Oxidation of Isoniazid by Manganese and *Mycobacterium tuberculosis* Catalase−Peroxidase Yields a New Mechanism of Activation

Jacques Bodiguel,[†] Judit M. Nagy,[‡] Katherine A. Brown,[‡] and Brigitte Jamart-Grégoire*,[†]

MAEM UMR mixte CNRS-UHP no 7567
Faculté des Sciences
Université H. Poincaré Nancy I
Bld. des Aiguillettes BP 239
F-54506 Vandoeuvre-lès-Nancy, France
Department of Biochemistry
Imperial College of Science, Technology and Medicine
Exhibition Road, London, UK, SW7 2AY

Received July 20, 2000

Isoniazid (isonicotinic acid hydrazide, INH) is a frontline prodrug which forms part of the core treatment for tuberculosis. Although bacterial resistance to INH was observed soon after its introduction into clinical use in the 1950s,[1,2] reemergence of tuberculosis in the mid-1980s coupled with increasing numbers of INH-resistant bacterial strains[3,4] stimulated numerous studies which have sought to develop a better understanding of its mechanism of activation and in vivo targets. In particular, it is widely accepted that a heme-containing catalase−peroxidase (CP) enzyme, encoded by the *katG* gene in *Mycobacterium tuberculosis*, oxidizes INH[5,6] and that CP confers INH sensitivity in vivo.[7] The general requirement for hydrogen peroxide in in vitro assays under aerobic conditions suggests that the peroxidatic activity of CP is involved in INH turnover.[5,6] Studies of the related *Mycobaterium smegmatis* CP have also reported an enhancement of INH activation upon addition of exogenous Mn^{2+} which is enzymatically converted to Mn^{3+} prior to oxidizing INH.[8,9] Recent work has indicated that addition of Mn^{2+} can also enhance INH turnover by the *M. tuberculosis* CP although the effect is more limited.[10] Nevertheless, the reactive intermediates of manganese-dependent and -independent INH activation have yet to be identified, and the mechanisms for activation are thus still open to question.

In this communication we report our studies of the activation of INH by Mn cation and *M. tuberculosis* CP (in both the presence and absence of Mn^{2+}) in an effort to better define the reaction pathways leading to INH oxidation. It has been previously shown by HPLC analyses that activation of INH in reaction mixtures containing mycobacterial CP and Mn^{2+} or Mn^{2+} alone yields the acid and amide derivatives of the drug.[6,8,11] An aldehyde derivative of INH, pyridine-4-carboxaldehyde, was also isolated using the *M. tuberculosis* CP with Mn^{2+}.[6,11] This same study also analyzed the amide by mass spectrometry, when the reaction conditions were carried out using ^{15}N-labeled isoniazid, and concluded that this derivative arose from splitting of the N−N bond of INH. We therefore thought that similar experiments could be used to more rigorously compare the activation of INH, leading to formation of isonicotinamide by Mn cation and the *M. tuberculosis* CP enzyme. However, on the basis of mass spectrometry analyses of reaction products obtained using singly labeled ^{15}N-isoniazid isotopic isomers,[12,13] we suggest that formation of isonicotinamide involves splitting of the C−N bond of isoniazid, rather than the N−N bond as previously proposed.

Initially, INH was incubated with various combinations of Mn^{2+}, H_2O_2, and purified enzyme,[14,15] and formation of reaction products was followed and quantitated by HPLC. The absence of transformation of INH in Na_2HPO_4 buffer demonstrates that no significant levels of hydrolysis occur which could result in production of isonicotinic acid and hydrazine during the time course chosen here. Addition of Mn^{2+} leads to formation of isonicotinic acid as the main product with detectable levels of amide and aldehyde derivatives of INH. This result is in agreement with that obtained by Magliozzo and Marcinkeviciene[8] who also carried out this experiment under aerobic conditions. This type of metal-catalyzed oxidation of INH by Mn^{2+}, termed autoxidation, was reported many years ago and yielded isonicotinic acid as the main reaction product.[16] Manganese-catalyzed autoxidation of INH was also invoked as a means of generating lethal species of INH capable of causing oxidative damage to DNA.[17] Addition of H_2O_2 slightly reduced the quantities of amide and acid derivatives obtained from INH and Mn^{2+} and also resulted in no detectable levels of the aldehyde oxidation product. Interestingly, inhibition of the Mn-catalyzed autoxidation of INH by H_2O_2 was also observed by Winder and Denneny[16] who suggested that the presence of hydrogen peroxide decreased the amount of manganese species able to oxidize the drug.

Enzyme-dependent INH oxidation also results in the formation of acid, amide, and aldehyde reaction products in all variations of H_2O_2 and Mn^{2+} described here. When *tert*-butyl hydrogen peroxide (*t*-BuOOH) was used instead of H_2O_2, acid and amide products were formed as previously reported,[18] but we also observed that no aldehyde product was produced. Although it is possible that aldehyde may be degraded under the reaction conditions used, formation of this product may also be the result of a reversible side reaction as previously proposed[6] which could be disfavored using *t*-BuOOH. A 10-fold reduction in the amount of H_2O_2 or *t*-BuOOH used in the assays showed only a reduction in the rate of product formation, as one might expect from the presence of the competing catalytic reaction, but did not alter which products were formed. It is particularly interesting to note that under aerobic conditions *M. tuberculosis* CP is able to oxidize INH in the absence of H_2O_2 and Mn^{2+}, albeit at a reduced rate. The reason for this may be a low-level autoxidation catalysis by the enzyme's heme. This is consistent with data reported by Winder and Denneny[16] who also showed that metal-catalyzed autoxidation of INH could occur using Fe^{3+}, hemin, and Cu^{2+} as

* To whom correspondence may be addressed: Telephone/Fax: 00 33(0)-383912748. E-mail: Brigitte.Jamart@maem.uhp-nancy.fr.
[†] Université Henri Poincaré.
[‡] Imperial College of Science, Technology and Medicine.

(1) Middlebrook, G. *Am. Rev. Tuberc.* **1954**, *69*, 471−472.
(2) Youatt, *J. Am. Rev. Respir. Dis.* **1969**, *99*, 729−749.
(3) Stoeckle, M. Y.; Guan, L.; Riegler, N.; Weitzman, I.; Kreiswirth, B.; Kornblum, J.; Laraque, F.; Riley, L. W. *J. Infect. Dis.* **1993**, *168*, 1063−1065.
(4) Heym, B.; Alzari, P. M.; Honore, N.; Cole, S. *Mol. Microbiol.* **1995**, *15*, 235−245.
(5) Schoeb, H. A.; Bowman Jr., B. U.; Ottolenghi, A. C.; Merola, A. *J. Antimicrob. Agents Chemother.* **1985**, *27*, 408−412.
(6) Johnsson, K.; Schultz, P. G. *J. Am. Chem. Soc.* **1994**, *116*, 7425−7426.
(7) Zhang, Y.; Heym, B.; Allen, B.; Young, D.; Cole, S. *Nature* **1992**, *358*, 591−593.
(8) Magliozzo, R. S.; Marcinkeviciene, J. A. *J. Am. Chem. Soc.* **1996**, *118*, 11303−11304.
(9) Magliozzo, R. S.; Marcinkeviciene, J. A. *J. Biol. Chem.* **1997**, *272*, 8867−8870.
(10) Lei, B.; Wei, C.-J.; Tu, S.-C. *J. Biol. Chem.* **2000**, *275*, 2520−2526.
(11) Johnsson, K.; Froland, W. A.; Schultz, P. G. *J. Biol. Chem.* **1997**, *272*, 2834−2840.
(12) Jamart-Grégoire, B.; Brosse, N.; Bodiguel, J. *Synthesis* **1998**, 269−270.
(13) Brosse, N.; Pinto, M. F.; Jamart-Grégoire, B. *J. Chem. Soc., Perkin Trans. 1* **1998**, 3685−3688.
(14) Nagy, J. M.; Cass, A. E. G.; Brown, K. A. *J. Biol. Chem.* **1997**, *272*, 31265−31271.
(15) Recombinant *M. tuberculosis* CP was overproduced in *Escherichia coli* lacking endogenous catalase activities and purified to homogeneity as we have previously described.[14] Purified enzyme was shown not to contain adventitious Mn^{2+} by inductively coupled plasma-atomic emission spectroscopy analysis.[14]
(16) Winder, F. G.; Denneny, J. M. *Biochem. J.* **1959**, *73*, 500−507.
(17) Ito, K.; Yamamoto, K.; Kawanishi, S. *Biochemistry* **1992**, *31*, 11606−11613.
(18) Wengenack, N. L.; Hoard, H. M.; Rusnak, F. *J. Am. Chem. Soc.* **1999**, *121*, 9748−9749.

10.1021/ja002674f CCC: $20.00 © 2001 American Chemical Society
Published on Web 03/30/2001

well as Mn^{2+}. As expected, addition of H_2O_2 demonstrated an increased rate of CP-catalyzed INH oxidation, but the added presence of Mn^{2+} showed no significant rate enhancement under standard assay conditions. However, when a 20:1 excess of Mn^{2+} to enzyme was used, the rate of INH oxidation increased somewhat, but the rate of oxidation of INH by Mn^{2+} alone (in the absence of enzyme) also increased with the higher Mn^{2+} concentration. Mn^{2+} oxidation by the *M. tuberculosis* enzyme has been reported[18] which might suggest that the enhancement observed here could result from increased levels of Mn^{3+} and other oxidants[18] in solution. Although the studies here do not explicitly address this point, the fact that rate enhancement with increased Mn^{2+} concentration was observed in both the presence and absence CP may simply indicate the presence of enzyme peroxidatic INH oxidation and Mn^{2+} autoxidation of INH acting simultaneously together in solution. Furthermore, lack of a rate enhancement by Mn^{2+} addition to CP/INH and CP/INH/H_2O_2 mixtures under standard assay conditions may be due to competing interactions between CP and Mn^{2+}, as well as inhibition of autoxidation by the presence of hydrogen peroxide[16] in the case of the latter reaction mixture.

Taken together, these data suggest that Mn^{2+} does not play an essential part in the oxidation of INH by the *M. tuberculosis* CP enzyme. A similar conclusion was drawn from recent studies which monitored the formation of an inhibitor of the downstream INH target enoyl reductase, InhA.[10] This inhibitor, isonicotinic acyl NADH, is thought to be formed by reaction of an activated INH intermediate with NADH. A 30% increase in inhibitor formation was observed upon addition of Mn^{2+} to the assay mixture containing CP, INH, and NADH at a 2:1 ratio of Mn^{2+} to enzyme. However, in the absence of enzyme the rate of inhibitor production was significantly reduced. It is possible that the observed rate enhancement of inhibitor formation from the addition of Mn^{2+} to CP, based upon results obtained here, may be due in part to autoxidation of INH by Mn^{2+}. Notably the low level of InhA inhibitor formation observed in the absence of enzyme may, for example, represent an inhibition of INH activation by NADH which remains to be tested. In any case, the results presented here suggest that Mn^{2+} is not required for INH activation by the *M. tuberculosis* CP and tend to favor a peroxidatic pathway for activation by CP in vivo. This view disagrees with Wengenack and colleagues[18] who suggest that a superoxide-dependent mechanism may instead be critical for enzyme-mediated INH oxidation. Our studies also support the view of Lei and colleagues[10] in that this enzyme may be different in its mode of activation of INH compared to the *M. smegmatis* enzyme which shows an Mn^{2+}-dependent oxidation of INH[8,9] and only negligible oxidation of the drug in the absence of added Mn^{2+} cation.[8]

To investigate further the mechanism of INH activation, we carried out reactions using INH isotopic isomers which were ^{15}N-labeled at the hydrazide moiety. The resulting isonicotinamide reaction products were isolated by HPLC and subsequently analyzed for isotopic content by electrospray mass spectrometry.[19] Using doubly labeled drug, INH $^{15}N^{15}N$,[20] only isonicotinamide ^{15}N was obtained from the four reaction conditions used (Mn^{2+}, CP, Mn^{2+}/CP, or Mn^{2+}/CP/H_2O_2). The same level of ^{15}N incorporation was observed in previous studies which showed that only isonicotinamide ^{15}N was obtained from a reaction containing INH $^{15}N^{15}N$ and Mn^{2+}/CP/H_2O_2.[6,11] This result led to the proposal of a mechanism for the enzymatic oxidation of INH in which the amide reaction product is generated from splitting of the N−N bond through a possible disproportionation of the INH hydrazide moiety.[6] To further study this proposed mechanism, we characterized the isotopic content of isonicotinamide, isolated from the four reaction conditions already mentioned, using singly labeled INH $^{14}N^{15}N$ or INH $^{15}N^{14}N$.[12,13] In all cases, 50% of the reaction product was isonicotinamide-^{15}N and 50% was isonicotinamide-^{14}N. These data lead us to suggest that the amide product would be obtained from a C−N, rather than N−N, splitting of INH.

A reaction mechanism for production of the amide reaction product is proposed where C−N bond splitting of INH yields an acyl radical and diazene intermediates. Monitoring of the reaction using singly labeled INH $^{15}N^{14}N$[12,13] by ^{15}N NMR[21] did not demonstrate formation of the other isomer, INH $^{14}N^{15}N$. This result indicates that this first step appears to be essentially irreversible and that the 50% isotopic distribution of isonicotinamide obtained using electrospray mass spectrometry with singly labeled INH is unlikely to be an artifact arising from an equilibration process involving the isotopic label. We propose that subsequent generation of NH_3, used in reaction with the acyl radical to form the isonicotinamide, would result from successive transformations of first diazene into hydrazine, followed by hydrazine into NH_3. This mechanism thus predicts that hydrazine and ammonia would be intermediates along the reaction pathway. To test this hypothesis, increasing amounts of unlabeled hydrazine or ammonia were added to reaction mixtures containing INH $^{15}N^{15}N$ and Mn^{2+} which in turn yielded increasing amounts of unlabeled isonicotinamide. These results agree with our proposed C−N bond splitting of INH and differ from a previously proposed mechanism for Mn^{2+}-catalyzed autoxidation of the drug which also included an N−N splitting of the hydrazide to generate the amide reaction product.[16] Furthermore, no unlabeled INH was isolated from these experiments,[22] demonstrating that direct reaction of the acyl radical with hydrazine to generate isonicotinamide is unlikely, further supporting the proposed step which requires transformation of hydrazine prior to formation of the amide.

In conclusion, the *M. tuberculosis* CP enzyme does not appear to demonstrate significant levels of Mn-dependent peroxidase activity which could catalyze the oxidation of INH. The ability of Mn cation to catalyze INH oxidation in the absence of enzyme is consistent with an autoxidation process, but the reaction also has been shown to yield the same reaction products as the enzyme-catalyzed reaction at a comparable rate. Given the apparent importance of manganese in the *M. smegmatis* CP activation of INH,[9] and discoveries of metal transporters in *M. tuberculosis*,[23,24] it is possible that intracellular Mn^{2+} concentrations may influence the efficiency of INH activation in vivo, particularly under aerobic conditions. In addition, the mechanism proposed here may help to develop a better understanding of the molecular events responsible for both metal- and enzyme-catalyzed INH activation and can stimulate new studies to identify the nature of the activated form of INH and its reactions with downstream targets.

Acknowledgment. We thank Dr.Pierre Mutzenhardt for carrying out the NMR studies and the reviewers for their criticisms and useful suggestions. This work has been supported by Grant BMH4-CT96-1492 (Commission of European Communities).

Supporting Information Available: 400 MHz ^{15}N NMR spectrum of INH $^{14}N^{15}N$ ($CDCl_3$). 400 MHz ^{15}N NMR spectrum ($CDCl_3$) of recovered INH obtained after incubation for 2 h of $^{15}N^{14}N$ INH with CP; Table 1: INH oxidation catalyzed by Mn^{2+} and *M. tuberculosis* catalase−peroxidase; Scheme 1: proposed reaction mechanism for production of the isonicotinamide (PDF). These materials are available free of charge via Internet at http://pubs.acs.org.

JA002674F

(19) Isonicotinamide was separated by HPLC, collected, and identified by mass spectrometry in the positive electrospray ionization mode.

(20) Doubly labeled INH was synthesized by condensing the corresponding ethyl ester with ^{15}N-labeled hydrazine hydrate obtained from ^{15}N-labeled hydrazine sulfate (99% isotopic purity, Aldrich).

(21) A reaction mixture containing INH $^{15}N^{14}N$/CP/H_2O_2 was incubated for 2 h at 37 °C. The enzyme was removed by ultrafiltration, and the recovered INH was isolated by HPLC. The solvent was removed, and the recovered INH was dissolved in $CDCl_3$ and analyzed by ^{15}N NMR spectroscopy using $^{15}NH_4OH$ as external standard.

(22) Hydrazine hydrate was incubated overnight at 37 °C with doubly labeled isoniazid and $MnCl_2$. Isonicotinamide was isolated by HPLC. The solvent was removed, and the amide was dissolved in EtOH and analyzed by mass spectrometry using a GC−MS spectrometer.

(23) Calder, K.; Horowitz, M. A. *Microb. Pathog.* **1998**, *24*, 133−143.

(24) Agranoff, D.; Monohan, I. M.; Mangan, J. A.; Butcher, P. D.; Krishna, S. *J. Exp. Med.* **1999**, *190*, 717−724.