Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Expression, purification, crystallization and preliminary X-ray analysis of the Met244Ala variant of catalase–peroxidase (KatG) from the haloarchaeon *Haloarcula marismortui*

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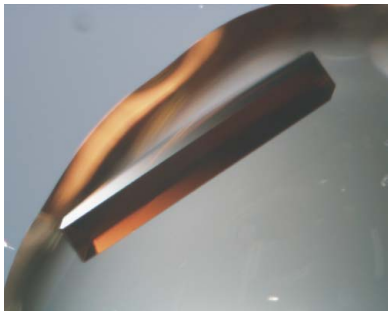
Received 31 July 2007
Accepted 21 September 2007

The covalent modification of the side chains of Trp95, Tyr218 and Met244 within the active site of *Haloarcula marismortui* catalase–peroxidase (KatG) appears to be common to all KatGs and has been demonstrated to be particularly significant for its bifunctionality [Smulevich *et al.* (2006), *J. Inorg. Biochem.* **100**, 568–585; Jakopitsch, Kolarich *et al.* (2003), *FEBS Lett.* **552**, 135–140; Jakopitsch, Auer *et al.* (2003), *J. Biol. Chem.* **278**, 20185–20191; Jakopitsch *et al.* (2004), *J. Biol. Chem.* **279**, 46082–46095; Regelsberger *et al.* (2001), *Biochem. Soc. Trans.* **29**, 99–105; Ghiladi, Knudsen *et al.* (2005), *J. Biol. Chem.* **280**, 22651–22663; Ghiladi, Medzihradzky *et al.* (2005), *Biochemistry*, **44**, 15093–15105]. The Met244Ala variant of the *H. marismortui* KatG enzyme was expressed in haloarchaeal host cells and purified to homogeneity. The variant showed a complete loss of catalase activity, whereas the peroxidase activity of this mutant was highly enhanced owing to an increase in its affinity for the peroxidatic substrate. The variant was crystallized using the hanging-drop vapour-diffusion method with ammonium sulfate and NaCl as precipitants. The reddish-brown rod-shaped crystals obtained belong to the monoclinic space group $C2$, with unit-cell parameters a = 315.24, b = 81.04, c = 74.77 Å, β = 99.81°. A crystal frozen using lithium sulfate as the cryoprotectant diffracted to beyond 2.0 Å resolution. Preliminary X-ray analysis suggests the presence of a dimer in the asymmetric unit.

1. Introduction

The catalase–peroxidase KatG, which is widely found in bacteria and archaea, is a member of the class I peroxidase superfamily, which also includes cytochrome *c* peroxidase (CcP) and ascorbate peroxidase (APX) (Fraaije *et al.*, 1996; Levy *et al.*, 1992). As suggested by its name, KatG is a bifunctional enzyme that exhibits both catalase and peroxidase activities with the function of preventing damage to cellular components by exogenous H_2O_2 and its deprotonation products. The catalytic reaction of KatG is explained as follows (Hillar *et al.*, 2000). The ferric enzyme (Fe^{III}–Por) reacts with a hydroperoxide molecule and forms compound I, an oxyferryl radical cation intermediate (Fe^{IV}═O–$Por^{\cdot+}$ or Fe^{IV}═O–Por–$aa^{\cdot+}$, where aa is the side chain of an amino acid). The reaction process is common among the haem-containing hydroperoxidases, including class I, class II (fungus lignin peroxidases) and class III (classical secretory peroxidases, an example being horseradish peroxidase) enzymes and mammalian liver catalases. In the catalase reaction, compound I is reduced by the second hydroperoxide molecule and returns to the ferric state. In the peroxidase reaction, reduction of compound I to the ferric state proceeds through the intermediate compound II (Fe^{IV}═O–Por) in successive two-step reductions by reducing substrates. All peroxidases except KatG possess only peroxidase activity and show no catalase activity, whereas mammalian catalases do not display any peroxidase activity (Welinder, 1991).

Haloarcula marismortui KatG is a homodimer with a monomer molecular weight of 81 kDa (Yamada *et al.*, 2002). The topological arrangements of the N-terminal (residues 18–430) and C-terminal (residues 431–731) domains of the enzyme are identical to those of CcP and APX, suggesting that the enzyme was generated by duplication of the gene encoding the common ancestor of class I



peroxidases, as proposed previously by Zamocky *et al.* (2000). The architecture of the active site, including the haem pocket composed of the 'distal triad' (Arg92, Trp95 and His96) and the proximal haem ligand His259 and its hydrogen-bonding partners Asp372 and Trp311, is also well conserved.

The covalent modifications of the side chains of three amino acids (Trp95, Tyr218 and Met244) close to the active site are also conserved (Yamada *et al.*, 2002). This covalent adduct has been shown to be generated by an autocatalytic process (Ghiladi, Knudsen *et al.*, 2005). These residues are conserved in all KatGs but not in other class I peroxidases. Mutagenesis experiments indicated that the covalent adduct is essential for catalase activity but not for peroxidase activity (Jakopitsch, Kolarich *et al.*, 2003; Jakopitsch, Auer *et al.*, 2003; Jakopitsch *et al.*, 2004; Singh *et al.*, 2004).

In this paper, we report the expression, purification, crystallization and preliminary X-ray analysis of the Met244Ala variant of *H. marismortui* KatG. The variant showed no catalase activity, but its peroxidase activity was highly enhanced. The crystal structure of the mutant KatG will provide a structural basis for elucidating the molecular mechanism of the catalase activity associated with the covalent adduct in the enzyme.

2. Expression and purification of the enzyme

2.1. Construction of plasmids and strains

A DNA fragment including the *katG* gene from *H. marismortui* ATCC43049 and its promoter (90 bp at the 5′-terminus of the *katG* gene) and putative transcription terminator signal regions (130 bp at the 3′-terminus of the gene) were amplified from the *H. marismortui* chromosomal DNA using KOD-plus polymerase (Takara Shuzo Co. Ltd, Kyoto, Japan). The oligonucleotides 5′-GGGATCCTGTTT-TTCACACGATGCTATTTC-3′ and 5′-GCTTCTAGAAAGGCGG-TAAAATGGCAGATG-3′ were used as PCR primers (the restriction sites introduced for *Bam*HI and *Xba*I digestion are underlined). The 2.4 kbp product was cloned into a pUC119 vector and the resultant vector, designated pHK, was used as the target for nucleotide substitutions and insertions. Site-directed mutagenesis was performed to insert $(CAC)_6$ into the 3′-terminus of the *katG* gene using the oligonucleotide 5′-TTCGACCTCGA**G***CACCACCAC-CACCACCAC*TAAGCCGGAGC-3′ (the inserted sequence is italicized, the restriction site for *Xho*I is underlined and the substitution from A to G is shown in bold) according to a previously described method (Kunkel, 1985). The resulting plasmid pHKH6 was subjected to further mutagenesis to produce the Met244Ala substitution in KatG to yield pHKM244AH6 using the oligonucleotide 5′-TCGACC-GCGCAGCGATGAAC-3′ (the restriction site for *Acc*II is underlined). Finally, the manipulated DNA fragments were excised from pHKH6 and pHKM244AH6 and introduced into the *Bam*HI/*Xba*I restriction site of the *Escherichia coli–Haloferax volcanii* shuttle plasmid pWL102 harbouring both ampicillin- and pravastatin-resistance cassettes, giving rise to the expression vectors pWLHK and pWLHKM244A, respectively (Lam & Doolittle, 1989).

Transfection of the expression vectors into the haloarchaeal host cells was performed according to a previously published method developed for *Haloferax volcanii* (Cline & Doolittle, 1987). *Haloferax denitrificans* JCM8864^{T} cells were spheroplasted by treatment with EDTA under low ionic strength conditions. The spheroplasted cells were further treated with polyethylene glycol. The competent cells thus prepared were used for transfection of the expression vectors. Transformants were selected for resistance to pravastatin by plating them onto agar medium containing ATCC1434 medium and 5.0 µg ml^{-1} pravastatin.

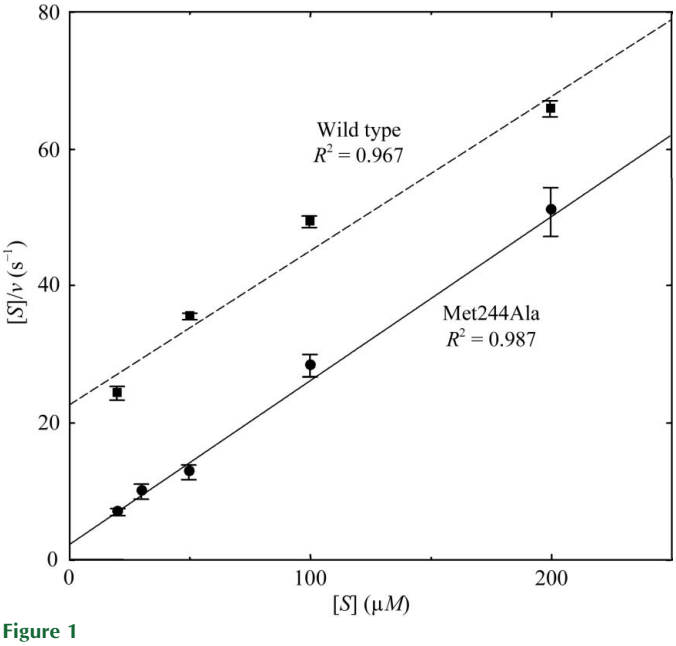


Figure 1
Binding affinity of the peroxidase substrate for the Met244Ala variant and wild-type protein (WT). Steady-state oxidation of *o*-dianisidine by the Met244Ala variant (solid line) and WT (dotted line) is shown. 10 µl of either the Met244Ala or WT enzyme solution was mixed with 1 ml reaction solution containing 100 m*M* sodium phosphate buffer pH 6.0, 2 *M* NaCl, 20 m*M* *tert*-butylperoxide and 20–200 µ*M* *o*-dianisidine. The ratio of the initial *o*-dianisidine concentration [*S*] to the reaction velocity *v* is plotted against substrate concentration [*S*]. Data were fitted to the Hanes–Woolf equation, yielding a K_m of 8.74 ± 0.57 µ*M* for Met244Ala and of 100.60 ± 2.36 µ*M* for WT, respectively. The multiple correlation coefficients R^2 of the models were calculated to describe the goodness of fit.

2.2. Purification of recombinants

The haloarchaeal host cells transformed with the pWLHKM244A plasmid were cultivated in ATCC1434 medium containing 10.0 µg ml^{-1} pravastatin at 310 K with vigorous aeration through an air filter. After 3–4 d cultivation, cells in the late-exponential growth phase were harvested from 10 l medium and used for purification of the Met244Ala variant of *Haloarcula marismortui* KatG. The cells obtained were suspended in 10 m*M* Tris–HCl buffer pH 8.0 containing 2.0 *M* NaCl (buffer *A*) and disrupted using an ultrasonic oscillation device. The cell extract was dialyzed against 10 m*M* Tris–HCl buffer pH 8.0 containing 60% saturated ammonium sulfate (buffer *B*) and the solution was centrifuged at 12 000*g* for 30 min to remove precipitants. The supernatant was mixed with butyl-Toyopearl 650M resin (Tosoh Co., Tokyo, Japan) that had been equilibrated with buffer *B*. After washing with buffer *B*, the recombinant KatG adsorbed on the resin was eluted with buffer *A*. The eluate was dialyzed against buffer *B* and was then adsorbed onto a Sepharose CL-4B column (2.5 × 20 cm, Amersham Pharmacia Biotech AB, Uppsala, Sweden) that had been equilibrated with buffer *B*. The recombinant KatG adsorbed on the column was eluted with a linear gradient between buffer *B* and buffer *A* over a 400 ml elution volume. The enzyme typically eluted between 50 and 55% buffer *B*. The fractions containing the enzyme were collected and dialyzed against 20 m*M* sodium phosphate buffer pH 7.2 containing 2.0 *M* NaCl (buffer *C*). The resulting solution was loaded onto a column (2.5 × 10 cm) packed with Ni^{2+}-chelating Sepharose Fast Flow resin (Amersham) which had been equilibrated with buffer *C*.

Table 1
Enzymatic activities of wild-type and Met244Ala variant *H. marismortui* KatG.

ND; not detected. The averages and standard deviations were obtained from three individual measurements.

	Wild type	Met244Ala
Catalase activity		
K_m (m*M*)	5.16 ± 0.18	ND
k_{cat} (s^{-1})	(3.0 ± 0.2) × 10^3	ND
k_{cat}/K_m (×10^6 M^{-1} s^{-1})	0.582 ± 0.013	ND
Peroxidase activity		
K_m (μ*M*)	100.60 ± 2.36	8.74 ± 0.57
k_{cat} (s^{-1})	4.43 ± 0.14	4.11 ± 0.32
k_{cat}/K_m (×10^6 M^{-1} s^{-1})	0.044 ± 0.003	0.47 ± 0.25

After washing the column with 100 ml buffer *C* containing 0.5 *M* sodium glutamate, the recombinant enzyme adsorbed on the column was eluted with a linear gradient between 0 and 50 m*M* imidazole over a 200 ml elution volume of buffer *C*. Protein-containing fractions that had an absorbance ratio ($A_{406\,nm}/A_{280\,nm}$) of more than 0.6 were eluted between 40 and 45 m*M* imidazole, collected (about 5 mg protein) and used as the purified preparation for enzyme characterization and crystallization. Purification of the wild-type recombinant protein that had been expressed in the pWLHK transformant followed the protocol described above.

2.3. Catalase and peroxidase assays

Catalase activity was monitored spectrophotometrically using an extinction coefficient of 0.0436 mM^{-1} cm^{-1} for H_2O_2 at 240 nm (Wei *et al.*, 2003), following the oxidation rate in 100 m*M* sodium phosphate buffer pH 6.0, 2 *M* NaCl, 1–10 m*M* H_2O_2 and 10 n*M* (haem concentration) enzyme at 298 K. The peroxidase activity was determined using 20 m*M* *tert*-butylperoxide and 20–200 μ*M* *o*-dianisidine (A_{460} = 11.3 mM^{-1} cm^{-1}; Wei *et al.*, 2003), following the oxidation rate of *o*-dianisidine in 100 m*M* sodium phosphate buffer pH 6.0, 2 *M* NaCl and 40 n*M* wild-type or 5 n*M* Met244Ala variant protein.

Steady-state kinetic parameters (K_m, k_{cat} and k_{cat}/K_m) were determined by fitting the averaged data at each concentration to the Michaelis–Menten equation (Higuchi *et al.*, unpublished results). As shown in Fig. 1, a Hanes–Woolf plot was used to determine the kinetic parameters K_m, V_{max} and V_{max}/K_m. Equation (1) can be derived from the Michaelis–Menten equation,

$$[S]/v = [S] \times (1/V_{max}) + (K_m/V_{max}), \quad (1)$$

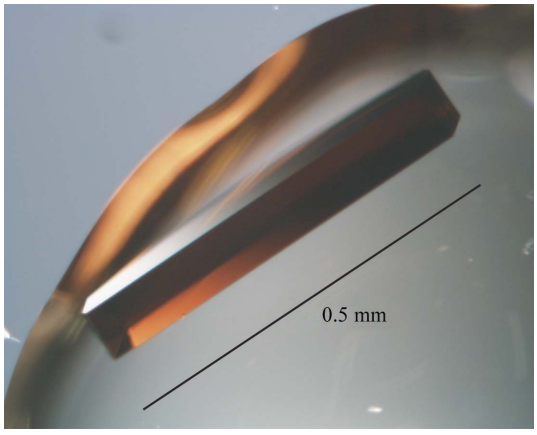


Figure 2
Monoclinic crystals of the Met244Ala variant of *H. marismortui* KatG.

Table 2
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Space group	*C*2
Resolution (Å)	34.01–2.01 (2.08–2.01)
Unit-cell parameters (Å, °)	*a* = 315.24, *b* = 81.04, *c* = 74.77, β = 99.81
No. of measured reflections	464899
No. of unique reflections	117578
Completeness (%)	95.1 (92.8)
R_{merge}† (%)	15.5 (46.8)
Average $\langle I/\sigma(I)\rangle$	4.7 (2.5)
Redundancy	3.95 (4.01)

† $R_{merge} = \sum_i \sum_{hkl} |I_i - \langle I\rangle| / \sum_i \sum_{hkl} I_i$, where I_i and $\langle I\rangle$ are the observed intensity and the average intensity obtained from multiple measurements, respectively.

which yields a straight line of slope $1/V_{max}$, a *y* intercept of K_m/V_{max} and an *x* intercept of $-K_m$. As seen in other KatG enzymes, substitution of alanine at position 244 led to a complete loss of catalase activity. On the other hand, a concomitant 11-fold enhancement of the peroxidase activity (k_{cat}/K_m) and the affinity for the peroxidase substrate (K_m) was observed in the mutant enzyme, whereas the catalytic constant (k_{cat}) was little affected (Table 1). Data fitting by the nonlinear regression least-squares method, which is significantly more accurate, is now in progress.

The absorption spectrum of the variant in the visible region was almost identical to that of the wild type, suggesting that the electronic structure of the haem was little affected by the mutation (data not shown).

3. Crystallization and data collection

The purified Met244Ala protein was dialyzed against 20 m*M* sodium phosphate pH 6.0 containing 1.50 *M* ammonium sulfate and 0.5 *M* NaCl and then concentrated to 35 mg ml^{-1} using a Microcon Ultracel YM-100 (Millipore, Billerica, MA, USA). A 1 μl droplet was equilibrated against 1 ml 20 m*M* sodium phosphate pH 6.0 containing 2.67 *M* ammonium sulfate by the hanging-drop vapour-diffusion method. A reddish-brown rod-shaped crystal of approximate dimensions 0.9 × 0.3 × 0.1 mm appeared after 1–2 weeks at 288 K (Fig. 2). Although wild-type *H. marismortui* KatG was crystallized at pH 8.0 (Yamada *et al.*, 2001, 2002), crystals of the recombinant enzyme were obtained at a lower pH, probably owing to the six histidine residues that were added at the C-terminus of the enzyme. The crystallization condition that was used to produce crystals of the WT enzyme was used as a starting point for the Met244Ala variant (Yamada *et al.*, 2001, 2002). This condition was refined by varying the ammonium sulfate concentration (from 2.67 to 3.03 *M*), the pH (from 5.5 to 6.1), the protein concentration (from 20 to 23 mg ml^{-1}) and the temperature (from 283 to 293 K). Each of these parameters was tested at a low, intermediate and high value within the range. The results of the optimization experiments suggest that the optimal conditions depend on either a combination of pH and temperature or a combination of the ammonium sulfate and protein concentrations, using the regression analysis, analysis of variance and response-surface method in the same manner as previously reported by Saijo *et al.* (2005)

Crystals were soaked in 1 *M* lithium sulfate in addition to the ingredients of the reservoir solution because neither powder-diffraction rings nor ice rings could be removed from the low-temperature diffraction pattern using the common cryoprotectants glycerol and polyethylene glycol. Diffraction intensities were

collected from a flash-frozen single crystal on a Rigaku R-AXIS IV image-plate detector using a Rigaku RU300 rotating-anode generator at 50 kV and 80 mA with Cu $K\alpha$ radiation. The diffraction data were indexed, integrated and merged using the software program *CrystalClear* v.1.3.6 (Rigaku/MSC, The Woodlands, Texas, USA; see Table 2).

The unit-cell parameters were determined to be a = 315.24, b = 81.04, c = 74.77 Å, β = 99.81° in space group $C2$, as shown in Table 1. A noncrystallographic twofold axis was found tilted almost 30° from the crystallographic twofold axis. Assuming the presence of two molecules in the asymmetric unit, the V_M value as defined by Matthews (1968) was 3.00 Å^3 Da^{-1}. The asymmetric unit of this mutant crystal contains two crystallographically independent molecules (designated subunits *A* and *B*) related by noncrystallographic twofold symmetry.

The crystal structure was solved by molecular replacement using the program *MOLREP* (Vagin & Teplyakov, 1997) with the native molecule (PDB code 1itk) as the search model. Structure refinement and model building are now in progress using *REFMAC*5 (Murshudov *et al.*, 1997; Vagin *et al.*, 2004) and *XtalView* (McRee, 1999).

4. Conclusions

The Met244Ala variant of *H. marismortui* was crystallized from sodium phosphate pH 6.0 and ammonium sulfate at 288 K and the crystals diffracted to beyond 2.0 Å resolution. The diffraction data show that the crystals appear to contain a dimer of subunits in the crystal asymmetric unit, corresponding to a solvent content of 59.0%. This crystal form is well suited to structural studies of the Met244Ala mutant of KatG. The crystal structure of this variant will help answer questions as to why it shows enhanced peroxidase activity but does not exhibit catalase activity.

This work was supported in part by Japan Space Forum Grant 180 (to TF).

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