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Crystal structure of the catalase–peroxidase KatG W78F mutant from *Synechococcus elongatus* PCC7942 in complex with the antitubercular pro-drug isoniazid

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ABSTRACT

Isoniazid (INH) is a pro-drug that has been extensively used to treat tuberculosis. INH is activated by the heme enzyme catalase–peroxidase (KatG), but the mechanism of the activation is poorly understood, in part because the INH binding site has not been clearly established. Here, we observed that a single-residue mutation of KatG from *Synechococcus elongatus* PCC7942 (*Se*KatG), W78F, enhances INH activation. The crystal structure of INH-bound KatG-W78F revealed that INH binds to the heme pocket. The results of a thermal-shift assay implied that the flexibility of the *Se*KatG molecule is increased by the W78F mutation, allowing the INH molecule to easily invade the heme pocket through the access channel on the γ-edge side of the heme.

1. Introduction

Isoniazid (INH) is a pro-drug that has been extensively used as a first-choice drug for the tuberculosis chemotherapy. A decade-long genetic and biochemical investigations revealed that INH is activated by the catalase–peroxidase KatG of *Mycobacterium tuberculosis* [1–3]. Originally, KatG proteins were identified as bi-functional heme enzymes belonging to the class I peroxidase family. In contrast to monofunctional peroxidases, these enzymes exhibit both catalase activity and broad-spectrum peroxidase activity. The INH activation ability of KatG was identified by analysis of an INH-resistant *M. tuberculosis* species [1]. During activation, the INH molecule is cleaved by KatG to generate an isonicotinyl radical (IN·); subsequently, in the presence of superoxide, the IN· radical combines with NAD^+ to generate an isonicotinyl-NAD (IN·NAD) covalent adduct [4]. The IN·NAD adduct inhibits the InhA enzyme, which is involved in the synthesis of mycolic acid [2], a major unique component of the mycobacterial cell wall. In contrast to the inhibitory mechanism of InhA by IN·NAD adduct [2], which has been clearly elucidated, the detailed mechanisms of INH activation by KatG remain unclear, and the exact binding site of INH on KatG is unknown.

The mechanisms of INH activation have been explored not only in *M. tuberculosis* KatG (*Mt*KatG) [5–7] but also in other KatGs such as those of *Burkholderia pseudomallei* (*Bp*KatG) [4,8–10], *Synechocystis* PCC 6803 (*Syn*KatG) [11–13] and *Synechococcus elongatus* PCC7942 (*Se*KatG) [14]. These KatGs share at least 54% sequence identity, and their overall structures are very similar [15–17]. In addition, the configuration of the residues around the heme, including the unique covalent bonds among the side chains of the Met–Tyr–Trp residues of KatG, located on the distal side of the heme, are found in KatG proteins from all three species. Therefore, the environments of the heme pocket in *Mt*KatG, *Bp*KatG, *Syn*KatG and *Se*KatG are believed to be almost identical.

To date, information regarding the INH binding site of KatG has primarily been obtained by spectroscopic studies. The results of

Abbreviations: APX, ascorbate peroxidase; ARP, *Arthromyces ramosus* peroxidase; *Bp*KatG, *Burkholderia pseudomallei* KatG; CCP, cytochrome *c* peroxidase; HRP, horseradish peroxidase; INH, isoniazid; *Mt*KatG, *Mycobacterium tuberculosis* KatG; *Se*KatG, *Synechococcus elongatus* PCC7942 KatG; *Syn*KatG, *Synechococcus* PCC6803 KatG

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the experiments performed so far suggest that the INH binding/activation process occurs in the vicinity of the heme: Raman spectroscopy intensities reveal that upon treatment with INH, the binding of INH perturbs bound CO in the distal heme pocket of *Mt*KatG [18]; similarly, the characteristic UV–visible spectrum of the oxoferryl species (Fe(IV)=O) in *Bp*KatG changes rapidly upon addition of INH [4]. In addition, electron paramagnetic resonance spectroscopy demonstrated that the heme-derived signal changes upon addition of INH [19].

Interestingly, electron paramagnetic resonance (EPR) experiments clearly demonstrated that KatG has several sites of the radical intermediate in the enzymatic reaction, and the site was differ among the KatGs; in the *Mt*KatG [Fe(IV)=O Trp$^{\cdot}$] species was the reactive intermediate with the INH reaction [20], whereas in the *Syn*KatG the first committed intermediate, identified as the Trp$^{\cdot}$ and a Tyr$^{\cdot}$, formed subsequently to the stable [Fe(IV=O) Porphyrin$^{\cdot+}$] species by intermolecular electron transfer [13]. More recently, we determined the structure of the INH-bound *Se*KatG [14], which revealed that three INH molecules are weakly bound to the molecular surface (Fig. 1); one INH molecule was bound at the entrance to the ε-edge side of heme (Site 1). Another was bound at the entrance to the γ-edge side of heme (Site 2), and the other was bound to the loop structures in front of the heme propionate side chain (Site 3). All of the interactions between KatG and the bound INH seemed to be weak, mediated mainly by van der Waals contacts. In contrast, crystallographic analysis of INH-bound *Bp*KatG demonstrated that one INH molecule binds to the molecular surface at the dimer interface, a site that is ~20 Å distant from the entrance to the heme cavity [4]. Subsequent mutation experiments demonstrated that the residues involved in INH binding, as revealed by the structure, had no effect on INH activation; therefore, this remote site is not the only binding site for INH [4]. Taken together, the INH-binding sites of *Se*KatG and *Bp*KatG are probably different, and the exact binding site of INH on KatG is still controversial.

During the course of a mutational study of *Se*KatG, aimed at investigating the localization of the radical during the reaction, we observed that a single-residue mutation in *Se*KatG, W78F, enhanced INH activation. The mutated tryptophan residue (W78), which is highly conserved in all KatG orthologues, is located at the entrance of the γ-edge side of the heme (Site 2) [15–17,21]. The side chain of the tryptophan shielded the γ-edge–side heme-access channel, and thus seemed to function as the "lid" of the heme pocket. In order to gain a better understanding of INH recognition/binding, we determined the crystal structures of the *Se*KatG-W78F mutant protein. The single-residue mutation resulted in dramatic alteration of the INH-binding site relative to that of the wild type: in *Se*KatG-W78F, the INH molecule bound in the heme pocket, whereas in wild-type *Se*KatG, three INH molecules bound on the molecular surface.

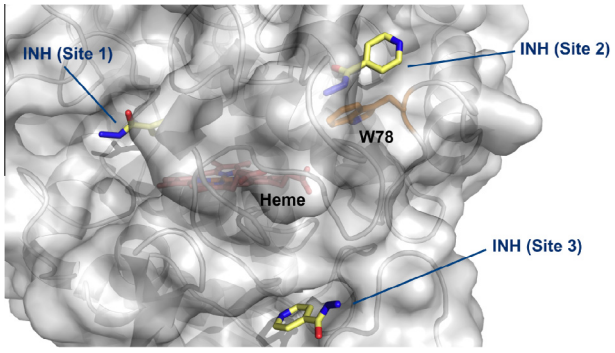

Fig. 1. The locations of the INH-binding sites in the wild-type *Se*KatG. The wild-type *Se*KatG has three low-affinity INH binding sites (Site 1–3). Three INH molecules bound on the molecular surface via van der Waals contacts. The molecular surface drawing is overlain with the cartoon drawing of the INH-bound wild-type *Se*KatG (PDB ID: 3WXO). The bound INH molecules and heme are shown in yellow and pink. Trp78 is shown in orange.

2. Materials and methods

2.1. Expression, purification, and characterization

Oligonucleotide-directed mutagenesis of Trp78 to Phenylalanine in *Se*KatG was performed by overlap extension PCR using the pET-3a plasmid containing the *Se*KatG gene as the template [17]. The oligonucleotide pair 5′-CCAAGACTGGTTTCCGGCAG-ACTG-3′ and 5′-CAGTCTGCCGGAAACCAGTCTTGG-3′ was used to produce the *Se*KatG-W78F mutant. The plasmids were transformed into *Escherichia coli* strain BL21(DE3). Protein overexpression and purification were carried out as described for native *Se*KatG [22].

2.2. Crystallization, X-ray diffraction data collection, and structure determination

Crystals of *Se*KatG-W78F were successfully obtained under the same conditions (4.3 M sodium formate, with 0.1 M sodium citrate [pH 6.3] as a precipitant) used for the native *Se*KatG. INH-bound crystals were prepared by soaking the crystals of *Se*KaG-W78F in crystallization solution containing 100 mM INH for 12 h. On beamline BL44XU at SPring-8 (Japan Synchrotron Radiation Research Institute), X-ray diffraction data of INH-bound and INH-free *Se*KatG-W78F were obtained to 3.2 Å and 2.65 Å resolution, respectively, from a flash-cooled crystal, which did not require

Table 1
Data collection and crystallographic analysis.*

	INH-bound KatG-W78F	INH-free KatG-W78F
Data collection		
Beamline	SPring-8 BL44XU	SPring-8 BL44XU
Space group	$P4_12_12$	$P4_12_12$
Unit cell		
a, *b*, *c* (Å)	107.7, 107.7, 204.8	108.3, 108.3, 203.3
α, β, γ (°)	90.0, 90.0, 90.0	90.0, 90.0, 90.0
Wavelength (Å)	0.9000	0.9000
Resolution (Å)	50.0–3.20 (3.31–3.20)	50.0–2.65 (2.74–2.65)
Completeness (%)	99.8 (99.8)	99.9 (99.4)
Redundancy	5.2 (4.6)	6.8 (6.4)
I/σ (I)	16.3 (3.0)	10.4 (5.7)
R_{merge}†	0.111 (0.532)	0.169 (0.491)
Refinement		
Resolution (Å)	50.0–3.20	50.0–2.65
No. of reflections	19354	33798
R/R_{free}‡	0.180/0.260	0.196/0.254
RMSD bond lengths (Å)	0.01	0.017
RMSD bond angles (°)	1.52	1.92
Average *B* value (Å^2)	73.1	49.3
Ramachandran plot		
Most favored region (%)	90.8	87.9
Additionally allowed regions (%)	7.8	12.1
Generously allowed regions (%)	1.4	0
PDB ID	4PAE	3X16

* The statistics in the highest-resolution shell are given in parentheses.
† $R_{merge} = \Sigma_{hkl}\ \Sigma_i\ |I_i(hkl) - \langle I(hkl)\rangle|/\Sigma_{hkl}\Sigma_i\ I_i(hkl)$, where $I_i(hkl)$ is the observed intensity and $\langle I(hkl)\rangle$ is the average intensity for multiple measurements.
‡ $R = \Sigma_{hkl}\ ||F_{obs}| - |F_{calc}||/\Sigma_{hkl}\ |F_{obs}|$, where F_{obs} is the observed structure factor and F_{calc} is the calculated structure factor. R_{free} is the same as *R*, except calculated using 5% of the data that were not included in any refinement calculations.