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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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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

ABSTRACT

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.

67 **Key words:** tuberculosis, resistance, isoniazid, catalase-peroxidase, KatG, enzymatic activities

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100 INTRODUCTION

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

111 KatG is a heme enzyme of the class I superfamily of fungal, plant, and bacterial heme peroxidases
112 which exhibits both high catalase activity and a broad-spectrum peroxidase activity [11, 12]. In *M.*
113 *tuberculosis*, the catalase-peroxidase is responsible for activating the prodrug INH [1]. Although the
114 details of this chemical transformation are still under investigation, it is hypothesized that INH is
115 converted into an isonicotinoyl radical which binds to NADH/NAD⁺/NAD[•], resulting in the
116 formation of an isonicotinoyl-NAD (IN-NAD) adduct which acts as a potent inhibitor of InhA and
117 interferes with cell wall biosynthesis [2, 13-16].

118 KatG is a functional homodimer in which each monomer is composed of two domains (Figure 1).
119 The N-terminal domain contains a heme binding site in which the heme is surrounded by a proximal
120 pocket (made up in part by His270 and Trp321 in Figures 1 and 2A) and a distal pocket (Trp107
121 and His108 in Figures 1 and 2A). A peculiar structural feature unique to KatG enzymes is the
122 presence of two covalent bonds bridging the side chains of amino acids Trp107, Tyr229 and
123 Met255 in the distal pocket, which is required for the catalase but not the peroxidase activity [17-
124 20]. The KatG heme is accessible to solvent through a narrow channel connecting the distal heme
125 pocket to the outside of the protein. This pocket, which is bordered by amino acid Ser315 and filled
126 by a network of organised water molecules (in cyan in Figures 1, 2A and 2B), has been shown to
127 bind one INH molecule in the crystallographic structures of various KatG enzymes in complex with
128 INH (NIZ-803 in Figures 2A and 2B) [17, 21-23]. Other potential INH binding sites, although
129 remote from the heme, have been reported in various KatG enzymes, such as NIZ-802 and NIZ-804
130 from *Synechococcus elongatus* and NIZ 749 from *Burkholderia pseudomallei* (Figures 1 and 2B)
131 [24, 25].

132 At the kinetic level, KatG belongs to the class I family of peroxidases [7] and is thus capable of
 133 utilizing either hydrogen peroxide or alkyl hydroperoxides to catalyze the oxidation of various
 134 substrates, including INH, via high-valent intermediates such as the oxoferryl porphyrin π -cation
 135 radical, [KatG Por⁺•-FeIV=O] and the ferric heme coupled with a protein radical in KatG, [KatG•
 136 Por-FeIII] generally referred to as compounds I and II, respectively [21, 22, 26-28]. In this
 137 pathway, the two intermediate compounds I/II of KatG that are produced by oxidation of the
 138 enzyme with peroxides, can oxidize each one molecule of INH before returning to the resting state
 139 [19, 26, 29, 30]. Additional pathways have been suggested to be involved in the activation process
 140 of INH, in which the superoxide moiety, O₂^{•-}, would be involved in the formation of the IN-NAD
 141 adduct from a ferric-superoxo form of KatG termed compound III ([KatG Por-FeIII-O₂^{•-}]) [31, 32].
 142 In *M. tuberculosis*, INH-R is mainly due to the presence of a mutation in the *katG* gene leading to
 143 the replacement of Ser315 by a threonine. The substitution S315T has been previously suggested to
 144 modify the main INH binding site which is located on the edge of the narrow funnel-shaped cavity
 145 leading to the heme (corresponding to NIZ-803 in Figures 2A and 2B). In fact several hypotheses
 146 have been made from the crystallographic structure of KatG S315T [28] to explain the INH-
 147 resistance of KatG S315T: a) a narrowing of the channel leading to the heme cavity conferring to
 148 the mutant KatG S315T a reduced affinity for INH, b) a reduction of water occupancy in KatG
 149 S315T which exhibits a reduced tendency to form six coordinate heme, with a less favorable
 150 binding to INH, c) an inability of oxyferrous KatG S315T to oxidize isoniazid, d) a slower turnover
 151 of INH linked to the hindrance presented by the narrower channel in Thr315, e) a disruption of the
 152 electron transfer network and f) a loss of a hydrogen bond between the side chain of S315 and a
 153 heme carboxyl group [17, 24, 28, 31-37]. The KatG mutation S315T impairs the conversion of INH
 154 to the IN-NAD adduct but preserves in part the catalase activity which is a key virulence factor in
 155 *M. tuberculosis*. The S315T substitution accounts for INH-R in ~70% of the clinical isolates of *M.*
 156 *tuberculosis* [6, 8, 9, 38-40]. However, in approximately 8 to 10% of the INH-R isolates, other
 157 mutations in KatG are associated with resistance by decreasing the ability of KatG to produce INH
 158 radicals, but the mechanisms by which such mutations alter the KatG activity remain partly
 159 uncharacterized.
 160 In this study, we compare the four main enzymatic activities (catalase, peroxidase, free radical
 161 production and isonicotinoyl-NAD adduct formation) of 13 mutated proteins for which biochemical
 162 activities were never investigated, to those of the wild-type (WT) KatG enzyme of *M. tuberculosis*
 163 and three previously characterized KatG mutants, S315T [8, 9, 38, 39, 41, 42], A110V [38, 41, 42]
 164 and R463L [38, 41-43]. To our knowledge, 8 mutants are reported here for the first time (G118D,

165 G121D, L141S, A162E, D189G, R249H, H270R, Q461), while 5 were previously described but not
166 studied at the enzymatic level (G494D [38], R595STOP [38], F658V [38], L336P [9],
167 W341G [38]).

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169

170 MATERIALS AND METHODS

171

172 Clinical isolates

173 The *M. tuberculosis* isolates were isolated at the National Reference Centre (NRC) for
174 Mycobacteria from TB cases diagnosed in France. They were selected on the basis of their INH-R
175 with (i) a mutation in KatG outside codon 315 and (ii) no mutation in the other genes known to be
176 involved in INH-R (*fabG1-inhA* operon and its upstream region, *furA* and its upstream region, *ndh*),
177 except for the KatG A110V mutant with a -15C→T mutation in the *inhA* promoter. In this study,
178 *katG*, *fabG1-inhA* operon and its upstream region, *furA* and its upstream region, and *ndh* were
179 amplified and sequenced as described before [38, 44, 45]. The mutations included S315T, A110V,
180 G118D, G121D, L141S, A162E, D189G, R249H, H270R, L336P, W341G, Q461P, G494D,
181 R595STOP and F658V detected in INH-R clinical isolates, and the polymorphism R463L found in
182 INH-susceptible isolates. The isolates with KatG A110V, S315T, W341G, G494D, R595STOP,
183 F658V and R463L are from previous studies [6, 38]. The others (G118D, G121D, L141S, A162E,
184 D189G, R249H, H270R, L336P and Q461P) are from the NRC collection and have not been
185 described before, except L336P [9]. Isolates with KatG G118D, L141S, R249H, H270R, L336P,
186 Q461P, G494D, R595STOP were INH mono-resistant, while isolates with KatG A110V, G121D,
187 A162E, D189G, S315T, W341G, F658V were multidrug-resistant.

188

189 Drug susceptibility testing

190 Susceptibility testing was performed using the proportion method on Löwenstein-Jensen medium
191 [46] at INH concentrations of 0.1, 0.2, 1 and 10 mg/l. A low level of resistance was defined as
192 resistance to ≥ 0.2 but < 1 mg/l, while a high level of resistance was defined as resistance to ≥ 1 mg/l
193 of INH. In the present study we distinguished between isolates resistant to ≥ 1 mg/l but < 10 mg/l
194 and isolates resistant to ≥ 10 mg/l.

195

196 Plasmid preparation

197 Cloning of the *katG* gene to produce hexahistidine-tagged KatG proteins.

198 For KatG WT, the coding region of *katG* from H37Rv was amplified by PCR with the primers
199 LIC1-*katG* (5'-GAC GAC GAC AAG ATG CCC GAG CAA CAC CCA CC-3') and LIC2-*katG*
200 (5'-GAG GAG AAG CCC GGT TCA GCG CAC GTC GAA CCT-3') and cloned in the pET30
201 vector in NovaBlue GigaSingles^{TM} competent *E. coli* cells using the pET-30 Ek/LIC Vector Kit
202 (Novagen) to produce hexahistidine-tagged proteins (His₆-KatG). To obtain resistant mutants,
203 mutagenesis was performed according to the manufacturer's protocol using the QuikChange® site-
204 directed mutagenesis kit from Stratagene with the primers listed in Table 1. The mutated plasmids
205 were introduced by transformation into XL1-Blue supercompetent *E. coli* cells.

206 Cloning of the *katG* gene to produce non-His₆-KatG proteins.

207 From the plasmid pET30-KatG WT, we performed site-directed mutagenesis to remove the
208 restriction site of *NdeI* in *katG* at nucleotide 75 (Table 1). After PCR amplification of the *katG* gene
209 with a primer containing the restriction site for *NdeI* and the 5' end of *katG* (primer KatGNdeI : 5'-
210 CAT ATG CCC GAG CAA CAC C-3') and a primer containing the restriction site for *HindIII* and
211 the 3' end of *katG* (primer KatGHindIII : 5'-AAG CTT TCA GCG CAC GTC G-3'), subcloning of
212 the amplified product in TOPO was achieved with the TOPO TA cloning® kit (Invitrogen). Then,
213 the plasmid pCR®2.1-TOPO®-KatG was digested with *NdeI* and *HindIII*, and the digestion product
214 cloned into pET29 digested with the same enzymes. The ligation product was introduced into TOP-
215 10 *E. coli* cells by electroporation.

216 XL1-Blue (for His₆-KatG) or TOP-10 (for non-His₆-KatG) *Escherichia coli* cells with plasmids
217 pET30-*katG* and pET29-*katG*, respectively, were grown on Luria Bertani (LB) agar plates
218 containing 30 µg/ml (30γ) kanamycin. Liquid cultures of LB-kanamycin 30γ were inoculated with
219 individual colonies, grown overnight at 37°C and the bacteria pelleted by centrifugation. Plasmid
220 DNA was extracted and purified from the bacterial pellet using the QIAprep Spin Miniprep® kit
221 (Qiagen). Sequencing of double-stranded plasmid DNA was used to confirm the desired nucleotide
222 substitutions and the absence of secondary mutations.

223

224 **Production of the KatG proteins**

225 The recombinant vectors pET30-His₆-*katG* and pET29-*katG* were introduced by transformation into
226 BL-21(DE3)pLysS *E. coli* cells (Stratagene) and the cells plated onto LB-kanamycin 30γ plates and
227 grown overnight at 37°C [31, 32]. Starter cultures (LB-kanamycin 30γ; 4 ml each) were inoculated
228 with a single colony and grown to an optical density of 0.5-1. Each starter culture was further used
229 to inoculate 50 ml of LB medium containing 30 mg/l hemin (dissolved in 0.2N NaOH) and 30γ
230 kanamycin. The addition of hemin ensures stoichiometric incorporation of the heme cofactor during

231 overexpression in *E. coli* for maximal holoenzyme isolation [47]. For BL21 cells with pET29-*katG*,
232 additional cultures were used to inoculate 500 ml of the same LB medium. Expression of the cloned
233 genes was then induced with isopropyl β -D-1-thiogalactopyranoside (1 mM final concentration) and
234 the cultures were grown overnight at 18°C. After centrifugation, the cell pellet was resuspended and
235 lysed by sonication in an ice bucket. Cellular debris was pelleted at 18,000 x *g* for 60 min, resulting
236 in a viscous, red-brown crude extract (except for the H270R mutant that had no color).

238 **Purification of the catalase/peroxidase KatG**

239 Hexahistidine-tagged KatG.

240 KatG proteins were purified from the crude extracts using the Ni-NTA His·Bind® resin and the
241 His·Bind® Buffer Kit from Novagen, following the manufacturer's instructions.

242 Non-hexahistidine-tagged KatG

243 Nucleic acids were precipitated by the addition of spermine (1% w/v, final concentration) to the
244 supernatant (15 min in ice), and the solution was centrifuged for 1 hr at 48,000 x *g* to pellet the
245 nucleic acids. Dialysis against 20 mM Bis Tris, pH 6.0, was carried out overnight and the dialysate
246 filtered (pore size, 0.22 μ m). The clear supernatant was applied to a Q-Sepharose HiTrap™ Q HP 5
247 ml (GE Healthcare Life Sciences) column equilibrated with 20 mM Bis Tris, pH 6.0. The adsorbed
248 proteins were eluted using a linear 0 to 1-M NaCl gradient. Fractions containing catalase activity,
249 which eluted between 0.3 and 0.4 M NaCl, were pooled, dialyzed at 4°C overnight against 20 mM
250 Bis Tris, pH 6.0, and applied to a DEAE-Sepharose (GE Healthcare Life Sciences) anion-exchange
251 column equilibrated with Bis Tris 20 mM, pH 6.0. The adsorbed proteins were eluted using a linear
252 0 to 1-M NaCl gradient. The active fractions were pooled, concentrated on Amicon® Ultra filters
253 (Millipore) and subjected to gel filtration on a Superdex™ 200 (GE Healthcare Life Sciences)
254 column in 20 mM Bis Tris, 50 mM NaCl, pH 6.0 [11, 26, 48].

256 **In-vitro KatG enzymatic activities**

257 Protein concentration was determined using the heme extinction coefficient $\epsilon_{407\text{nm}} = 100 \text{ mM}^{-1} \text{ cm}^{-1}$
258 [28], except for the H270R mutant that contained no heme and for which the protein concentration
259 was determined using the Bradford method with a Nanodrop® spectrophotometer (Thermo
260 Scientific). All assays were performed in triplicate.

261 The catalase activity was determined spectrophotometrically by measuring the decrease in H₂O₂
262 concentration at 240 nm ($\epsilon_{240\text{nm}} = 0.0435 \text{ mM}^{-1} \text{ cm}^{-1}$). The reaction mixture contained 50 mM
263 sodium phosphate buffer, pH 7.5, 25 mM H₂O₂ and 30 nM KatG (up to 2 μ M for mutants with low

264 catalase activity) [48, 49]. One unit (U) of catalase activity corresponded to the consumption of 1
265 $\mu\text{mole H}_2\text{O}_2/\text{min}/\text{mg}$ of protein. The apparent K_m and k_{cat} values were obtained from non-linear
266 regression of Michaelis-Menten [32].

267 The peroxidase activity was determined spectrophotometrically by measuring the rate of oxidation
268 of 0.1 mM *O*-dianisidine, at 460 nm ($\epsilon_{460\text{nm}} = 11.3 \text{ mM}^{-1} \text{ cm}^{-1}$) in the presence of 23 mM tert-butyl
269 hydroperoxide (tBHP) in 50 mM sodium acetate buffer, pH 5.5 and 100 nM KatG (up to 2 μM for
270 KatG H270R) [48, 50]. One U of peroxidase activity was defined as the oxidation of 1 mmole *O*-
271 dianisidine/min/mg of protein.

272 The free radical production was followed at 560 nm using the INH-dependent reduction of nitroblue
273 tetrazolium (NBT) to mono- and diformazan ($\epsilon_{560\text{nm}} = 15,000 \text{ M}^{-1} \text{ cm}^{-1}$ for monoformazan) [49, 50].
274 The reaction mixture consisted of 0.2 mM NBT in 50 mM Tris HCl buffer (pH 8.5) containing INH
275 (7.5 mM) and enzyme (30 nM, or up to 2 μM for KatG H270R). The reactions were initiated by the
276 addition of H_2O_2 (500 μM). One U of free radical production activity was defined as 1 nmole NBT
277 reduced to monoformazan/min/nmole of heme.

278 The rate of isonicotinoyl-NAD (IN-NAD) adduct formation was determined spectrophotometrically
279 at 326 nm using the extinction coefficient of isonicotinoyl-NAD ($\epsilon_{326\text{nm}} = 6.900 \text{ M}^{-1} \text{ cm}^{-1}$) [13, 28,
280 31, 50-52]. The reaction was carried out using KatG (2 μM), NAD^+ (240 μM), the H_2O_2 -generating
281 system glucose/glucose oxidase (G/Gox) [glucose oxidase (66.6 mU/mL), glucose (16.7 mM)] [31]
282 and INH (200 μM) in 50mM sodium phosphate, pH 7.5). The reference cuvette contained all
283 components except NAD^+ to correct for background activity, as previously described [28, 37]. The
284 generation of adduct was initiated by the addition of INH (200 μM). One U of isonicotinoyl-NAD
285 formation activity was defined as the production of 1 nmole isonicotinoyl-NAD/min/nmole of
286 heme.

287

288 **Three-dimensional modeling**

289 The location of the mutations detected in KatG was investigated using the crystal structure of the *M.*
290 *tuberculosis* KatG protein (PDB entry 2CCA) [28] with PyMol software [53].

291

292 **Nucleotide sequence accession numbers.**

293 The nucleotide sequences determined for the *katG* mutants were deposited in the GenBank database
294 under accession numbers **KC122363** to **KC122378**.

295

296

297 **RESULTS**

298

299 **Purification of KatG proteins**

300 In this study, two non-His₆-KatG proteins (KatG WT and the S315T mutant) and 17 His₆-KatG
301 proteins (KatG WT, the S315T mutant, the polymorphism R463L mutant, and 14 other KatG
302 mutants) were produced. The His₆-tag was introduced to facilitate the purification of the protein by
303 nickel affinity chromatography, and the two non-His₆-KatG proteins, KatG WT and the S315T
304 mutant, were purified in order to verify that the presence of the His₆-tag did not alter the
305 biochemical and the enzymatic properties of KatG. The two non-tagged proteins had an optical
306 purity ratio (Reinheitszahl: Rz) (heme Soret absorbance/total protein absorbance: A_{408}/A_{280}) [54] of
307 0.58 and 0.55, respectively and were obtained with typical yields of ~6 mg per liter of culture. They
308 displayed a single band of ~80,000 daltons on SDS-PAGE, and of ~160,000 daltons on non-
309 denaturing PAGE (data not shown). The His₆-KatG proteins were purified using immobilized metal
310 affinity chromatography which yielded brown protein solutions with an $R_z > 0.5$, except for the
311 colorless KatG H270R mutant for which R_z was < 0.01 . The typical yield of purified protein was
312 ~25 mg per 100 ml of culture, except for the two mutants R595STOP and Q461P for which 2 mg of
313 protein were obtained. All mutant proteins had an apparent molecular weight of ~80,000 daltons on
314 SDS-PAGE, except R595STOP with ~65,000 daltons (data not shown).

315

316 **Enzymatic activities**

317 Before studying the enzymatic activities of the KatG mutants, we tested whether the His₆-tag
318 introduced to facilitate the purification of the proteins affected these activities. The K_m and k_{cat}
319 values for catalase activity of His₆-KatG WT were nearly identical to those of non-tagged KatG WT
320 (~10 mM and ~4.000 s⁻¹, respectively). These values were in accordance with previously reported
321 values, ranging from 0.6 to 30 mM and from 2,300 to 10,000 s⁻¹, respectively [11, 31, 32, 48, 49,
322 54-56]. We also comparatively assayed for the WT and the S315T mutant the catalase and
323 peroxidase activities, free radical production and IN-NAD adduct formation of the His₆- and non-
324 His₆-KatG proteins which yielded very similar values (Table 2).

325 The catalase activity of the His₆-KatG WT enzyme was 2023±58 U and its peroxidase activity was
326 0.91±0.03 U. As for free radical production (NBT reduction), we noted a significant background
327 activity of NBT reduction in the absence of isoniazid with the WT enzyme. However, NBT
328 reduction was substantially enhanced in the presence of isoniazid, allowing us to determine after
329 subtraction of the background activity [43, 56] the net INH-dependent reduction of NBT (free

radical production) which was 2.01 ± 0.15 U (Table 2). Finally, isonicotinoyl-NAD adduct formation in the absence of KatG was undetectable with the H_2O_2 -generating G/Gox system (data not shown) [31]. This adduct formation by His₆-KatG WT was 0.78 ± 0.02 , consistent with previously reported values [33, 37, 50, 57].

Fifteen KatG mutants from INH-R clinical isolates and the R463L mutant found in INH-susceptible clinical isolates (R463L is a phenotypically silent polymorphism) were studied comparatively to the WT enzyme (Table 2). As mentioned above, 8 have never been reported before (G118D, G121D, L141S, A162E, D189G, R249H, H270R, Q461), 5 were previously described but not studied (G494D [38], R595STOP [38], F658V [38], L336P [9], W341G [38]) and 3 were reported and their biochemical activities were investigated (S315T [8, 9, 38, 39, 41, 42], A110V [38, 41, 42], R463L [38, 41-43]). Here we report their enzymatic activities relative to those of KatG WT. The 16 KatG mutants could be categorized into six distinct groups based on the activity profiles given in Table 2. First, H270R, which was purified from a very high-level INH-R clinical isolate, was the only mutant for which no enzymatic activity was detectable (Table 2). The second group included five mutants (R595STOP, L336P, W341G, G118D, L141S) obtained from very high-level INH-R isolates and one mutant (G121D) from a high-level INH-R isolate. Overall, the six mutants were characterized by a drastic decrease in their enzymatic activities. Catalase activity was virtually undetectable in four (R595STOP, L336P, W341G and G118D), while the remaining two mutants (G121D and L141S) had very low residual activity (i.e. 14 and 2% of the KatG WT activity, respectively) (Table 2). Concomitantly, the six mutants displayed a marked alteration of free radical production activity, with values ranging from 7 to 19% (Table 2). On the other hand, the mutants, which were all impaired in their peroxidase activity and their capacity to form IN-NAD adducts, retained significant residual activities ranging from 21 to 45% for the peroxidase activity, and from 12 to 41% for the IN-NAD formation (Table 2).

The third group included two mutants, S315T and A162E, from high-level and very high-level INH-R isolates, respectively. Both were characterized by a sharp decrease in free radical production efficiency (residual activities of 9 and 26%, respectively) but were only moderately affected in their catalase and peroxidase activities (55 and 70% and 70 and 118%, respectively). S315T displayed a drastic decrease in IN-NAD formation activity (10%), while A162E appeared to be less affected (56%).

Five mutants were included in the fourth group. One of them (G494D) was identified in a high-level INH-R isolate, and four (Q461P, D189G, R249H, and F658V) were found in low-level INH-R isolates (Table 2). Overall, these five mutants showed moderately impaired enzymatic activities

when compared to the abovementioned mutants, with values ranging from 43 to 83% for catalase activity, 45 to 88% for free radical production, 72 to 109% for peroxidase activity, and 60 to 78% for IN-NAD formation (Table 2).

KatG mutant A110V was considered to form a group of its own because it showed moderately decreased catalase and free radical production activities (71 and 73%, respectively) while its peroxidase activity was markedly increased (198% compared to KatG WT). This mutant was obtained from a low-level INH-R isolate (Table 2).

Finally, mutant R463L, obtained from an INH-susceptible clinical isolate, represented a phenotypically silent polymorphism that can be found in INH-susceptible and INH-R isolates [38, 55]. When compared to KatG WT, R463L showed no variation in free radical production and the three other activities were only slightly lower (Table 2).

DISCUSSION

Analysis of the enzymatic parameters allowed the distinction of several categories of mutants that are discussed below (Table 3).

Mutants with no detectable enzymatic activity

All enzymatic activities of the KatG mutant H270R found in a very high-level INH-R isolate were abolished. The histidine residue at position 270 of KatG WT is located in the proximal pocket and forms an essential link of coordination with the iron of the heme (Figure, 2A). In the H270R protein, the mutation creates a steric hindrance and causes loss of the coordination link to the heme. Consequently, the heme cannot bind to KatG, as demonstrated by the Rz value <0.01 found for the purified H270R protein. This heme defect in KatG accounts for the complete loss of enzymatic activity of the H270R mutant.

Mutants with drastically decreased catalase and free radical production activities

The KatG R595STOP mutation, associated with a very high level of INH-R, creates a truncated protein in which 145 amino acid residues are deleted in the C-terminal domain (shown in orange in Figure 1). The yield of purification of the His₆-KatG R595STOP protein was lower than those obtained for the other proteins (2 mg versus 25 mg, respectively), indicating that the folding and/or the stability of the C-terminally truncated protein could be markedly altered. Nevertheless, the Rz value of >0.5 indicated that there is no alteration of heme binding in the active site. The catalase

activity of the R595STOP mutant was completely abolished, showing that the C-terminal region is important for this activity, even if it is generally considered to make no direct contribution to the catalytic mechanism [58]. By contrast, this mutant exhibited drastically reduced but still measurable peroxidase, free radical production and IN-NAD formation activities (Table 2), indicating that secondary oxido-reduction pathways decoupled from the catalase mechanism are still functional in the mutant protein, as previously suggested [11, 17, 31, 55].

The two mutants W341G and L336P were characterized by undetectable catalase activity and drastically reduced free-radical production activity (Table 2). They also displayed significant reduction in their peroxidase and IN-NAD formation activities. The two residues are located in the proximal pocket in the vicinity of Met377 (mean distance of ~ 4.1 Å) (Figure 2A) which is at 3.9 Å to Trp321 in the essential triad His270, Trp321, Asp381 (Figure 2A). Previous studies have shown that mutations in the 3 latter amino acids of the proximal triad of KatG in different bacteria are generally shown to be responsible for less than 0.1% catalase activity compared to the WT enzyme [42, 59]. In the 2 mutants W341G and L336P, which are both drastically affected in their catalase activity, the replacement of Trp341 by a glycine creates a cavity exposing Met377 to the solvent, while the introduction of a proline at position 336 probably alters the folding of the alpha-helix holding this residue and generates more room around Met377. As a consequence, both mutations induce critical structural alterations in a region essential for the catalase activity of KatG and the formation of compound I [KatG Por⁺•FeIV=O] required for the oxidation of INH to IN•.

G118D and G121D are above the distal pocket containing the essential catalytic residue His108 (Figure 2B). Both mutants were associated with high to very high levels of INH-R and had very similar enzymatic properties with a dramatic decrease in catalase and free radical production activities (Table 2). In the 3D structure of KatG, Gly118 and Gly121 are localized at the solvent-exposed face of a long tunnel constituting the back door entrance of the distal pocket connecting the catalytic residue His108 to the outside of the protein, which contains an extended network of hydrogen-bonded water molecules and could be an equivalent of the access channel of H₂O₂ in the mono-functional catalases (Figure 2B). In both mutants, the replacement of Gly118 and Gly121 by an aspartic acid could disrupt the integrity of this channel by creating steric clashes and possibly electrostatic interactions with the side chains of two neighbouring arginine residues, Arg484 (with Asp118) and Arg418 (with Asp121) (Figure 2B), the latter acting like a catalytic switch that can be oriented toward the solvent or toward the essential Met255-Tyr229-Trp107 triad (see the two orientations in Figure 2B) [60]. One can note that G118D and G121D mutants maintained a significant level of peroxidase activity, while the catalase activity was very low (Table 2),

429 suggesting that oxidation of the heme by tBHP involves oxydo-reduction pathways distinct from
430 those involved during H₂O₂ oxidation.

431 The L141S mutant, which was detected in a very high-level INH-R isolate, displays an extremely
432 low level of catalase activity and a drastic reduction of free radical and IN-NAD production.
433 Leu141 is located next to the distal pocket at a close distance (3.8 Å) to the ring of the key catalytic
434 residue His108 (Figure 2B). It is therefore likely that Leu141 prevents the side chain of His108 to
435 move away from the iron atom and the Trp107-Tyr229-Met255 triad which participates in the
436 stabilization of the radical formed after initial oxidation by H₂O₂. The replacement of Leu141 by a
437 serine not only creates room allowing His108 to shift from its optimal position, but also introduces
438 a polar side chain in the heme region that may disturb the electron transfer pathways required for
439 the catalase activity. Of note is the fact that the mutant displayed a significant level of peroxidase
440 activity like the other mutants described above.

441

442 **Mutants with drastically decreased free radical production activity but retaining significant** 443 **catalase/peroxidase activity**

444 Mutants S315T and A162E, which were respectively found in isolates with high- and very high-
445 levels of INH-R displayed a unique enzymatic behaviour in the sense that there is a marked
446 decoupling in both mutants between the catalase and peroxidase activities, which decreased slightly,
447 and the activities of IN• production and formation of the IN-NAD adduct which decreased sharply
448 (Table 2).

449 S315T, which is the most commonly occurring mutation in clinical isolates (found in ~70% of INH-
450 R isolates), has been extensively studied in the past. In concordance with previous reports [28, 31,
451 32, 41, 49, 54], we observed that S315T showed a moderate reduction in catalase and peroxidase
452 activities, contrasting with the dramatic reduction in IN• and IN-NAD formation activities (Table
453 2), indicating that S315T specifically affects the binding and/or processing of INH. Accordingly,
454 S315T is located in the immediate vicinity of the heme, in the narrowest part of a short funnel-
455 shaped channel connecting the heme pocket filled with water molecules (in cyan on Figure 2B) to
456 the outside of the protein. This channel has been previously suggested to contain the primary INH
457 binding site (NIZ-803 on Figure 2B) and the bulkier side chain of Thr315 would impede the access
458 of INH to the heme pocket [28]. This model readily explains the decoupling of the effects observed
459 for the catalase/peroxidase activities on one hand, which are in part preserved, and the loss of
460 activation of INH on the other. However, one has to note here that alternative hypotheses have been

461 proposed which relate the Ser315 mutation to structural alterations affecting the porphyrin moiety
462 and the electron transfer processes (as detailed in the Introduction section) [17, 24, 28, 31-37].
463 The behaviour of the A162E mutant is more puzzling. In the structure of KatG, Ala162 is located at
464 the N-terminal part of an alpha-helix, at a very long distance to the heme pocket (Figure 2B). The
465 side chain of Glu162, which lies very close to the solvent-exposed face of the long tunnel leading to
466 the distal pocket, creates steric clashes with nearby residues, in particular Gly123 and Gly124
467 located downstream of Gly118-Gly121. It is therefore conceivable that the mutation A162E
468 decreases the rate of free radical production (Table 2), as observed in the 2 mutants G118D and
469 G121D. On the other hand, the limited effects observed on the catalase and peroxidase activities,
470 are more difficult to explain.

471

472 **Mutants showing moderately decreased enzymatic activities**

473 We studied five mutants (G494D, Q461P, D189G, R249H, and F658V) that were associated with
474 low-level of INH-R (except G494D found in a high-level resistant isolate) and that had similar
475 catalytic effects, i.e. a moderate (G494D, Q461P and D189G) to weak (R249H and F658V) overall
476 reduction of all measured activities that decreased within the range of 6 to 57% (Table 2). In the
477 KatG structure, the mutated residues are remote from the heme active site, except R249H which is
478 found in the middle of an alpha-helix located at the edge of the heme pocket (Figure 1). Despite its
479 critical position with respect to the heme, the replacement of Arg249 by an histidine has limited
480 impact on the enzymatic activities of KatG (Table 2), probably because the corresponding side
481 chain is oriented toward the solvent (Figure 1). The four remaining mutations, G494D, Q461P,
482 D189G and F658V are distant from the heme pocket, G494D, Q461P and F658V being located in
483 the C-terminal domain of KatG (Figure 1). Interestingly, the purification yield of the Q461P mutant
484 was significantly lower when compared to that of the other proteins (2 mg versus ca 25 mg,
485 respectively), suggesting that the introduction of a proline at position 461 significantly impairs the
486 folding and/or the stability of KatG. In contrast, the replacements in the 3 remaining mutants did not
487 modify the purification yield or the Rz value. Nevertheless, the mutations gave rise to an overall
488 impairment of the enzymatic activities (by ~50%) which may be ascribed to steric effects (F658V)
489 and/or to modifications of ionic and polar contacts with neighboring residues (G494D and D189G).

490

491 **Mutants displaying a moderate effect on catalase and INH activation, with an increased** 492 **peroxidase activity**

493 The A110V mutant was obtained from a low-level INH-R isolate, which also harboured a -15C→T
494 mutation in the *inhA* promoter known to confer a low-level of INH-R. It is therefore difficult to
495 establish the contribution of the KatG A110V mutation in the INH-R of this isolate [6, 7]. The
496 residue Ala110 is located at the level of the heme pocket, not far from the catalytic residue His108
497 and the Trp107 of the Met255-Tyr229-Trp107 adduct (Figure 2B). Although inexplicable at the
498 molecular level at the present state of our knowledge, our kinetic results are consistent with
499 published observations [17, 40, 56] and confirm that the bulkier side chain of Val110 restricts the
500 degree of freedom of the helix bearing His108, which moderately reduces the catalase and the IN•
501 and IN-NAD formation activities (Table 2), but increases by 2 fold the peroxidase activity
502 compared to that of WT KatG. Additionally, they confirm that there is decoupling of enzymatic
503 functions in KatG.

504

505 **Mutants that do not modify the free radical production activity and do not confer INH-R**

506 The R463L mutation is frequently detected in INH-susceptible isolates. The residue is located far
507 away from the porphyrin ring (56 Å) (Figure 1). As shown by our enzymatic measurements, this
508 mutation has a very limited impact on the four enzymatic activities (Table 2). These results are
509 consistent with published data and with the view that this mutation is an instance of a
510 phenotypically silent polymorphism [31, 41, 54, 55].

511

512

513 **CONCLUSION**

514 Considering the current state of our knowledge, reaching a comprehensive understanding of the role
515 of KatG mutations in the enzymatic perturbations observed in KatG remains a demanding task.
516 Concluding from the observations made with the mutations identified in high-level INH-R isolates
517 (Table 3), 7 mutants from very high-level (H270R, R595STOP, L336P, W341G, G118D, and
518 L141S) or high-level (G121D) INH-R showed a drastic decrease in their catalase activity, resulting
519 in the impairment of compound I [KatG Por⁺•-FeIV=O] formation in the presence of hydrogen
520 peroxide. As a consequence, the rate of IN• production in the presence of H₂O₂ (i.e. free radical
521 production), which requires the formation of compound I, is dramatically affected when catalase
522 activity is lost. However, one can note that these mutants generally retained significant peroxidase
523 activity, an observation which sustains the hypothesis that oxidation of the heme in KatG would
524 occur through two distinct routes according to the type of oxidizing molecule used in the reaction,
525 i.e. the H₂O₂ pathway on one hand and the alkyl peroxide pathway (tBHP in this study) on the

526 other. Two other mutants from high- or very high-level INH-R isolates (S315T and A162E,
527 respectively) exhibited an optimal balance between drastic decrease in free radical production and
528 relative conservation of catalase and peroxidase activities, a property that may confer a survival
529 advantage to the bacterial cell and that has been suggested previously to account for the global
530 spread of isolates with the KatG S315T mutation [61]. Conversely, the mutants associated with
531 low-level of INH-R, such as Q461P, D189G, R249H and F658V, were generally characterized by a
532 moderate reduction in the four catalytic activities. Finally, it is worth highlighting that there was no
533 strict correlation between the level of INH-R and the IN-NAD activities of the KatG mutants in our
534 study, in accordance with previous reports [31]. However, the mutants from high- (HL1) to very
535 high-level (HL10) INH-R isolates displayed relative activities below 50%, while those from low-
536 level resistant isolates had relative activities above that value (Table 2).

537

538

539

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543

544

545 **COMPETING INTERESTS**

546 None declared.

547

548

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552

553

554 **ETHICAL APPROVAL**

555 Not required.

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753 **Table 1. Mutagenic primers employed in the site-directed mutagenesis of WT KatG using the**
754 **QuickChange XL kit. The changed bases are in boldface.**

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A110V	5'-GGC GTG GCA CGC TGT CGG CAC CTA CCG CAT CC-3' 3'-GG ATG CGG TAG GTG CCG ACA GCG TGC CAC GCC-5'
G118D	5'-CCG CAT CCA CGA CGA CCG CGG CGG C-3' 5'-G CCG CCG CGG TCG TCG TGG ATG CGG-3'
G121D	5'-CGA CGG CCG CGG CGA CGC CGG GGG CGG C-3' 3'-G CCG CCC CCG GCG TCG CCG CGG CCG TCG-3'
L141S	5'-CCC GAC AAC GCC AGC TCG GAC AAG GCG CG-3' 5'-CG CGC CTT GTC CGA GCT GGC GTT GTC GGG-3'
A162E	5'-GCT CTC ATG GGA GGA CCT GAT TGT TTT CGC-3' 5'-GCG AAA ACA ATC AGG TCC TCC CAT GAG AGC-3'
D189G	5'-GCT TCG GCC GGG TCG GCC AGT GGG AGC-3' 5'-GCT CCC ACT GGC CGA CCC GGC CGA AGC-3'
R249H	5'-CGG CGG TCG ACA TTC ACG AGA CGT TTC GG-3' 5'-CC GAA ACG TCT CGT GAA TGT CGA CCG CCG-3'
H270R	5'-CGT CGG CGG TCG CAC TTT CGG TAA GAC CC-3' 5'-GG GTC TTA CCG AAA GTG CGA CCG CCG ACG-3'
S315T	5'-GGA CGC GAT CAC CAC CGG CAT CGA GGT CG-3' 5'-CG ACC TCG ATG CGG GTG GTG ATC GCG TCC-5'
L336P	5'-CCT CGA GAT CCC GTA CGG CTA CGA GTG GG-3' 5'-CC CAC TCG TAG CCG TAC GGG ATC TCG AGG-3'
W341G	5'-CCT GTA CGG CTA CGA GGG GGA GCT GAC GAA GAG CC-3' 5'-GGC TCT TCG TCA GCT CCC CCT CGT AGC CGT ACA GG-3'
Q461P	5'-CCA GCC TTA AGA GCC CGA TCC GGG CAT CGG G-3' 3'-C CCG ATG CCC GGA TCG GGC TCT TAA GGC GGT-3'
R463L	5'-GCC AGC CTT AAG AGC CAG ATC CTG GCA TCG GGA TTG ACT G-3' 5'-C AGT CAA TCC CGA TGC CAG GAT CTG GCT CTT AAG GCT GGC-3'
G494D	5'-GGC GGC GCC AAC GAT GGT CGC ATC CGC CTG C-3' 3'-G CAG GCG GAT GCG CCA ATC GTT GGC GCC GCC-5'
R595STOP	5'-GGC AGA TGG CTT CTG AAA CTA CCT CGG AAA GGG-3'

	5'-CCC TTT CCG AGG GAT TTT CAG AAG CCA TCT GCC-3'
F658V	5'-CAC TGA CCA ACG ACT TCG TCG TGA ACC TGC TCG-3'
	5'-CGA GCA GGT TCA CGA CGT AGT CGT TGG TCA GTG-3'
WT	5'-CCC GTC GTG GGT CAC ^a ATG AAA TAC CCC GTC G-5'
	5'-CGA CGG GGT ATT TCA TGT GAC CCA CGA CGG G-3'
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757	^a (nt 75: T→C)
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784 **Table 2. Enzymatic activities of KatG wild-type and 16 KatG mutants**

KatG ^b	Enzymatic activity (U) ^a				Level of INH resistance ^c
	Catalase	Peroxidase	Free radical production	Isonicotinoyl-NAD formation	
WT	2023±58	0.91±0.03	2.01±0.15	0.78±0.02	S
non-His ₆ WT	2225±101	0.89±0.04	2.60±0.04	0.73±0.02	S
H270R	<2 (<0.1%)	<0.01 (<0.1%)	<0.01 (<0.1%)	<0.01 (<0.1%)	R (HL10)
R595STOP	<2 (<0.1%)	0.19±0.01 (21%)	0.28±0.02 (14%)	0.09±0.02 (12%)	R (HL10)
L336P	<2 (<0.1%)	0.23±0.02 (25%)	0.38±0.04 (19%)	0.12±0.01 (15%)	R (HL10)
W341G	<2 (<0.1%)	0.31±0.03 (34%)	0.26±0.02 (13%)	0.32±0.02 (41%)	R (HL10)
G118D	<2 (<0.1%)	0.27±0.01 (30%)	0.16±0.04 (8%)	0.23±0.03 (29%)	R (HL10)
G121D	283±4 (14%)	0.40±0.01 (44%)	0.14±0.02 (7%)	0.16±0.03 (21%)	R (HL1)
L141S	40±4 (2%)	0.41±0.04 (45%)	0.28±0.10 (14%)	0.24±0.02 (31%)	R (HL10)
A162E	1416±40 (70%)	1.07±0.02 (118%)	0.52±0.06 (26%)	0.44±0.01 (56%)	R (HL10)
S315T	1112±40 (55%)	0.64±0.04 (70%)	0.18±0.02 (9%)	0.08±0.01 (10%)	R (HL1)
Non-His ₆ S315T	951±121 (47%)	0.47±0.01 (52%)	0.18±0.02 (9%)	0.07±0.01 (9%)	R (HL1)
G494D	870±40 (43%)	0.65±0.02 (72%)	1.04±0.04 (52%)	0.55±0.03 (70%)	R (HL1)
Q461P	890±20 (44%)	0.53±0.01 (58%)	0.90±0.06 (45%)	0.55±0.04 (71%)	R (LL)

D189G	1072±182 (53%)	0.54±0.01 (59%)	0.98±0.08 (49%)	0.47±0.01 (60%)	R (LL)
R249H	1497±61 (74%)	0.70±0.02 (77%)	1.63±0.14 (81%)	0.54±0.02 (69%)	R (LL)
F658V	1679±40 (83%)	0.99±0.03 (109%)	1.77±0.14 (88%)	0.61±0.02 (78%)	R (LL)
A110V	1436±40 (71%)	1.80±0.02 (198%)	1.47±0.04 (73%)	0.31±0.02 (40%)	R (LL)
R463L	1598±101 (79%)	0.81±0.03 (89%)	2.05±0.06 (102%)	0.67±0.01 (86%)	S

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786 ^a (%) = Normalized to the value obtained for WT KatG as 100%.

787 ^b WT= wild-type

788 ^c S= susceptible; R= resistant; LL= low-level resistant, defined as resistant to >0.1 but <1 mg/l of
789 INH; HL= high-level resistant, defined as resistant to ≥1 mg/l; HL1= resistant to ≥1 mg/l but <10
790 mg/l; HL10= resistant to ≥10 mg/l.

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808 **Table 3. Correlation between the different classes of mutants and changes in amino acids in**
809 **KatG**

Classes of mutants	KatG	Location of the mutation	Level of INH resistance
Mutants with no detectable enzymatic activity	H270R	Loss of the coordination link between H270 and the iron of the heme	R (HL10)
Mutants with drastically decreased catalase and free radical production activities	R595STOP	Deletion of the C-terminal domain	R (HL10)
	W341G	In the proximal pocket, close to the proximal triad	R (HL10)
	L336P	In the proximal pocket, close to the proximal triad	R (HL10)
	G118D	In the long tunnel connecting the entrance to the distal pocket	R (HL10)
	G121D	In the long tunnel connecting the entrance to the distal pocket	R (HL1)
Mutants with drastically decreased free radical production activity but retaining significant catalase/peroxidase activity	L141S	Next to the distal pocket and the key catalytic residue H108	R (HL10)
	S315T	In the short channel connecting the heme pocket to the outside of the protein	R (HL1)
Mutants showing moderately decreased enzymatic activities	A162E	In the long tunnel connecting the entrance to the distal pocket	R (HL10)
	G494D	Remote from the heme	R (HL1)
	Q461P	Remote from the heme	R (LL)
	D189G	Remote from the heme	R (LL)
	R249H	At the edge of the heme pocket, with a solvent-exposed side-chain	R (LL)
Mutants displaying a moderate effect on catalase and INH activation, with an increased peroxidase activity	F658V	Remote from the heme	R (LL)
	A110V	At the level of the heme pocket, not far from the catalytic residue H108 and the M255-Y229-W107 adduct	R (LL)
Mutants that do not modify the free radical production activity and do not confer INH-R	R463L	Far from the heme	S

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811 **Figure 1. Three-dimensional representation (ribbon model) of KatG of *M. tuberculosis* (PDB**
812 **entry 2CCA).**

813 The heme prosthetic group and the catalytic His108 are shown in black. The mutated residues are
814 represented using red sticks for His270, Trp341, Leu336, Leu141, Ser315, Ala162, Gln461,
815 Asp189, Arg249, Phe658, Ala110 and Arg463, while red spheres are used to represent Gly118,
816 Gly121 and Gly494. The segment of polypeptide chain deleted in the Arg595STOP mutant is
817 shown in orange. The numerous tryptophan, tyrosine and methionine residues present in KatG are
818 shown as thin blue lines. Water molecules (cyan and blue spheres) indicate the location of the two
819 access channels to the heme: a long and wide channel (filled by water molecules represented in
820 blue) that extends from the surface of the protein to the catalytic residue His108, and a narrower
821 short channel connecting the distal heme pocket to the protein surface at the level of residue S315
822 (corresponding to the water molecules represented in cyan). The yellow INH molecules (NIZ)
823 indicate four potential INH binding sites in KatG which have been positioned by superimposing the
824 3D structures of KatG of *M. tuberculosis*, *S. elongatus* and *B. pseudomallei* [24, 25, 28]. The
825 binding site corresponding to NIZ-803, which is generally considered to be the main INH binding
826 site in KatG, has been inferred from crystallographic structures of class I, II, III peroxidases in
827 complex with INH or other small aromatic compounds of similar structure to INH [17, 21-23]. It is
828 located in the narrowest part of the channel connecting the distal heme pocket to the protein surface
829 at the level of S315, hence at the entrance to the ϵ -edge side of the heme [25]. Structural
830 comparisons have revealed that the identity and configuration of the residues in the binding site
831 corresponding to NIZ-803 are very similar among *S. elongatus* KatG, *B. pseudomallei* KatG, and
832 *M. tuberculosis* KatG. NIZ-802 and NIZ-804 have been recently identified in KatG of *S. elongatus*,
833 at the level of the entrance to the γ -edge side of the heme and in front of the heme propionate side
834 chain, respectively [25], and NIZ-749 in KatG of *B. pseudomallei* at the entrance of a long channel
835 connecting the distal pocket to the outside of the protein [24]. In contrast to the binding site
836 corresponding to NIZ-803, the environment of NIZ-802 and NIZ-804 are structurally more diverse
837 among the three KatG proteins of *S. elongatus*, *B. pseudomallei*, and *M. tuberculosis*. Image
838 constructed using PyMOL [53].

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844 **Figure 2. Close-ups showing structural details in three regions of KatG.**

845 The legend is the same as in Figure 1. **2A**, view of the mutated residues in the proximal pocket
846 (His270, Leu336 and Trp341, in red). Trp321 (forming a transient tryptophan radical), Asp381 and
847 Met377 are depicted as blue sticks. Distances (in Å) are indicated by black dotted lines. **2B**, view of
848 the mutated residues in the distal pocket (Gly118, Gly121, Leu141, Ala162, Ser315, Asp189 and
849 Ala110, in red). Two Arg residues (Arg418 and Arg484 potentially interacting with residues 118
850 and 121), are shown as blue sticks. The Met255-Tyr229-Trp107 adduct is visible close to His108.
851 The two arrows indicate the opening of the two access channels. H-bonds are depicted as blue
852 dotted lines.

Figure 1

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Figure 2