Tuberculosis 100 (2016) 15–24

Contents lists available at ScienceDirect

Tuberculosis

journal homepage: http://intl.elsevierhealth.com/journals/tube

DRUG DISCOVERY AND RESISTANCE

Comparative study of enzymatic activities of new KatG mutants from low- and high-level isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis*

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ARTICLE INFO

Article history:
Received 31 January 2016
Received in revised form
8 May 2016
Accepted 5 June 2016

Keywords:
Tuberculosis
Resistance
Isoniazid
Catalase-peroxidase
KatG
Enzymatic activities

SUMMARY

Resistance to isoniazid (INH-R) in *Mycobacterium tuberculosis* is mainly due to mutations at position 315 (S315T) of the catalase-peroxidase KatG. We identified 16 mutations (including 13 biochemically uncharacterized mutations) in KatG from INH-R clinical isolates of *M. tuberculosis* showing mutations other than S315T. The KatG enzymatic activities (catalase, peroxidase, free radical production and isonicotinoyl-NAD formation) of wild-type KatG and the 16 mutants were determined and correlated to their spatial location in a KatG model structure. Of all mutations studied, H270R, which conferred a high level of INH-R and results in the disruption of a coordination bond with the heme, caused complete loss of all enzymatic KatG activities. The mutants generally associated with a very high level of INH-R were all characterized by a drastic reduction in catalase activity and a marked decrease in INH activation activities. One mutant, A162E, displayed a behavior similar to S315T, i.e. a moderate decrease in catalase activity and a drastic decrease in the formation of the radical form of INH. Finally, the mutants associated with a low level of INH-R showed a moderate reduction in the four catalytic activities, likely stemming from an overall alteration of the folding and/or stability of the KatG protein.

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1. Introduction

Mycobacterium tuberculosis, the causative agent of tuberculosis, is the second leading cause of death worldwide among known infectious diseases. Isoniazid (INH), the cornerstone of front-line tuberculosis (TB) treatment, is a prodrug that needs activation by the *katG*-encoded catalase-peroxidase [1,2]. The activated form of INH targets the NADH-dependent enoyl-acyl carrier protein reductase InhA of the fatty acid biosynthesis type II system which is involved in the synthesis of mycolic acids [3,4]. Resistance to INH (INH-R) has been previously reported to result mainly from mutations altering the activator protein KatG (~70% of INH-R isolates have a S315T mutation in KatG) [8–10], and secondarily to mutations in the InhA protein, which prevent the activated forms of the drug to bind to the target [3,5], and in the *inhA* promoter that cause overexpression of the target InhA [6,7].

KatG is a heme enzyme of the class I superfamily of fungal, plant, and bacterial heme peroxidases which exhibits both high catalase activity and a broad-spectrum peroxidase activity [11,12]. In *M. tuberculosis*, the catalase-peroxidase is responsible for activating the prodrug INH [1]. Although the details of this chemical transformation are still under investigation, it is hypothesized that INH is

Abbreviations: G/Gox, glucose/glucose oxidase; IN•, isonicotinoyl acyl radical; INH, isoniazid; IN-NAD, isonicotinoyl-NAD; NBT, nitroblue tetrazolium; R, resistance; Rz, optical purity ratio of Reinheitszahl; TB, tuberculosis; tBHP, tert-butyl hydroperoxide; WT, wild-type.

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http://dx.doi.org/10.1016/j.tube.2016.06.002
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converted into an isonicotinoyl radical which binds to NADH/NAD^+/NAD•, resulting in the formation of an isonicotinoyl-NAD (INNAD) adduct which acts as a potent inhibitor of InhA and interferes with cell wall biosynthesis [2,13–16].

KatG is a functional homodimer in which each monomer is composed of two domains (Figure 1). The N-terminal domain contains a heme binding site in which the heme is surrounded by a proximal pocket (made up in part by His270 and Trp321 in Figures 1 and 2A) and a distal pocket (Trp107 and His108 in Figures 1 and 2A). A peculiar structural feature unique to KatG enzymes is the presence of two covalent bonds bridging the side chains of amino acids Trp107, Tyr229 and Met255 in the distal pocket, which is required for the catalase but not the peroxidase activity [17–20]. The KatG heme is accessible to solvent through a narrow channel connecting the distal heme pocket to the outside of the protein. This pocket, which is bordered by amino acid Ser315 and filled by a network of organized water molecules (in cyan in Figures 1, 2A and 2B), has been shown to bind one INH molecule in the crystallographic structures of various KatG enzymes in complex with INH (NIZ-803 in Figure 2A and B) [17,21–23]. Other potential INH binding sites, although remote from the heme, have been reported in various KatG enzymes, such as NIZ-802 and NIZ-804 from *Synechococcus elongatus* and NIZ 749 from *Burkholderia pseudomallei* (Figures 1 and 2B) [24,25].

At the kinetic level, KatG belongs to the class I family of peroxidases [7] and is thus capable of utilizing either hydrogen peroxide or alkyl hydroperoxides to catalyze the oxidation of various substrates, including INH, via high-valent intermediates such as the oxoferryl porphyrin π-cation radical, [KatG Por^{+}•-FeIV=O] and the ferric heme coupled with a protein radical in KatG, [KatG• Por-FeIII] generally referred to as compounds I and II, respectively [21,22,26–28]. In this pathway, the two intermediate compounds I/II of KatG that are produced by oxidation of the enzyme with peroxides, can oxidize each one molecule of INH before returning to the resting state [19,26,29,30]. Additional pathways have been suggested to be involved in the activation process of INH, in which the superoxide moiety, $O_2^{\bullet -}$, would be involved in the formation of the IN-NAD adduct from a ferric-superoxo form of KatG termed compound III ([KatG Por-FeIII-$O_2^{\bullet -}$]) [31,32].

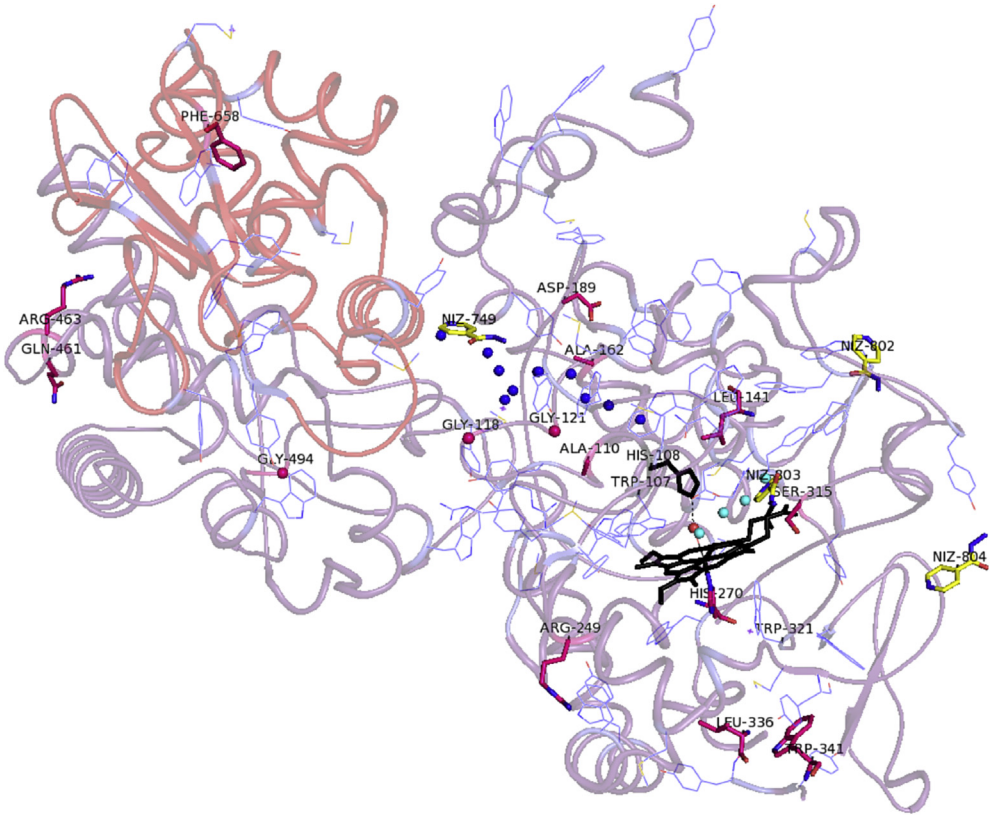

Figure 1. Three-dimensional representation (ribbon model) of KatG of *M. tuberculosis* (PDB entry 2CCA). The heme prosthetic group and the catalytic His108 are shown in black. The mutated residues are represented using red sticks for His270, Trp341, Leu336, Leu141, Ser315, Ala162, Gln461, Asp189, Arg249, Phe658, Ala110 and Arg463, while red spheres are used to represent Gly118, Gly121 and Gly494. The segment of polypeptide chain deleted in the Arg595STOP mutant is shown in orange. The numerous tryptophan, tyrosine and methionine residues present in KatG are shown as thin blue lines. Water molecules (cyan and blue spheres) indicate the location of the two access channels to the heme: a long and wide channel (filled by water molecules represented in blue) that extends from the surface of the protein to the catalytic residue His108, and a narrower short channel connecting the distal heme pocket to the protein surface at the level of residue S315 (corresponding to the water molecules represented in cyan). The yellow INH molecules (NIZ) indicate four potential INH binding sites in KatG which have been positioned by superimposing the 3D structures of KatG of *M. tuberculosis*, *S. elongatus* and *B. pseudomallei* [24,25,28]. The binding site corresponding to NIZ-803, which is generally considered to be the main INH binding site in KatG, has been inferred from crystallographic structures of class I, II, III peroxidases in complex with INH or other small aromatic compounds of similar structure to INH [17,21–23]. It is located in the narrowest part of the channel connecting the distal heme pocket to the protein surface at the level of S315, hence at the entrance to the ε-edge side of the heme [25]. Structural comparisons have revealed that the identity and configuration of the residues in the binding site corresponding to NIZ-803 are very similar among *S. elongatus* KatG, *B. pseudomallei* KatG, and *M. tuberculosis* KatG. NIZ-802 and NIZ-804 have been recently identified in KatG of *S. elongatus*, at the level of the entrance to the γ-edge side of the heme and in front of the heme propionate side chain, respectively [25], and NIZ-749 in KatG of *B. pseudomallei* at the entrance of a long channel connecting the distal pocket to the outside of the protein [24]. In contrast to the binding site corresponding to NIZ-803, the environment of NIZ-802 and NIZ-804 are structurally more diverse among the three KatG proteins of *S. elongatus*, *B. pseudomallei*, and *M. tuberculosis*. Image constructed using PyMOL [53].

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