Catalase-peroxidases (KatG) exhibit NADH oxidase activity

by Rahul Singh¹, Ben Wiseman¹, Taweewat Deemagarn¹, Lynda J. Donald², Harry W. Duckworth², Xavi Carpena¹, Ignacio Fita³ and Peter C. Loewen¹*

¹ Department of Microbiology, University of Manitoba, Winnipeg, MB R3T 2N2
² Department of Chemistry, University of Manitoba, Winnipeg, MB R3T 2N2
³ Consejo Superior de Investigacione Cientificas, Parc Cientific, Josep Samitier 1-5. 08028 Barcelona, Spain

*Corresponding author:

Department of Microbiology

University of Manitoba

Winnipeg, MB R3T 2N2

204 474-8334

204 474-7603 (fax)

peter_loewen@umanitoba.ca

Running title: NADH oxidase activity in catalase-peroxidases

Key words: catalase-peroxidase, NADH oxidase, isoniazid, catalytic mechanism
SUMMARY

Catalase-peroxidases (KatG) produced by *Burkholderia pseudomallei*, *Escherichia coli* and *Mycobacterium tuberculosis* catalyze the oxidation of NADH to form NAD$^+$ and either H$_2$O$_2$ or superoxide radical depending on pH. The NADH oxidase reaction requires molecular oxygen, does not require hydrogen peroxide, is not inhibited by superoxide dismutase or catalase, and has a pH optimum of 8.75, clearly differentiating it from the peroxidase and catalase reactions with pH optima of 5.5 and 6.5, respectively, and from the NADH peroxidase-oxidase reaction of horse radish peroxidase. *B. pseudomallei* KatG has a relatively high affinity for NADH ($K_M = 12$ µM), but the oxidase reaction is slow ($k_{cat} = 0.54$ min$^{-1}$) compared to the peroxidase and catalase reactions. The catalase-peroxidases also catalyze the hydrazinolysis of INH in an oxygen and H$_2$O$_2$ independent reaction, and KatG-dependent radical generation from a mixture of NADH and INH is 2 to 3 times faster than the combined rates of separate reactions with NADH and INH alone. The major products from the coupled reaction, identified by HPLC fractionation and mass spectrometry, are NAD$^+$ and isonicotinoyl-NAD, the activated form of isoniazid that inhibits mycolic acid synthesis in *M. tuberculosis*. Isonicotinoyl-NAD synthesis from a mixture of NAD$^+$ and INH is KatG-dependent and is activated by manganese ion. *M. tuberculosis* KatG catalyzes isonicotinoyl-NAD formation from NAD$^+$ and INH more efficiently than *B. pseudomallei* KatG.
INTRODUCTION

Isonicotinic acid hydrazide (isoniazid or INH) is a widely used pro-drug effective against *Mycobacterium tuberculosis* (1 and other reviews therein). Formation of isonicotinoyl-NAD, the active form of the drug, involves removal of hydrazine from INH by KatG (2) and ligation of the isonicotinoyl group with NAD\(^+\) (3). Isonicotinoyl-NAD interferes with the synthesis of mycolic acid, and therefore, cell wall synthesis, by binding to InhA, an enoyl-acyl carrier protein (ACP) reductase (4) and possibly to KasA, a β-ketoacyl ACP synthase (5), blocking their NADH binding sites. The central role of KatG in INH activation is evident in the significant fraction of INH resistant cases of tuberculosis attributable to mutations in *katG*, and in biochemical studies that have demonstrated a direct role for KatG in the generation of various isonicotinoyl derivatives (6). Much of the literature related to the activation of INH by KatG has focused on the fate of INH and possible intermediates involved in the process (6 - 8). With NAD\(^+\) included in the mix, the generation of the isonicotinoyl-NAD adduct was observed both with and without KatG present (7 - 9), leading to the suggestions that the role of KatG is limited to the hydrazinolysis of INH, and that the subsequent reaction of the isonicotinoyl radical with NAD\(^+\) is a non-enzymatic event involving a homolytic aromatic substitution (7, 10). Reactive oxygen species have been implicated in INH activation both in vivo (11, 12) and in vitro (6), and an elevated level of superoxide (13) was identified as a possible reason for the high sensitivity of *M. tuberculosis* to INH (1). However, the absence of H\(_2\)O\(_2\) involvement in INH activation implies that a reaction different from either the peroxidase or the catalase reactions is involved and some reports have suggested that the active participation of KatG in INH activation involves more than just hydrazinolysis (9).

NADH oxidases are widely distributed in nature, being found in species ranging from bacteria (14, 15) and archaebacteria (16) to mammals (17). The enzyme can be both soluble and membrane
bound, and while NADH is the common electron donor, water, hydrogen peroxide or superoxide may be formed as products depending on the enzyme. The physiological role of NADH oxidases in many instances is unknown, but the reaction has been described as a detoxifier of oxygen in nitrogen fixing organisms and archaebacteria (16) and as a possible sensor of oxygen levels in the regulation of muscle contractility (17). A variation of the NADH oxidase reaction, a "peroxidase-oxidase" reaction has been well characterized in horse radish peroxidase (HRP) to require a catalytic amount of H$_2$O$_2$ which is alternately used and regenerated during the reaction of NAD radicals with molecular oxygen (18 - 20 and reviewed in 21). Plant peroxidases, including HRP, have about 20% sequence similarity and remarkable structural similarity to the N-terminal domain of KatG proteins, particularly in the vicinity of the heme active site, raising the possibility that KatG proteins may also exhibit an NADH peroxidase-oxidase activity. Indeed, there are reports of NADH being oxidized to NAD$^+$ by KatG in both peroxidase (22) and an oxidase-like (7) reactions, but the reaction of KatG with NADH has not been characterized. Given the potential importance of any reaction involving NADH in isonicotinoyl-NAD formation, this report investigates the interactions of KatG with NADH, demonstrating an oxygen-dependent NADH oxidase activity and a direct role for KatG in the formation of isonicotinoyl-NAD.

**EXPERIMENTAL PROCEDURES**

*Strains and plasmids* - The plasmids pAH1 (23), pBpKatG (24), pBT22 (25), were used as the source of catalase-peroxidases from, respectively, *M. tuberculosis, Burkholderia pseudomallei*, and *Escherichia coli*. All plasmids were transformed into the catalase-deficient E. coli strain UM262 (*pro leu rpsL hsdM hsdR endI lacY katE1 katG17:: Tn10 recA*) (26) and grown in Luria broth containing 10
g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl for expression of the catalase-peroxidases.

Subsequent purification of the enzymes was essentially as described (24).

**Enzyme and protein determination** - Catalase activity was determined by the method of Rørth and Jensen (27) in a Gilson oxygraph equipped with a Clark electrode. One unit of catalase is defined as the amount that decomposes 1 µmol of H$_2$O$_2$ in 1 min in a 60 mM H$_2$O$_2$ solution at pH 7.0 at 37°C. Peroxidase activity was determined spectrophotometrically using ABTS (2,2’-azinobis(3-ethylbenzothiazolesulfonic acid)) (28). One unit of peroxidase is defined as the amount that decomposes 1 µmol of ABTS in 1 min in a solution of 0.3 mM ABTS ($\epsilon = 36,800$ M$^{-1}$ cm$^{-1}$) and 2.5 mM H$_2$O$_2$ at pH 4.5 and 25°C. The peroxidatic substrate o-dianisidine was also tested but the data are not reported. Free radical production was assayed by NBT (nitroblue tetrazolium) reduction to a monoformazan ($\epsilon = 15,000$ M$^{-1}$ cm$^{-1}$). NADH oxidase activity was determined spectrophotometrically at 340 nm using $\epsilon = 6,300$ M$^{-1}$ cm$^{-1}$ for NADH. One unit of NADH oxidase is defined as the amount that produces 1 nmol of radical or that decomposes 1 nmol of NADH in a solution of 250 µM NADH at 25°C. Protein was estimated according to the methods outlined by Layne (29).

**Polyacrylamide gel electrophoresis and visualization of enzymatic activities.** Gel electrophoresis of purified proteins was carried out under denaturing conditions on SDS-polyacrylamide gels as previously described (30, 31) Gel electrophoresis was carried out under nondenaturing conditions according to Davis (32), except in pH 8.1 Tris-HCl. Following electrophoresis, peroxidase activity was visualized by the method of Gregory and Fridovich (33) and catalase was visualized as described by Clare et al (34), but using 20 mM H$_2$O$_2$ for better contrast. KatG mediated oxidation of INH and NADH was visualized using NBT as described by Hillar and Loewen (23).

**Mass spectrometry** BpKatG was dialysed into 5 mM ammonium acetate and NADH was dialysed against three changes of 1L 0.1 M ammonium bicarbonate after which the concentration was
determined by A<sub>340</sub>. The matrix solution (DHB) was freshly prepared each day at 160 mg/mL 2,5-dihydroxybenzoic acid in 3:1 water:acetonitrile, 2% formic acid solution. 20 µL reaction mixtures contained 200 µg/mL BpKatG, 128 µM NADH, and 10 mM INH in 0.1 M NH₄HCO₃ at room temperature. 0.5 µL aliquots were removed at 1 min, 2 h, 4 h, and 18 h and immediately mixed with 0.5 µL DHB solution on a metal target. Samples were analyzed on a QqTOF mass spectrometer.

**Determination of conserved residue locations** The locations of residues conserved in >95% of 52 available KatG sequences were determined using the program CONRES. The program identifies conserved residues in an alignment file from CLUSTALW and extracts the equivalent residues from the structure of one of the aligned proteins. The sequence alignment file included 52 catalase peroxidase sequences and the structure file for *B. pseudomallei* KatG (1MWV.pdb) was used as the model. The accession numbers for the sequences included in the comparison are listed by Klotz and Loewen. The source code or IRIX 6.5 executable for CONRES can be obtained from the authors on request.

**RESULTS**

**NADH oxidation by KatG** NAD<sup>+</sup> replaces the hydrazine moiety of the anti-tubercular pro-drug INH to generate the active form of the drug, isonicotinoyl-NAD. In an attempt to define more precisely the role of KatG in the formation of isonicotinoyl-NAD, the potential utilization of NADH and NAD<sup>+</sup> as substrates was investigated revealing that NADH supports BpKatG-mediated radical generation (Fig. 1a) in the absence of H<sub>2</sub>O<sub>2</sub> using NBT as radical sensor at a rate approximately equal to the rate of NADH disappearance (Fig. 1b). NADPH supports a slower rate of radical generation and NAD<sup>+</sup> does not support any radical production (Table 1). NAD<sup>+</sup> was confirmed as the product of the reaction with NADH by HPLC analysis (Fig. 2). Both superoxide dismutase and a lower molecular oxygen
concentration reduce radical generation (Table 1) from NADH. The pH optima for both radical production and NADH disappearance are 8.75, but the curves are not perfectly super-imposable (Fig 1c), suggesting two different, pH dependent, fates for the reduced oxygen, most likely H$_2$O$_2$ at lower pH and superoxide radical at higher pH. Unfortunately, the rapid degradation of H$_2$O$_2$ by both the catalase and peroxidase activities of BpKatG precluded its identification or even the observation of any spectral changes in the enzyme during the reaction. The optimum pH for the oxidase reaction is significantly different from the optimum pHs for the peroxidase (pH 4.5) and catalase (pH 6.5) reactions (Fig. 1c). These results are consistent with BpKatG having an NADH oxidase activity producing NAD$^+$ and either superoxide ion or H$_2$O$_2$ and with a similar activity existing in MtKatG and EcKatG, but at a much lower level.

The kinetic parameters of the BpKatG mediated NADH oxidase reaction (Table 2) reveal a relatively high affinity for NADH but a very slow turnover rate in comparison to the catalase and peroxidase reactions. NAD$^+$ and pyridoxine act as competitive inhibitors of the NADH oxidase reaction (data not shown). Purified BpKatG migrates as a single band (apparent mass of 78 kDa) on denaturing gels (Fig. 3a) with only a small amount of slower migrating dimer. On a non-denaturing gel (Fig. 3b-g), catalase, peroxidase and oxidase activities all co-migrate with the main bands of protein (lanes b to e). The presence of two bands of catalase-peroxidase with the same mass on non-denaturing gels has been noted previously, but not explained.

Horse radish peroxidase (HRP) catalyzes an unusual NADH peroxidase-oxidase reaction in which H$_2$O$_2$ is required to initiate the reaction after which there is a cycling of O$_2^-$, H$_2$O$_2$ and compound III (reviewed in 21). Given the sequence similarity between plant peroxidases and catalase-peroxidases, particularly in the active site, the possibility that the NADH oxidase activity of KatG is similar to the peroxidase-oxidase reaction was investigated (Table 3). The HRP peroxidase-oxidase
reaction is characterized by a need for a catalytic amount of H$_2$O$_2$, by inhibition by SOD or catalase, and by long lag periods in the presence of low [NADH] and high [HRP]. NADH oxidation by BpKatG does not exhibit any of these characteristics (Table 3). For comparison, NADH oxidation by the W111F variant, which lacks catalase activity, is reduced slightly by catalase, but not SOD, and is significantly enhanced by added H$_2$O$_2$, attributable to NADH serving as a peroxidatic substrate. No lag in the initiation of NADH oxidation was evident over a broad range of NADH concentrations or enzyme concentrations for either BpKatG or the W111F variant.

**INH hydrazinolysis by BpKatG** The activation of INH as an anti-tubercular drug by catalase-peroxidases involves removal of the hydrazine moiety and proceeds via a radical producing reaction that can be monitored using NBT as a radical sensor (23). The H$_2$O$_2$ independence (Fig. 4a) and enhanced rates at alkaline pH (Fig. 4b) of the KatG-mediated INH hydrazinolysis reaction differentiate it from the catalase and peroxidase reactions (Fig 1c). There is a pH-dependent, non-enzymatic generation of radicals from INH, but in the pH 7 to 9 range, the contribution of the enzymatic reaction is clear and suggests a pH optimum between 8 and 9, albeit not well defined (Fig. 4b). To minimize the non-enzymatic contribution, all subsequent assays and reactions involving INH were carried out at pH 8. Oxygen levels do not affect the KatG-mediated radical generation from INH, and superoxide dismutase does not reduce radical production (Table 1) confirming that molecular oxygen does not have a role in the reaction. MtKatG catalyzes INH hydrazinolysis at about the same rate as BpKatG, but the EcKatG-mediated reaction is slower (Fig 1 and Table 1). Despite having a pyridine ring as part of its structure, NAD$^+$ does not inhibit INH hydrazinolysis (data not shown).

**Interaction of KatG with a mixture of INH and NADH** Radical generation in a mixture of BpKatG, INH and NADH is about 2 times faster than the cumulative rate of the individual reactions of INH and NADH (Fig. 5a). This rate is further enhanced by the inclusion of 2 µM manganese ion
(either Mn$^{+2}$ or Mn$^{+3}$) (Fig. 5b). The rate of NADH disappearance, as measured by the decrease in A$_{340}$, is similarly increased (data not shown). NAD$^+$ is not a substrate for radical generation and the rate of radical formation from a mixture of NAD$^+$ and INH is the same as with INH alone (Fig 4a) and is enhanced by Mn$^{+2}$ (Fig. 5c). The MtKatG and EcKatG mediated reactions of INH and NADH are both slower in the absence of Mn$^{+2}$ (Fig. 4a), but approach or exceed the BpKatG mediated reaction in the presence of Mn$^{+2}$ (Fig. 5b).

Based on HPLC retention times (Fig. 2) combined with MS and tandem MS analysis (data not shown), the main products of the combined NADH and INH reaction with KatG are NAD$^+$ and isonicotinoyl-NAD with other products being present in smaller amounts. Isonicotinoyl-NAD elutes from the column as four peaks, all with exhibiting an ion at m/z 771, consistent with the report of Wilming and Johnsson (7) who explained the elution pattern as the result of two stereo-isomers, arising from addition of the isonicotinoyl group to opposite faces of the nicotinamide ring, each with two rotamers, resulting from restricted rotation of the isonicotinoyl group. The time course of appearance and identity of isonicotinoyl-NAD were confirmed by MS analysis of the reaction mixture and the product ion at m/z 771 (inset to Fig. 6c), coincident with the mass of isonicotinoyl-NAD, was evident only in mixtures containing all three of KatG, INH and NADH or NAD$^+$. Tandem mass spectrometry measurements of the ions at m/z 753 and 771 produced almost identical fragmentation patterns confirming that the ion at m/z 753 is a product of the parent ion at m/z 771 (Table 4). The difference in mass between the two ions, 18.014 Da, suggests the loss of H$_2$O (18.016 Da) but offers no clue as to the structural basis for the decay or its cause. Comparison with the fragmentation pattern of NADH clearly confirms the presence of NAD in the pattern of fragments from the m/z 771 and 753 ions (Table 4) degraded from the nicotinamide end, and by inference allowing for the isonicotinoyl group, also after degradation from the adenine end (Table 4).
**NAD\(^+\) as precursor for isonicotinoyl-NAD** The facile KatG-mediated oxidation of NADH and lack of apparent reaction with NAD\(^+\) initially suggested that NADH, not NAD\(^+\), is the precursor to isonicotinoyl-NAD. However, isonicotinoyl-NAD is formed in a KatG-mediated reaction more efficiently from a mixture of NAD\(^+\) and INH (Fig. 6) than from a mixture of NADH and INH, both with 2 µM Mn\(^{+2}\) (Fig. 2). Therefore, NAD\(^+\) is the probable precursor for isonicotinoyl-NAD as concluded earlier (7), but NADH oxidation by KatG to NAD\(^+\) will lead to the same product in an NADH mixture. The presence of 2 µM Mn\(^{+2}\) greatly speeds the NAD\(^+\)-dependent formation of isonicotinoyl-NAD by all three katGs, BpKatG, MtKatG and EcKatG.

**NADH oxidation can be uncoupled from superoxide formation** It has been well documented that changing any one of a number of residues in KatG reduces catalase activity with minimal effect on peroxidase activity (38-42). A number of variants of BpKatG with residues changed in the active site cavity and in the Met-Tyr-Trp cross-linked structure (43, 44) were assayed for oxidase and hydrazinolysis activities for comparison with their catalase and peroxidase activities (Table 5). The hydrazinolysis reaction was largely unaffected by any of the changes except the change of His112 to Ala. By contrast, the oxidase reaction was affected in several of the variants and two groups with different properties can be discerned. One group, including those with changes in any of the three residues in the cross-linked structure of Trp111, Tyr238 and Met264, exhibits normal rates of NADH disappearance but significantly reduced rates of radical production. The apparent uncoupling of NADH oxidation from superoxide generation could be a result of a broken electron tunnel or of a change in the reaction chemistry to favor H\(_2\)O\(_2\) over superoxide formation. Unfortunately, the unambiguous identification or quantification of H\(_2\)O\(_2\) formed in the reaction is not possible because it is immediately utilized in a peroxidatic reaction. Indeed, an inconclusive 13% hypochromic and 3-5 nm red shift in the Soret band and little change in the 500 - 700 nm region is observed for W111F and
Y238F variants during the oxidase reaction, whereas no change is observed with native BpKatG. The second group exhibits reduced rates of both NADH disappearance and superoxide formation, consistent with reduced NADH oxidation. This group includes those with changes in the active site residues Arg108, His112 and Asp141. The changes in catalase and peroxidase activities caused by the sequence changes are consistent with those reported previously for KatGs from *Synechocystis* (38, 40, 41), *M. tuberculosis* (39) and *E. coli* (42).

**Location of conserved residues**  Because the in vivo peroxidase substrate remains unidentified, possible substrate binding sites for as yet unidentified substrates was a topic for speculation in the original report of the BpKatG structure (44). Unambiguous identification of such sites will have to await a crystal structure determination of protein-ligand complexes, but further evidence for a diversity of binding sites in KatGs is evident in the high frequency of highly conserved residues (Table 6) and their broad distribution throughout the subunit including on the surface (Fig. 7). Over 27% and 18% of the residues, respectively, in the catalytic N-terminal domain and non-catalytic C-terminal domain of the KatG subunit are identical in more than 95% of the sequences. This is compared to the 8 to 14% frequency of nearly identical residues, all located near the active sites in pyruvate kinases, catalases, peroxidases, CuZn SODs and FeMn SODs (data not shown).

**DISCUSSION**

The existence of catalase, peroxidase and oxidase activities in a single protein makes KatG a complex and fascinating enzyme, an assessment further enhanced by INH hydrazinolysis and isonicotinoyl-NAD synthesis activities. A summary of these various activities (Fig. 8) illustrates the independence of the closely related catalase and peroxidase activities from the oxidase, hydrazinolysis and synthase activities. Not only are the pH optima very different, but, unlike the catalase and
peroxidase reactions, the oxidase, hydrazinolysis and synthase reactions do not require H$_2$O$_2$ or involve the formation of identifiable oxidized heme intermediates. The hydrazinolysis and synthase reactions are closely linked because hydrazinolysis must occur before the ligation of NAD$^+$ to the isonicotinoyl radical, but the oxidase reaction is not mechanistically linked to any other reaction except that it very likely shares with the synthase the same NAD$^+/$/NADH binding site. In addition the oxidase reaction may serve as a source of NAD$^+$ for the synthase reaction and H$_2$O$_2$ for the catalase and peroxidase reactions (Fig. 8).

The catalase and peroxidase functions of KatG are rationalized as protection against H$_2$O$_2$, and the NADH oxidase activity may present a complementary protection against molecular oxygen or a means of maintaining low cytoplasmic levels of oxygen. However, the turnover rate of the oxidase is very slow compared to the catalase and peroxidase reactions, and the oxidase activity may simply be a residual vestige of what was once a more substantial activity with metabolic significance in a particular environmental niche. Certainly, the high affinity of the enzyme for NADH is consistent with there being, or having been, a physiological significance to the activity, and even among the three KatGs in this study, the variation in oxidase activity, highest in BpKatG and lower in MtKatG and EcKatG, might be interpreted as the result of an environmentally determined differential loss of activity.

While the KatGs can utilize NADH as a peroxidatic substrate, the NADH oxidase characterized here is clearly not a peroxidatic reaction, and it is also different in several key respects from the peroxidase-oxidase activity associated with HRP. H$_2$O$_2$ does not have a catalytic role; SOD and catalase do not inhibit the reaction; and lag periods in reaction initiation are not evident in the presence of high enzyme or low NADH concentrations. In addition, the pH dependence of superoxide radical formation in the oxidase reaction suggests the unusual possibility of two reaction outcomes for molecular oxygen depending on proton availability. Below pH 8, with protons more readily available,
a two electron transfer to oxygen takes place generating H$_2$O$_2$ (reaction 1) while at higher pH, two one
electron transfers to two oxygen molecules generate two superoxide ions (reaction 2). The protonation
state of the imidazole ring of the distal side His112 may determine the reaction pathway through
presentation of a proton to the bound oxygen in the active site. Any H$_2$O$_2$ produced via reaction 1
would rapidly oxidize the heme to compound I and subsequent reduction in catalase or peroxidase
reactions would give rise to H$_2$O.

\[
\begin{align*}
\text{NADH} + O_2 + H^+ & \rightarrow \text{NAD}^+ + H_2O_2 \quad (1) \\
\text{NADH} + 2O_2 & \rightarrow \text{NAD}^+ + 2O_2^- \quad (2)
\end{align*}
\]

The enhancement of INH hydrazinolysis by KatG and its importance in INH pro-drug
activation is well documented. A single mutation in Ser315 of MtKatG is sufficient to reduce the
enzyme's affinity for INH (39) and to prevent isonicotinoyl-NAD formation (45) leading to in vivo
isoniazid resistance. The fact that NAD$^+$ is a competitive inhibitor of NADH oxidation but does not
inhibit the hydrazinolysis reaction, and that an INH-NADH mixture supports a rate of radical
production greater than the cumulative rates of the individual reactions, suggest that NADH/NAD$^+$ and
INH have separate binding sites and that there is an element of synergy between the two reactions.
Manganese ion (both Mn$^{+2}$ and Mn$^{+3}$) enhances both non-enzymatic and enzymatic hydrazinolysis to
the extent that Mn-mediated isonicotinoyl radical formation is faster than the KatG-mediated reaction.
However, despite this high rate of Mn-mediated hydrazinolysis, Mn-mediated isonicotinoyl-NAD
formation is negligible compared to its rapid formation in the presence of KatG. The data in this report
do not dispute the observation that there can be a non-enzymatic origin of isonicotinoyl-NAD (7), but
under the conditions employed in this work, KatG is a much more effective catalyst of isonicotinoyl-
NAD synthesis than manganese ion.
The regulatory systems controlling KatG expression, involving OxyR in *E. coli*, are generally oxidative response systems, supporting the conjecture that the primary role of KatG is the detoxification of H$_2$O$_2$ as a catalase. The physiological role and importance of the peroxidatic reaction in the anti-oxidant process remains unclear, in large part because the identity of the in vivo peroxidatic substrate(s) remains unknown. The wide variety of in vitro substrates that are utilized by KatG, and the wide variety of substrates used by the closely related plant peroxidases, ranging from whole proteins to simple carbohydrates and metal ions, have not helped in the identification. Furthermore, the structure of KatGs present several potential substrate binding sites in its surface topography, and there is an abnormally high percentage of highly conserved residues close to the protein surface consistent with the idea of important features residing in other than the heme pocket. Of the known substrates, H$_2$O$_2$ binds at the heme iron which is also the most likely site for O$_2$ binding; INH binds in the heme cavity or entrance channel near Ser324; and the peroxidatic substrates probably also bind in the vicinity of Ser324 as suggested by benzhydroxamic acid binding in HRP (46), although the substrates used here are larger and would not fit into as small a cavity as benhydroxamic acid. Trying to identify the NADH binding site would be pure conjecture at this point, but the possibility that the cross-linked side chains of W111, Y238 and M264 may have a role in electron transfer from NADH to O$_2$, and the observations that INH does not inhibit NADH oxidation and NAD$^+$ does not inhibit hydrazinolysis, suggest that the NADH binding site is some distance from the heme pocket. However, this surmise must be tempered by the logic of having NAD$^+$ bind in close proximity to the site of isonicotinoyl radical formation. A structural definition of the binding sites is needed.

ACKNOWLEDGEMENTS
This work was supported by a grant OGP9600 from the Natural Sciences and Engineering Research Council of Canada (to PCL), by the Canadian Research Chair Program (to PCL) and by fellowship EX-2003-0866 from the Ministerio de Educación Cultura y Deporte, Spain (to XC). The cooperation of Drs. K. G. Standing and W. Ens in arranging use of the mass spectrometers in the TOF Laboratory (supported by NIH grant GM59240 to KGS) of the Department of Physics, University of Manitoba is also appreciated.

REFERENCES


Table 1  BpKatG mediated radical production in the presence of different substrates.

<table>
<thead>
<tr>
<th></th>
<th>No enzyme(^a) (pmole/min)</th>
<th>BpKatG(^b) (pmole/min/nmole)</th>
<th>MtKatG(^b) (pmole/min/nmole)</th>
<th>EcKatG(^b) (pmole/min/nmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH</td>
<td>3.0 ± 0.2</td>
<td>640 ± 96</td>
<td>100 ± 15</td>
<td>13 ± 4</td>
</tr>
<tr>
<td>NADH + Mn(^{+2})</td>
<td>2.0 ± 0.1</td>
<td>540 ± 23</td>
<td>99 ± 4</td>
<td>10 ± 4</td>
</tr>
<tr>
<td>NADH - O(_2)(^c)</td>
<td>1.0 ± 0.2</td>
<td>320 ± 15</td>
<td>9 ± 2</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>NADH + SOD</td>
<td>1.0 ± 0.1</td>
<td>350 ± 8</td>
<td>58 ± 6</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>NADPH</td>
<td>1.0 ± 0.1</td>
<td>260 ± 2</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>NAD(^+)</td>
<td>3.0 ± 0.6</td>
<td>nd(^d)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>NADH + INH</td>
<td>8.0 ± 2</td>
<td>1140 ± 76</td>
<td>260 ± 21</td>
<td>100 ± 6</td>
</tr>
<tr>
<td>NADH + INH + Mn(^{+2})</td>
<td>290 ± 16</td>
<td>2940 ± 29</td>
<td>1830 ± 21</td>
<td>930 ± 91</td>
</tr>
<tr>
<td>NAD(^+) + INH</td>
<td>4.0 ± 0.3</td>
<td>82 ± 1</td>
<td>79 ± 17</td>
<td>74 ± 19</td>
</tr>
<tr>
<td>NAD(^+) + INH + Mn(^{+2})</td>
<td>140 ± 4</td>
<td>230 ± 12</td>
<td>340 ± 8</td>
<td>220 ± 9</td>
</tr>
<tr>
<td>INH</td>
<td>5.0 ± 0.2</td>
<td>76 ± 2</td>
<td>78 ± 5</td>
<td>38 ± 2</td>
</tr>
<tr>
<td>INH + Mn(^{+2})</td>
<td>190 ± 18</td>
<td>410 ± 3</td>
<td>460 ± 9</td>
<td>450 ± 2</td>
</tr>
<tr>
<td>INH - O(_2)(^c)</td>
<td>6.0 ± 2</td>
<td>75 ± 5</td>
<td>78 ± 5</td>
<td>40 ± 3</td>
</tr>
<tr>
<td>INH + SOD</td>
<td>5.2 ± 1</td>
<td>90 ± 2</td>
<td>65 ± 5</td>
<td>38 ± 3</td>
</tr>
<tr>
<td>INH + pyridoxine</td>
<td>4.0 ± 1</td>
<td>9.0 ± 2</td>
<td>20 ± 5</td>
<td>18 ± 3</td>
</tr>
</tbody>
</table>

\(^a\) All reactions contained 200 µM NBT in 50 mM Tris pH 8.75, supplemented with 10 mM INH, 250 µM NADH, 250 µM NDPH, 250 µM NAD\(^+\), 200µg Superoxide dismutase as indicated. O\(_2\) was reduced by flushing the reaction mixture with N\(_2\) gas.

\(^b\) KatG was added to 1.2 µM

\(^c\) For anaerobic reactions, the reaction mixtures were flushed with nitrogen to remove dissolve oxygen.

\(^d\) nd: not detected
Table 2  Kinetic constants for enzymatic activities associated with BpKatG

<table>
<thead>
<tr>
<th></th>
<th>Vmax</th>
<th>$K_M$</th>
<th>$k_{cat}$</th>
<th>$k_{cat}/K_M$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmol.min⁻¹.µmol heme⁻¹</td>
<td>µM</td>
<td>(sec⁻¹)</td>
<td>(M⁻¹.sec⁻¹)</td>
</tr>
<tr>
<td>Oxidase (ΔA₃₄₀)</td>
<td>540 ± 96 x 10⁻³</td>
<td>12.5 ± 2.0</td>
<td>9.0 ± 0.4 x 10⁻³</td>
<td>7.2 x 10²</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>1083 ± 29</td>
<td>330 ± 30</td>
<td>10.1 ± 0.27</td>
<td>3.1 x 10⁴</td>
</tr>
<tr>
<td>Catalase</td>
<td>902 ± 20 x 10³</td>
<td>7,700 ± 400</td>
<td>8.4 ± 0.2 x 10³</td>
<td>1.1 x 10⁶</td>
</tr>
</tbody>
</table>

a  [NADH] for the oxidase reaction, [ABTS] for the peroxidase reaction and [H₂O₂] for the peroxidase and catalase reactions.
Table 3 Comparison of NADH oxidation by HRP, BpKatG and the W111F variant.

<table>
<thead>
<tr>
<th></th>
<th>HRP</th>
<th>BpKatG</th>
<th>W111F</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH</td>
<td>110 ± 18b</td>
<td>490 ± 16</td>
<td>500 ± 41</td>
</tr>
<tr>
<td>NADH + H₂O₂</td>
<td>360 ± 22</td>
<td>490 ± 24</td>
<td>2160 ± 180</td>
</tr>
<tr>
<td>NADH + HPII</td>
<td>30 ± 4</td>
<td>480 ± 16</td>
<td>392 ± 16</td>
</tr>
<tr>
<td>NADH + HPII + H₂O₂</td>
<td>54 ± 4</td>
<td>480 ± 16</td>
<td>290 ± 16</td>
</tr>
<tr>
<td>NADH + SOD</td>
<td>86 ± 16</td>
<td>400 ± 24</td>
<td>460 ± 16</td>
</tr>
<tr>
<td>NADH + SOD + H₂O₂</td>
<td>540 ± 29</td>
<td>400 ± 16</td>
<td>2200 ± 190</td>
</tr>
</tbody>
</table>

a All reactions contained 100 µM NADH, 100 µg/ml protein in 50 mM Tris pH 8.75.

b Data are expressed as pmol.min⁻¹.nmol⁻¹ heme.
Table 4  Comparison of expected and observed m/z values of ions in the MS/MS spectra of NADH and the product of the reaction of KatG with INH and NADH identified as isonicotinoyl-NAD.

<table>
<thead>
<tr>
<th>Product ions (m/z) from parent ion(^a)</th>
<th>Assigned structure(^b)</th>
<th>Formula to calculate expected ion</th>
<th>Expected (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>m/z 771</td>
<td>IN-NRPPA</td>
<td>C(<em>{27}H</em>{33}N_8O_{15}P_2)</td>
<td>771.154</td>
</tr>
<tr>
<td>m/z 753</td>
<td>IN-NRPPA - H(_2)O</td>
<td>C(<em>{27}H</em>{31}N_8O_{14}P_2)</td>
<td>753.144</td>
</tr>
<tr>
<td>m/z 666</td>
<td>NHRPPA (NADH)</td>
<td>C(<em>{21}H</em>{30}N_7O_{14}P_2)</td>
<td>666.133</td>
</tr>
</tbody>
</table>

A: Parent ions

<table>
<thead>
<tr>
<th>Parent ions</th>
<th>Formula to calculate expected ion</th>
<th>Expected (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>771.154</td>
<td>C(<em>{27}H</em>{33}N_8O_{15}P_2)</td>
<td>771.154</td>
</tr>
<tr>
<td>753.140</td>
<td>C(<em>{27}H</em>{31}N_8O_{14}P_2)</td>
<td>753.144</td>
</tr>
<tr>
<td>666.090</td>
<td>C(<em>{21}H</em>{30}N_7O_{14}P_2)</td>
<td>666.133</td>
</tr>
</tbody>
</table>

B: Fragmentation from the adenine end ("y" ions)

<table>
<thead>
<tr>
<th>Fragmentation</th>
<th>Assigned structure(^b)</th>
<th>Formula to calculate expected ion</th>
<th>Expected (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>530.061</td>
<td>NHRPR</td>
<td>C(<em>{16}H</em>{24}N_2O_{14}P_2)</td>
<td>530.070</td>
</tr>
<tr>
<td>514.059</td>
<td>NHRPPR - NH(_3)</td>
<td>C(<em>{16}H</em>{22}NO_{14}P_2)</td>
<td>514.052</td>
</tr>
<tr>
<td>424.093</td>
<td>IN-NRP</td>
<td>C(<em>{17}H</em>{19}N_3O_{8}P)</td>
<td>424.091</td>
</tr>
<tr>
<td>319.073</td>
<td>NHRP</td>
<td>C(<em>{11}H</em>{16}N_2O_{7}P)</td>
<td>319.070</td>
</tr>
<tr>
<td>229.044</td>
<td>IN-N</td>
<td>C(<em>{12}H</em>{10}N_3O_{7}P)</td>
<td>229.085</td>
</tr>
<tr>
<td>123.063</td>
<td>NH</td>
<td>C(<em>{6}H</em>{7}N_2O)</td>
<td>123.056</td>
</tr>
</tbody>
</table>

C: Fragmentation from the nicotinamide end ("b" ions)

<table>
<thead>
<tr>
<th>Fragmentation</th>
<th>Assigned structure(^b)</th>
<th>Formula to calculate expected ion</th>
<th>Expected (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>649.109</td>
<td>NHRPPA - NH(_3)</td>
<td>C(<em>{21}H</em>{27}N_6O_{14}P_2)</td>
<td>649.106</td>
</tr>
<tr>
<td>664.126</td>
<td>NRPPA</td>
<td>C(<em>{21}H</em>{28}N_7O_{14}P_2)</td>
<td>664.117</td>
</tr>
<tr>
<td>542.078</td>
<td>RPPA</td>
<td>C(<em>{15}H</em>{22}N_5O_{13}P_2)</td>
<td>542.069</td>
</tr>
<tr>
<td>524.059</td>
<td>RPPA - H(_2)O</td>
<td>C(<em>{15}H</em>{20}N_5O_{12}P_2)</td>
<td>524.058</td>
</tr>
<tr>
<td>428.041</td>
<td>PPA</td>
<td>C(<em>{10}H</em>{16}N_5O_{10}P_2)</td>
<td>428.037</td>
</tr>
<tr>
<td>348.073</td>
<td>PA</td>
<td>C(<em>{10}H</em>{15}N_5O_{7}P)</td>
<td>348.071</td>
</tr>
<tr>
<td>250.094</td>
<td>A</td>
<td>C(<em>{10}H</em>{12}N_5O_{3})</td>
<td>250.094</td>
</tr>
<tr>
<td>136.070</td>
<td>Adenine</td>
<td>C(<em>{5}H</em>{6}N_5)</td>
<td>136.062</td>
</tr>
</tbody>
</table>

\(^a\) The parent ions at m/z 771.154, 753.166 and 666.090 were selected individually and fragmented by tandem mass spectrometry.

\(^b\) Abbreviations: A, adenosine; N, nicotinamide; IN, isonicinoyl group; R, ribose
Table 5  Comparison of catalase, peroxidase and oxidase activities in BpKatG and its variants.

<table>
<thead>
<tr>
<th></th>
<th>Catalase (units/mg)</th>
<th>Peroxidase</th>
<th>Oxidase$_{(340)}$</th>
<th>Oxidase$_{(560)}$</th>
<th>Hydrazinolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>BpKatG</td>
<td>4100 ± 200</td>
<td>4.4 ± 0.5</td>
<td>6.9 ± 1.0</td>
<td>7.7 ± 1.2</td>
<td>0.9 ± 0.02</td>
</tr>
<tr>
<td>R108A</td>
<td>1250 ± 110</td>
<td>1.0 ± 0.1</td>
<td>3.7 ± 0.5</td>
<td>2.2 ± 0.4</td>
<td>0.3 ± 0.01</td>
</tr>
<tr>
<td>R108K</td>
<td>320 ± 20</td>
<td>0.9 ± 0.1</td>
<td>2.2 ± 0.2</td>
<td>1.3 ± 0.1</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td>H112A</td>
<td>1 ± 0.2</td>
<td>0.1 ± 0.01</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>H112N</td>
<td>2 ± 0.6</td>
<td>0.1 ± 0.01</td>
<td>3.1 ± 0.2</td>
<td>1.3 ± 0.5</td>
<td>0.2 ± 0.06</td>
</tr>
<tr>
<td>W111F</td>
<td>2 ± 0.1</td>
<td>3.3 ± 0.6</td>
<td>7.7 ± 2.4</td>
<td>nd</td>
<td>0.5 ± 0.04</td>
</tr>
<tr>
<td>D141A</td>
<td>60 ± 10</td>
<td>5.8 ± 0.4</td>
<td>2.2 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>D141N</td>
<td>390 ± 20</td>
<td>5.4 ± 0.3</td>
<td>2.8 ± 0.2</td>
<td>1.2 ± 0.2</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>D141E</td>
<td>3290 ± 40</td>
<td>6.3 ± 1.7</td>
<td>3.4 ± 0.1</td>
<td>2.8 ± 0.2</td>
<td>0.8 ± 0.02</td>
</tr>
<tr>
<td>Y238A</td>
<td>2 ± 0.1</td>
<td>6.1 ± 0.1</td>
<td>10.8 ± 1.9</td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.04</td>
</tr>
<tr>
<td>Y238F</td>
<td>6 ± 1.1</td>
<td>2.8 ± 0.3</td>
<td>10.3 ± 2.0</td>
<td>nd</td>
<td>1.1 ± 0.05</td>
</tr>
<tr>
<td>M264A</td>
<td>6 ± 1.1</td>
<td>7.0 ± 1.4</td>
<td>4.3 ± 0.1</td>
<td>nd</td>
<td>0.6 ± 0.04</td>
</tr>
<tr>
<td>M264L</td>
<td>2 ± 0.9</td>
<td>6.1 ± 1.3</td>
<td>11.0 ± 1.5</td>
<td>4.9 ± 0.6</td>
<td>0.6 ± 0.04</td>
</tr>
<tr>
<td>MtKatG</td>
<td>3550 ± 160</td>
<td>9.7 ± 0.2</td>
<td>2.4 ± 0.8</td>
<td>1.2 ± 0.2</td>
<td>1.0 ± 0.06</td>
</tr>
<tr>
<td>EcKatG</td>
<td>2320 ± 230</td>
<td>9.9 ± 0.3</td>
<td>1.1 ± 0.4</td>
<td>0.2 ± 0.05</td>
<td>0.5 ± 0.02</td>
</tr>
</tbody>
</table>
Table 6  Comparison of the percentage of highly conserved residues (>95% identical) in KatGs compared to other families of proteins.

<table>
<thead>
<tr>
<th></th>
<th>KatG (N-term)</th>
<th>KatG (C-term)</th>
<th>Catalase (Cu-Zn)</th>
<th>SOD (Fe-Mn)</th>
<th>SOD (Cu-Zn)</th>
<th>Peroxidase</th>
<th>Pyruvate kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. in family</td>
<td>52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>228&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25&lt;sup&gt;c&lt;/sup&gt;</td>
<td>24&lt;sup&gt;d&lt;/sup&gt;</td>
<td>28&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Length</td>
<td>390</td>
<td>322</td>
<td>490</td>
<td>155</td>
<td>238</td>
<td>294</td>
<td>470</td>
</tr>
<tr>
<td>No. with &gt;95% identity</td>
<td>107</td>
<td>59</td>
<td>57</td>
<td>22</td>
<td>22</td>
<td>24</td>
<td>59</td>
</tr>
<tr>
<td>% with &gt;95% identity</td>
<td>27.4</td>
<td>18.3</td>
<td>11.6</td>
<td>14.2</td>
<td>9.2</td>
<td>8.2</td>
<td>12.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Accession numbers are described in Klotz and Loewen (36)

<sup>b</sup> Accession numbers for sequences used: AA054860, AAR15417, AB087845, AF312586, AF312588, AF318938, AJ581746, AY428604, AY434497, BAC96317, BC061861, CAC28938, Jo2658, J04087, L13778, M15175, M84013, NM011435, NM174615, NP012638, NP231223, NP940620, P24704, X97766, XM358882

<sup>c</sup> Accession numbers for sequences used: AAN16456, AA057908, AB093035, AB109302, AF318020, AY314980, J03511, L11707, L25675, M33119, M60401, M74242, M94879, M96560, NM013671, NM057577, NM059889, NP011872, NP231679, NP232322, NP704405, NP940564, Q8K6Y8, X03951
Accession numbers for sequences used: AB027752, AB009084, AB022273, AB024437, AB027752, AB027753, AF039027, AF109123, AF139910, AF149278, AF155124, AF159380, AF159629, AF175710, AJ003141, AJ011939, D11102, D11337, D14442, D83669, D84400, D90115, M60729, Y16773, Y17192

Accession numbers for sequences used: AAB47952, AAH16619, AAH19265, AAHY25737, AAH61541, AAO57788, AAQ57928, BAC91436, BAD01636, CAA24631, CAE07913, CAE14987, CAE20854, NP230139, NP231642, NP416191, NP416368, NP703926, NP721573, NP881869, NP934082, NP939895, Q8Z6K2, Q9Z9B4, Q89A18, XP224416, XP341924, ZP00026409, ZP00078743, ZP000122573
Figure Legends

Fig. 1. NADH oxidation by catalase-peroxidases. (a) The rates of radical production in a solution containing 100 µM NADH, 1.2 µM enzyme, 200 µM NBT and 50 mM Tris pH 8.75 are followed by formazan appearance measured at 560 nm. Separate assays contain no enzyme (control), BpKatG, MtKatG, or EcKatG. (b) The rates of NADH oxidation in a solution containing 100 µM NADH, 1.2 µM enzyme and 50 mM Tris pH 8.75 was followed by NADH disappearance measured at 340 nm. Separate assays contain no enzyme (control), BpKatG, MtKatG, or EcKatG. (c) pH dependence of NADH oxidation by BpKatG determined by radical formation at 560 nm (□) and NADH disappearance at 340 nm (●) compared to the pH dependence of the catalase (■) and peroxidase reactions (○). Buffered solutions contained 50 mM each of sodium acetate, pH 3, 4 and 5, potassium phosphate pH 6 and 7, Tris-HCl pH 8 and 9, and CHES pH 10.

Fig. 2. Elution profiles from reverse phase HPLC of reaction products from mixtures containing a) 100 µM NADH and 200 µM INH; b) 1.2 µM BpKatG and 100 µM NADH; c) 1.2 µM BpKatG, 100 µM NADH and 200 µM INH; d) 1.2 µM MtKatG, 100 µM NADH and 200 µM INH.

Fig. 3. Migration of purified BpKatG on a 8% SDS-polyacrylamide gel (lane a) and a non-denaturing 8% polyacrylamide gel (lanes b to g). Lane a (SDS polyacrylamide gel) was stained with Coomassie Brilliant blue dye. A single 200 µg amount of BpKatG was loaded in one large lane for lanes b to g and after electrophoresis, the gel was cut into 6 strips for separate staining. Lane b was stained for catalase activity (a clear band on a brown background). Lane c was
stained for peroxidase activity (brown bands on a clear background). Lane d was stained with Coomassie Brilliant blue. Lane e was stained for oxidase activity in a mixture of 200 µM NADH and 200 µM NBT. Lane f was stained for INH hydrazinolysis activity in a mixture of 10 mM INH and 200 µM NBT. Lane g was stained for combined INH hydrazinolysis and NADH oxidase activity in a mixture of 10 mM INH, 200 µM NADH and 200 µM NBT.

Fig. 4. Removal of hydrazine from isoniazid. a) Radical generation in a solution containing 10 mM INH, 200 µM NBT as radical sensor in 50 mM Tris pH 8.0 was followed by formazan appearance measured at 560 nm. Separate assays contain no enzyme (control), BpKatG, MtKatG, or EcKatG. (b) pH dependence of radical generation in a solution of 200 µM NBT with (■) and without BpKatG (●), and a solution of 200 µNBT and 10 mM INH with (□) and without BpKatG (○) is shown.

Fig. 5. Radical generation in a mixture of INH and either NADH (panels a and b) or NAD⁺ (panel c) mediated by catalase-peroxidases using NBT as radical sensor. The initial rates of radical production are followed for BpKatG, MtKatG, EcKatG and no enzyme (control). a) The mixture contained 1.2 µM enzyme, 10 mM INH and 100 µM NADH. b) The mixture was as in a) but with 2 µM Mn²⁺ added. c) The mixture contained 200 µM INH, 250 µM NAD⁺ and 2 µM Mn²⁺. Separate assays contain no enzyme (control), BpKatG, MtKatG, or EcKatG.

Fig. 6. Elution profiles from reverse phase HPLC of reaction products from mixtures of: (a) 100 µm NAD⁺, 200 µM INH and 2 µM Mn²⁺; b) 1.2 µM BpKatG, 100 µM NAD⁺, 200 µM INH and 2 µM Mn²⁺; c) 1.2 µM MtKatG, 100 µM NAD⁺, 200 µM INH and 2 µM Mn²⁺. The inset in
panel (c) shows part of a MALDI mass spectrum of a reaction mixture after 18 h incubation showing the isonicotinoyl-NAD product ion at $m/z$ 771 and the decay product at $m/z$ 753.

Fig. 7. Location of residues in BpKatG that are identical in more than 95% of the 53 catalase-peroxidase sequences available. The distribution in the N-terminal domain is shown in panel (a) and the distribution in the C-terminal domain is shown in panel (b). The domains are treated separately for a clearer representation of the differences.

Fig. 8. Scheme showing the relationship of the five activities of KatG. The locations of the INH hydrazinolysis, NADH oxidase, isonicotinoyl-NAD synthase, catalase and peroxidase reactions are indicated underlined. The two pH dependent options for the NADH oxidase reaction are also indicated. The isonicotinoyl (a) and hydrazide (b) radicals from the hydrazinolysis reaction are available to radical scavengers in the absence of NAD$^+$. The di-imide and proton products from the synthase reaction, indicated in parentheses with an asterisk, have not been confirmed as products but provide a convenient and logical way to balance the reaction. The catalase and peroxidase cycles are shown at the bottom and appear to be independent of the reactions at the top except for the possible metabolism of $H_2O_2$ generated in the oxidase reaction (dotted line) and the possibility that molecular oxygen may also bind to the heme.
**Fig. 1**

(a) Time course of NADH oxidation in the presence of KatG enzymes. 

(b) NBT reduction as a function of time.

(c) pH dependence of KatG activity.

- BpKatG
- MtKatG
- EcKatG
Fig. 2

A chromatogram showing absorbance at 260 nm over time (min).

- Panel a: Peaks labeled N-H, I.
- Panel b: Peaks labeled N.
- Panel c: Peaks labeled I-N.
- Panel d: Peaks showing complex absorbance pattern.

Absorbance (260 nm)

Time (min)
Fig. 3
Fig. 4

(a) Graph showing the time course of nmoles of NBT per nmole of subunit for different conditions:
- BpKatG/MtKatG
- EcKatG
- Control

(b) Graph showing the concentration of nmoles of NBT per min per nmole of subunit as a function of pH:
- pH values: 6, 7, 8, 9, 10

Fig. 4
Fig. 5

BpKatG
MtKatG
EcKatG

KatGs
Control

Time (sec)

nmol NBT.nmol⁻¹ subunit
INH \rightarrow \text{hydrazinolysis} \rightarrow \text{synthase} \rightarrow \text{KatG} \rightarrow \text{catalase} \\
\text{NADH} \rightarrow \text{oxidase} \rightarrow \text{peroxidase} \\
\text{IN-NAD} \rightarrow \text{SOD} \rightarrow \text{INH-NADH} \\
H_2O_2 + O_2 \rightarrow \text{Cpd I} \rightarrow \text{Cpd II} \rightarrow \text{KatG} \\
\text{H}_2\text{O} \rightarrow \text{A}^+ + \text{H}_2\text{O} \\
\text{H}_2\text{O}_2 \rightarrow \text{A}^+ + \text{AH}