sodium formate with 100 mM sodium citrate pH 6.0-6.5 as a precipitant). On beamline BL38B1 at SPRing-8 (Japan Synchrotron Radiation Research Institute), diffraction data were obtained to 2.2 Å resolution from a flash-cooled crystal, which did not require the addition of other cryoprotectants. The data set was processed and scaled using the HKL-2000 package (Otwinowski & Minor, 1997).

#### 2.2. Structure determination and refinement

Because the present crystal appeared to be nearly isomorphous to previously reported crystals, the coordinates of the protomer molecule of SeKatG in the asymmetric unit (PDB entry 1ub2) were refined by rigid-body refinement. The initial model was refined by several cycles of rigid-body positioning with B-factor optimization. The quality of the model was gradually improved by alternating rounds of refinement using REFMAC5 (Murshudov et al., 2011) in the CCP4 suite (Winn et al., 2011) and modification of the model, including sequence revision, using Coot (Emsley & Cowtan, 2004), until the *R* work and *R* free values were reduced to 22.4 and 26.2%, respectively. Water molecules were added to the model at locations with *F* o - *F* c densities higher than 3σ and hydrogen-bonding stereochemistry using the water-picking function of Coot. Two peaks exhibiting electron density higher than 7σ were assigned as sodium ions. Water molecules with *B* factors higher than 60 Å² were deleted from subsequent refinement rounds. The final refinement converged with *R* work and

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### Table 1

Summary of the data-collection and refinement statistics.

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<th>Data-collection statistics</th>
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*R* free values of 16.8 and 20.6%, respectively. The stereochemistry of the final model was analyzed with PROCHECK (Laskowski et al., 1993). A Ramachandran plot showed 90.1% of the residues in the most favoured regions and the remaining residues in additionally allowed regions. The data-collection and final refinement statistics are summarized in Table 1. The coordinates and X-ray data have been deposited in the Protein Data Bank with accession code 3wu. Illustrations were prepared with PyMOL (DeLano, 2002). The revised structure in this study (PDB entry 3wu) is essentially identical to the previously determined structure (PDB entry 1ub2) except for the mutated site.

### 3. Results

The SeKatG crystal structure was determined to a resolution of 2.2 Å, with *R* work and *R* free values of 16.8 and 20.6%, respectively (Fig. 1a). The final model comprises one subunit (residues 11-720) of a SeKatG molecule including a protoporphyrin IX haem moiety, two cations (modelled as sodium ions) and 522 water molecules. The crystal has a relatively high *V* M value (Matthews, 1968) of 3.77 Å³ Da⁻¹ and a solvent content of 0.67. The subunit is composed of two domains: N-terminal (residues 51-421) and C-terminal (residues 437-720). The N-terminal domain possesses the haem b prosthetic group and the KatG-typical covalent adduct Met248-Tyr222-Trp94, which is a key structural element for catalase activity (Fig. 1b). The crystallographic equivalent subunit is created by a twofold symmetry operation to form the functional dimer, as shown in Fig. 1. The dimer is formed by an interlocking association, in which the N-terminal hook region (residues 11-26) wraps around the neighbouring subunits. Ten amino-acid residues within the N-terminus were disordered and did not show sufficient electron density for assignment, possibly because they were located on the dimer surface. The accessible surface area (ASA)
buried at the dimer interface per subunit was calculated to be 1446 Å², corresponding to 16.8% of the total subunit ASA. The overall structure of the SrKatG dimer was quite similar to other known KatGs, e.g. HmkKatG, BpKatG, MkKatG and MagKatG; the root-mean-square (r.m.s.) deviations for Cα atoms ranged from 0.87 to 1.02 Å.

The SrKatG Fc – Fo map showed two peaks with electron densities higher than 7σ (Figs. 2a and 2b): one on the distal side and one on the proximal side of the N-terminal domain. The peaks on the distal and proximal sides were surrounded by five and four O atoms, respectively, with distances of 2.33–2.54 Å. The distances are equivalent to Na+–ligand distances (Harding, 2002); additionally, a carboxylate group was found around each of the peaks (Asp413 for the distal side in Fig. 2a, Asp322 for the proximal side in Fig. 2b). Thus, we further refined the structure by considering that these two sites were occupied by Na+ ions; accordingly, the Fc – Fo map did not show positive peaks when Na+ ions were assigned to these two sites.

In the four KatG structures reported to date, only BpKatG binds a Na+ ion on the distal side in the same location as that observed in the SrKatG structure. A comparison of the residues on the distal side involved in cation binding showed that the ligand configurations, including the water molecules, were very similar in SrKatG and BpKatG (Figs. 2a and 2c). In the other KatG structures the ligand residues were also conserved, but the locations of the water molecules around the distal cation site are diverse. These diversities are probably derived from the differences in the surrounding residues near the water ligands (data not shown). Furthermore, the crystallization conditions for SrKatG and BpKatG included sodium ion, whereas the other KatG crystals were not obtained in the presence of sodium ion. Thus, at the moment, it remains controversial whether the identity of the cation binding to the distal side depends upon the environment of the site or upon the crystallization conditions.

On the other hand, cation binding to the proximal haem pocket was only observed in SrKatG, even though the BpKatG crystals were prepared in a solution containing 0.1 M sodium citrate (Carpena et al., 2003). In the SrKatG proximal binding site, Asp322 was thought to play a significant role in holding the Na+ ion within the three-dimensional structure (Fig. 2b). In BpKatG, the corresponding position is replaced by the non-ionic residue Ser338 (Fig. 2d); therefore, the cation might not be able to bind to this site. Instead, in BpKatG, a water molecule is bound to the site at the same position as the Na+ ion in SrKatG.

Notably, the cation-binding site observed in the proximal haem pocket of SrKatG is conserved across a number of canonical peroxidases (Fig. 3); it is known that the structures of the N-terminal domains of KatGs are similar to the overall structure of the canonical peroxidases (Fig. 3a). The proximal cation-binding site was located near the haem (≥13 Å); the cation-binding site faces the haem across the helix containing the histidine residue which is the haemin proximal ligand (e.g. in SrKatG the backbone of Thr264, the next residue to the His263 haem ligand, was one of the coordinate residues involved in Na+ ion binding; Fig. 3c). In these peroxidases, the cation (Na+, K+ or Ca++) substantially affects the location of the radical species during the course of the peroxidase reaction (see below). In the case of the chloroplastic ascorbate peroxidase (APX), the site is occupied by a sodium ion (Fig. 3c; Wada et al., 2003), whereas in the cytosolic APX a potassium ion is bound in this site (Patterson & Poulos, 1995). In contrast, cytochrome c peroxidase (CCP) has no cation in this site (Finzel et al., 1984). As shown in Fig. 3, the residues surrounding the cation in the SrKatG and chloroplastic APX structures were similar; the coordinate atoms were composed of one carboxylate O atom (aspartate residue) and a main-chain/side-chain O atom (Figs. 3b and 3c). However, there were slight differences in the details of Na+ binding. Specifically, in SrKatG the carboxylate O atom of Asp322 directly interacted with the sodium ion, whereas in the chloroplastic APX Asp203 interacted via a water molecule.

### 4. Discussion

In crystal structures of the canonical peroxidases, such as ascorbate peroxidases (APXs), horseradish peroxidase (Gajhede et al., 1997),...
lignin peroxidase (Poulos et al., 1993) and Achromobacter peroxidase (Kunishima et al., 1994), various cation species (Na\(^+\) in cytosolic APX, K\(^+\) in chloroplastic APX or Ca\(^{2+}\) in the other peroxidases) have been identified in the proximal haem pocket. Although it has been reported that cytochrome c peroxidase (CCP) does not bind any cation at this site, engineered CCP, like cytosolic APX, bound potassium ion, and the lifetime of the Trp191 cation radical, the protein-based radical, decreased in inverse proportion to the concentration of potassium ion (Bhaskar et al., 2002). Furthermore, in APX the radical located to the \(\pi\)-orbital of the porphyrin ring (Patterson & Poulos, 1995), not to the tryptophan residue, probably owing to cation binding. These results indicated that the cation in the proximal site is the delocalization element for the radical location, at least in the case of the typical peroxidases; this has been attributed to the flexibility of the cation-binding site (Bhaskar et al., 2002). In catalase-peroxidases, extensive studies have demonstrated that the protein-based radical species are formed as the reactive intermediate of the peroxidase-like reaction of KatGs. Interestingly, BpKatG and Synechocystis sp. PCC 6803 KatG differ in the site of formation of tryptophanyl radical intermediates. Three distinct sites where radical intermediates can form, Trp330, Trp139 and Trp153, have been identified as the reactive intermediates of BpKatG (Colin et al., 2009). In contrast, in Synechocystis sp. PCC 6803 KatG, Trp106 (equivalent to Trp95 in BpKatG) has been identified as the unique site for the protein-based radical species, and the proximal Trp341 (equivalent to Trp330 in BpKatG (Fig. 3d) and Trp314 in SeKatG (Fig. 3a)) is not assigned as a radical site (Ivancich et al., 2003; Jakopitsch et al., 2006).

In this study, crystallographic analysis revealed that SeKatG possesses a cation-binding site in a position corresponding to that observed in canonical peroxidases. The amino-acid sequences of SeKatG and Synechocystis sp. PCC 6803 KatG share 76.2% identity and 94.7% similarity; moreover, the residues surrounding the proximal cation-binding pocket in SeKatG are completely conserved in Synechocystis sp. PCC 6803 KatG. This sequence similarity implies that Synechocystis sp. PCC 6803 KatG also has the proximal cation-binding site. Therefore, the EPR differences for radical intermediates between BpKatG and Synechocystis sp. PCC 6803 KatG might be caused by the binding of the cation in the proximal haem pocket, based on the engineered CCP analogy that cation binding in the proximal cation site tends to delocalize the radical. The behaviour of the radical is complicated because the fine structure of the overall

![Figure 2](image-url)

**Figure 2**
The coordination environments of the sodium ions. (a) The distal cation-binding site of SeKatG. An OMIT \(F_o - F_c\) map for sodium ion contoured at 7σ (red) is overlaid on the \(2F_o - F_c\) map contoured at 1.5σ (blue). The sodium ion and water molecules are shown as orange and cyan balls, respectively. Broken lines indicate coordinate or hydrogen bonds. Distances are in Å. (b) The proximal cation-binding site of SeKatG. (c) The distal cation-binding site of BpKatG (PDB entry 1mwv; Carpena et al., 2003). (d) The region corresponding to the proximal cation-binding site in BpKatG.

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protein structure could affect its motion. Thus, further biochemical, mutational and spectroscopic studies are needed to elucidate the detailed mechanism of the KatG reaction.

The synchrotron-radiation experiments were performed at SPring-8 and the Photon Factory. We thank the beamline staff at BL38B1 of SPring-8 (Harima, Japan) and at NE3A of PF-AR (Tsukuba, Japan) for providing data-collection facilities and support. This work was financially supported by the Program to Disseminate Tenure Tracking System from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to KW).

References


Figure 3

Structural comparison of the proximal haem pocket. (a) Superposition of the structures of SeKatG (dark green), chloroplastic APX (yellow; PDB entry 1iy7; Wada et al., 2003) and BpKatG (pink). (b–d) The proximal pockets of (b) SeKatG, (c) chloroplastic APX and (d) BpKatG. The sodium ion and water molecules are shown as orange and cyan balls, respectively.

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