A Fast and Efficient Metal-Mediated Oxidation of Isoniazid and Identification of Isoniazid – NAD(H) Adducts

Michel Nguyen, Catherine Claparols, Jean Bernadou,* and Bernard Meunier*[a]

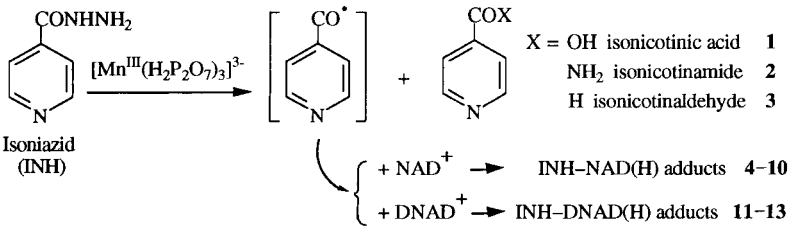
It is currently believed that isoniazid (INH) is oxidised inside Mycobacterium tuberculosis *to generate, by covalent attachment to the nicotinamide ring of NAD(H) (β-nicotinamide adenine dinucleotide), a strong inhibitor of InhA, an enzyme essential for mycolic acid biosynthesis. This work was carried out to characterise the InhA inhibitors (named INH – NAD(H) adducts) which are generated, in the presence of the nicotinamide coenzyme NAD^+, by oxidation of INH with manganese(III) pyrophosphate, a nonenzymatic and efficient oxidant used to mimic INH activation by the catalase – peroxidase KatG inside* M. tuberculosis. *The oxidation process is almost complete in less than 15 minutes (in comparison to the slow activation obtained in the KatG-dependent process (2.5 hours) or in the nonenzymatic O_2/Mn^{II}-dependent activation (5 hours)). The alkylation of NAD^+ by the postulated isonicotinoyl radical generates, in solution, a family of INH – NAD(H) adducts. Analyses with liquid chromatography/electrospray ionisation mass spectrometry (LC/ESI-MS) and experiments performed with ^{18}O- and ^2H-labelled substrates allowed us to propose two open and four hemiamidal cyclised dihydropyridine structures as the main forms present in solution; these result from the combination of the isonicotinoyl radical and the nicotinamide part of NAD^+. A small amount of a secondary oxidation product was also detected. Structural data on the forms present in solution should help in the design of inhibitors of enzymes involved in the biosynthesis of mycolic acids to act as potential antituberculosis drugs.*

KEYWORDS:

drug research · isoniazid · manganese · oxidation · structure elucidation

Introduction

Isoniazid (INH) is one of the oldest synthetic antituberculosis drugs and has been widely used in prophylaxis and treatment of tuberculosis.[1] It is now well accepted that INH acts as a prodrug[2–4] which requires an intracellular conversion by *Mycobacterium tuberculosis* catalase – peroxidase KatG to generate the active form responsible for the lethal effect on bacterial cells. None of the stable derivatives observed in KatG-dependent INH activation reaction, that is, isonicotinic acid (**1**), isonicotinamide (**2**) and isonicotinaldehyde (**3**; Scheme 1), have demonstrated bactericidal effect.[5] Recent studies[6, 7] have suggested that the activated form of INH, probably an isonicotinoyl radical, is capable of reacting with β-nicotinamide adenine dinucleotide (NAD^+/NADH) which is the cofactor of the long-chain enoyl – acyl carrier protein reductase InhA.[8] InhA is a key enzyme involved in the biosynthesis of long-chain fatty acids and of mycolic acids, specific components of the mycobacterial cell wall.[9] The formation of covalent adduct(s) INH – NAD(H) as competitive inhibitors might explain the inactivation of InhA. Recently, an isonicotinoyl – NADH adduct has been characterised within the active site of InhA from data obtained by X-ray crystallography.[6] In solution, UV absorption and mass spectral data on a crude mixture of free inhibitor(s) formed either by nonenzymatic O_2/Mn^{II} activation or by the KatG-dependent process[10] and some data on isonicotinoyl – NAD inhibitors[7] have also been reported. However, further experiments are needed to elucidate the exact nature of the observed products. The use of Mn^{III} to oxidise INH and form InhA inhibitors has been mentioned, but without any detail about experimental conditions.[7] Despite these recent efforts, the active form of INH and the activation mechanism of this drug are still a matter of debate.



Scheme 1. *INH oxidation products and adducts formed in the presence of NAD(H) and DNAD(H).*

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Several studies have been focussed on the role of the catalase – peroxidase KatG,[3, 4, 11] a hemoprotein with manganese-dependent peroxidase activity.[12, 13] KatG catalyses the conversion of Mn^{II} into Mn^{III}, and this latter metal ion is probably able to oxidise INH. (This mode of activation is reminiscent to the mechanism of action of the manganese peroxidase of the white rot fungus *Phanerochaete chrysosporium*.[14]) Consequently, studies on the oxidation of INH by manganese salts are biologically relevant. Since Mn^{II} is only slowly converted into Mn^{III} in aerobic conditions, the easily available Mn^{II} salts used in aerated solutions behave as a poor activating system.[15, 16] Direct INH activation by Mn^{III} salts appears to be more attractive since manganese(III) malonate or manganese(III) pyrophosphate have been shown to be capable of activating this drug, to form the oxidation products **1** and **2**[12] or induce DNA breaks.[16] In order to simplify in vitro mechanistic studies and because of our previous model studies on heme oxygenases[17] and manganese peroxidases,[18] we decided to mimic the KatG oxidation of INH either by catalytic peroxidase models, such as activated synthetic metalloporphyrins, or by the use of stoichiometric amounts of a stable manganese(III) derivative.

Table 1. *Yields of products obtained from INH oxidation.*[a]

Run	Oxidation system	INH conversion [%]	Yield [%] 1	2	3	Reaction time
1	control	3	0,1	< 1	< 1	50 h
2	$MnCl_2/O_2$	58	42	8	6	5 h
3	Mn-TMPyP/$KHSO_5$[c,d]	98	82	4	4	15 min
4	Mn-TMPyP/Na_2SO_3/O_2	98	51	13	20	15 min
5	Mn^{III} pyrophosphate (pH 4.5)	93	45	12	4	15 min
6	Mn^{III} pyrophosphate (pH 6.5)	66[b]	32	26	3	15 min
7	KatG protein[4]	n.d.	30	1.5	1.5	2 h 23 min
8	KatG protein/Mn^{II}[15]	76	66	13	n.d.	2 h 30 min

[a] The values are the mean of three determinations. Standard deviations were always lower than ±1% except for [b] (±3%). n.d. = not determined. [c] Under the same experimental conditions, $KHSO_5$ alone gave only 14% conversion of INH. [d] Mn-TMPyP pentaacetate was prepared according to ref. [25].

Results and Discussion

Metal-mediated oxidation of INH

The first system, a peroxidase/cytochrome P-450 model expected to oxidise INH in the same way as horseradish peroxidase,[19] consisted of the water-soluble manganese porphyrin catalyst Mn-TMPyP [Mn-*meso*-tetrakis(4-*N*-methylpyridiniumyl)porphyrin] associated to an oxygen atom source (either $KHSO_5$ or Na_2SO_3/O_2). Both cases involve a manganese(V) – oxoporphyrin as the active species.[20] The second system consisted of manganese(III) pyrophosphate,[21] a stable form of Mn^{III} ions in aqueous solution which was previously used in our model studies on the manganese peroxidase of *Phanerochaete chrysosporium*.[18] Since aquated Mn^{III} ions are an oxidizing agent in aqueous solution with a marked tendency to disproportionate into Mn^{II} and Mn^{IV}, we chose pyrophosphate as an oxidant-resistant oxygen-donor ligand able to stabilise Mn^{III} in the pH range of 4 – 6. An improved synthesis (in comparison to that reported by Archibald and Fridovich[21]) is described in the Experimental Section. The product is stable over several months at 4 °C. Use of other organic chelating agents such as malate, malonate, lactate, oxalate or tartrate produced manganese complexes which are not as stable over time and show storage problems.

As shown in Table 1, INH was not easily oxidised in air (run 1), even in the presence of an Mn^{II} salt (run 2). In contrast, catalytic oxidation with a metalloporphyrin in the presence of $KHSO_5$ (run 3) or Na_2SO_3/O_2 (run 4) and stoichiometric oxidation by manganese(III) pyrophosphate (runs 5 and 6) gave high conversions of INH within short reaction times. For example, INH conversion was 93% in 15 min with two molar equivalents of manganese(III) pyrophosphate. In addition, these methods gave the same stable oxidation products **1** – **3** as in the KatG protein assay (runs 7 and 8). Therefore, these metal-mediated systems are good candidates for modelling the KatG protein in vitro. However, since none of the stable oxidation products of INH are able to inhibit InhA,[5] one key point which remains to be determined is the ability of these chemical activating systems to produce short-lived reactive intermediates (for example, the isonicotinoyl radical) that can react on the NAD^+/NADH cofactor of InhA to generate inhibitors of this enzyme.

Formation of INH – NAD(H) and INH – DNAD(H) adducts

The three different drug-activating systems, Mn-TMPyP/$KHSO_5$, Mn-TMPyP/Na_2SO_3/O_2 and manganese(III) pyrophosphate, were tested in the presence of INH and NAD^+ for their ability to produce INH – NAD(H) adducts. In all cases and in addition to the degradation products of NAD^+ (ADP ribose and nicotinamide) and to the usual INH oxidation products **1** – **3** (**3** was not directly observed and needed a prior derivatisation), we detected a number of peaks (numbered 4 – 10 in Figure 1 A, for example) with retention times higher than NAD^+ and corresponding to the formation of adducts between an INH residue and NAD^+. In the case of the porphyrin catalyst, the overall yield of adducts did not rise above 5%, but in the case of the manganese(III) pyrophosphate system this yield ranged from 35 – 45% depending on the reaction conditions. So, only this last efficient system was used for further studies. Figure 1 shows two typical high-pressure liquid chromatography (HPLC) chromatograms, which illustrate the formation of these adducts when INH was oxidised by manganese(III) pyrophosphate in the presence of NAD^+ or $DNAD^+$ (deamido-NAD^+, nicotinic acid adenine dinucleotide), a nonnatural cofactor used to facilitate the elucidation of the adduct structures. As shown in chromatogram A, in the case of adducts formed with NAD^+, at least six peaks were detected with molecular masses compatible with those of structures including both INH and NAD(H) residues. We did not succeed in isolating these different compounds due to their partial degradation and/or interconversion during the HPLC collection and lyophilisation work-up. (A dynamic equilibrium between isomeric isonicotinoyl – NAD adducts was recently proposed.[7]) Therefore, we

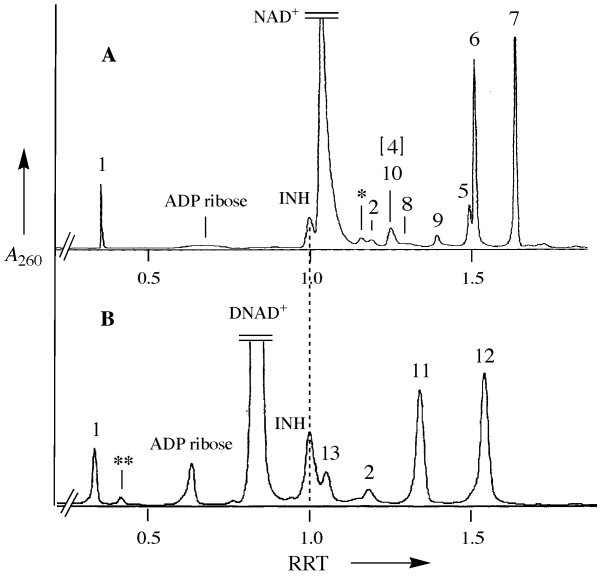
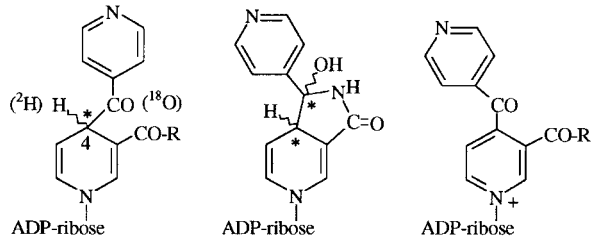


Figure 1. *HPLC profiles of reaction mixtures containing (A) NAD$^+$ (2 mM) or (B) DNAD$^+$ (2 mM) with INH (2 mM) and manganese(III) pyrophosphate (4 mM; introduced in 10 consecutive additions of 400 μM each, every 2 min) in phosphate buffer (100 mM; pH 7.5). * = Nicotinamide, ** = nicotinic acid; these come from decomposition of NAD$^+$ and DNAD$^+$, respectively. RRT = relative retention time (with INH as the reference).*

decided to characterise the compounds by direct liquid chromatography/electrospray ionisation mass spectrometry (LC/ESI-MS) analyses of crude reaction mixtures from experiments with labelled INH and NAD$^+$.

Table 2. *MS data and proposed structure for the INH – NAD(H) and INH – DNAD(H) adducts.*[a]



4, 5, 11, 12: open structure **6–9**: cyclized structure **10, 13**: oxidized structure

Run		Molecular peak			
		$[M+H]^+$	$[M+H]^+$	$[M+H]^+$	$[M]^+$
	R = NH$_2$				
	INH – NAD(H) adducts[b]	**4**, **5**	**6**, **7**	**8**, **9**	**10**[c]
1	[^{16}O]INH/[^{1}H]NAD$^+$	771	771	771	769
	dehydrated fragment	–	753	753	–
2	[^{18}O]INH/[^{1}H]NAD$^+$	n.d.	773[d]	773[d]	771[d]
	dehydrated fragment	–	753	753	–
3	[^{16}O]INH/[^{2}H]NAD$^+$	n.d.	772	772	769
	dehydrated fragment	–	754	754	–
	R = OH				
	INH – DNAD(H) adducts[b]	**11**, **12**			**13**
4	[^{16}O]INH/[^{1}H]NAD$^+$	772	–	–	770
	dehydrated fragment	–	–	–	–

[a] Only the modifications on the nicotinamide and the nicotinic acid moieties of NAD$^+$ and DNAD$^+$ are shown. New asymmetric carbon atoms are indicated by an asterisk. [b] Mass/charge values for monocharged species. [c] A cyclised hemiamidal structure cannot be excluded for compound **10**. [d] A partial exchange of the carbonyl oxygen with water was observed for all compounds **6** – **10**. (Such incorporation of oxygen from water was also reported in ref. [7].)

Characterisation of INH – NAD(H) and INH – DNAD(H) adducts

The two labelled compounds [^{18}O]INH and [^{2}H]NAD$^+$ were prepared as described in the Experimental Section. They exhibit a label on two crucial positions useful for the interpretations described hereafter, that is, on the oxygen atom of the carbonyl group of INH and at position 4 of the nicotinamide ring of NAD$^+$. The isotopic purities obtained were 90 – 92% for [^{18}O]INH and > 98% for [^{2}H]NAD$^+$.

In the case of NAD$^+$ adducts (Figure 1 A), the HPLC profile was rather complicated, with seven peaks attributed to INH – NAD$^+$ adducts on the basis of the LC/ESI-MS data (Table 2). Six of them (peaks 4 – 9) had a mass of 770 ($[M+H]^+ = 771$) corresponding to a combination of an isonicotinoyl radical with NAD$^+$; the last one (peak 10), with a mass of 769 ($[M]^+$), appears as an oxidised form of the preceding adducts and was only detected as a minor product of the reaction (< 5% compared to the sum of the other adducts **4** – **9**). By reference to previous works,[7] peak 10 could be attributed to a compound with structure **10** (Table 2) where position 4 of the nicotinamide ring of NAD$^+$ is substituted by an isonicotinoyl radical. The retention of the ^{18}O label (from the [^{18}O]INH residue, run 2) and the loss of the ^{2}H label (from the [^{2}H]NAD$^+$ moiety, run 3) both support this structure. Compound **10** appeared to be a secondary oxidation product since it increased when the reaction mixture was mildly heated (data not shown), while the pool of other adducts decreased. Since only one peak with a mass of 769 ($[M]^+$) was detected and no dehydratation fragment was formed during MS analysis, the formation of two diastereoisomeric cyclic hemidal structures (see below) is unlikely.

Attribution of peaks 4 – 9 was more delicate. All the products corresponding to the six peaks display UV spectra characteristic for dihydropyridine derivatives (λ_{max} values near 320 – 330 nm). The common molecular mass of 770 should simply correspond to a reduced dihydropyridine form of the structure **10**, but, in this case, only the two diastereoisomers **4** and **5** (Table 2) should be observed; they would result from the creation of one supplementary chiral center at position 4 of the nicotinamide ring. A careful examination of the mass spectra of the six products observed experimentally showed that, in the course of MS analysis (Figure 2, orifice voltage 20 V), two of them did not give any dehydrated fragment (**4**, **5**), two others were dehydrated to a rather low extent (**8**, **9**; the relative intensity of the dehydrated fragment was less than 50% that of the molecular peak) and the last two were easily dehydrated (**6**, **7**; the relative intensity of the dehydrated fragment is higher than 50% that of the molecular peak). The same differences in behaviour were observed with a higher orifice voltage (80 V, Figure 2, bottom): Compounds **4** and **5** were not dehydrated at all, the intensity of the dehydrated fragments of **8** and **9** increased but remained

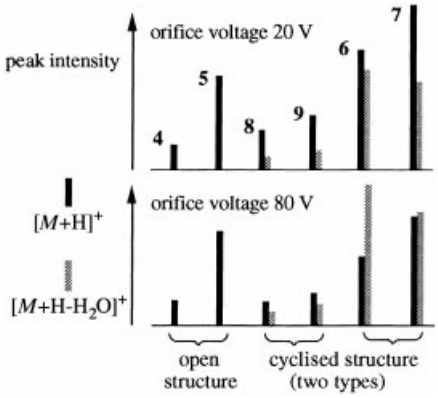
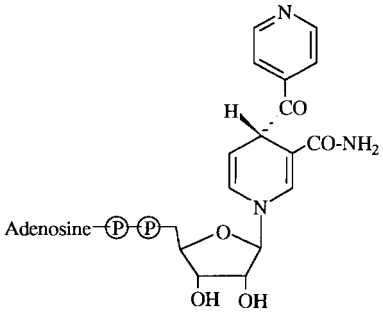


Figure 2. *Peak intensity of INH – NAD(H) adducts **4** – **9**: Three types of adducts are considered to be present, based on variation of orifice voltage in MS experiments and formation (or nonformation) of a dehydrated fragment.*

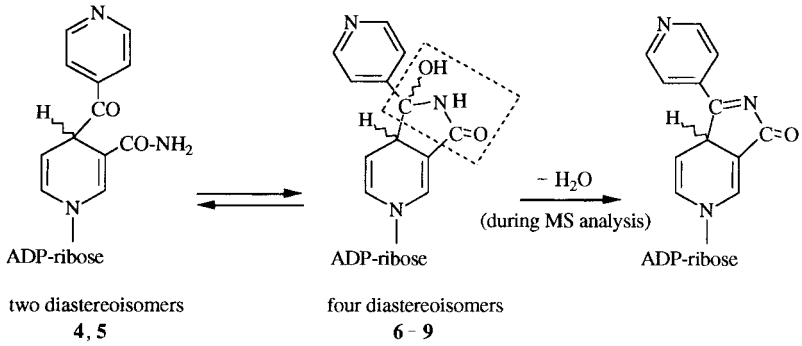
below 100% that of the corresponding molecular peaks and the intensity of the dehydrated fragments of **6** and **7** became higher than that of the molecular peaks. On the basis of these results, the two peaks 4 and 5 might be attributed to the open structures of the two diastereoisomers **4** (present in very low amount and only detected by LC/ESI-MS under the peak of compound **10**) and **5**. Either compound **4** or **5** corresponds to the INH – NADH adduct characterised within the active site of InhA from the X-ray crystallography data (Scheme 2).[6] Peaks 6 – 9 might correspond to the hemiamidal cyclised structures **6** – **9** (Table 2), which include a second new asymmetric center; this would explain the existence of four diastereoisomers.



Scheme 2. *Proposed structure of the INH – NADH adduct characterised within the active site of InhA with X-ray crystallography data.[6]*

The cyclised structures **6** – **9**, whose attributions are based on the dehydration observed during mass analyses, are furthermore supported by mass data from the labelling experiments shown in runs 2 and 3 of Table 2. Both the ^{18}O label from [^{18}O]INH and the ^{2}H label from [^{2}H]NAD$^+$ were retained in the respective molecular peaks and, with consideration of the dehydrated fragments, it is clear that dehydration involved the oxygen atom of the INH carbonyl but not the hydrogen in position 4 of the nicotinamide part. These results indicate that the loss of a water molecule concerned only the hemiamidal moiety with formation of a pyrrolinone ring and so confirmed the existence of the cyclised structures **6** – **9** (Scheme 3). Such hemiamidal structures in cyclised products have been previously observed for benzamides substituted at *ortho* positions with acyl groups.[22]

Since the amide function present in NAD$^+$ plays a crucial role in formation of the cyclised derivatives **6** – **9**, we checked that replacing NAD$^+$ by DNAD$^+$, where the amide group is missing, simplified the chromatographic profile. Effectively, as shown in Figure 1 B and Table 2, only three adducts could be detected, one of which corresponds to the oxidised compound **13** ($m/z = 770$, $[M]^+$) and two others which correspond to the two diastereoisomeric open structures **11** and **12** ($m/z = 771$, $[M+H]^+ = 772$). As expected, no dehydration was observed for any of these three uncyclised compounds (run 4, Table 2). In these last experiments, the other peaks were easily attributed to residual INH and



Scheme 3. *Cyclisation creates another asymmetric centre and renders possible the dehydration reaction suggested by mass analyses. The dashed box highlights the cyclic hemiamidal structure.*

DNAD$^+$, to stable oxidation products of INH (**1** and **2**) and to degradation products of DNAD$^+$ (ADP-ribose, nicotinic acid).

Treatment of crude reaction mixtures containing either INH – NAD(H) or INH – DNAD(H) adducts with phenylhydrazine gave, respectively, four new compounds ($m/z = 861$) and two new compounds ($m/z = 862$). Their formation corresponds to the disappearance of adducts **6** – **9** in the first case and of adducts **11** – **12** in the second (see Figure 1 and Table 1 in the Supporting Information). In the mass spectra of the four phenylhydrazine derivatives from INH – NAD(H) adducts, the presence of a weak but constant fragment at $m/z = 753$, which corresponds to the loss of one phenylhydrazine molecule, supports the cyclic structures **17** – **20** (Table 1 in the Supporting Information; structures **17** – **20** are analogous to structures **6** – **9**, but with the OH group replaced by the NH-NH-Ph group). In the case of INH – DNAD(H) adducts, both the existence of only two diastereoisomers and the absence of the fragment at $m/z = 753$ support the formation of the two phenylhydrazones **21** and **22** (Table 1 in the Supporting Information).

These results show that, with the carbonyl group of the isonicotinoyl moiety, as with its hydrazone derivative, only the nucleophilic amido function present in the NAD$^+$ residue can lead to a cyclisation process that creates a new chiral center and

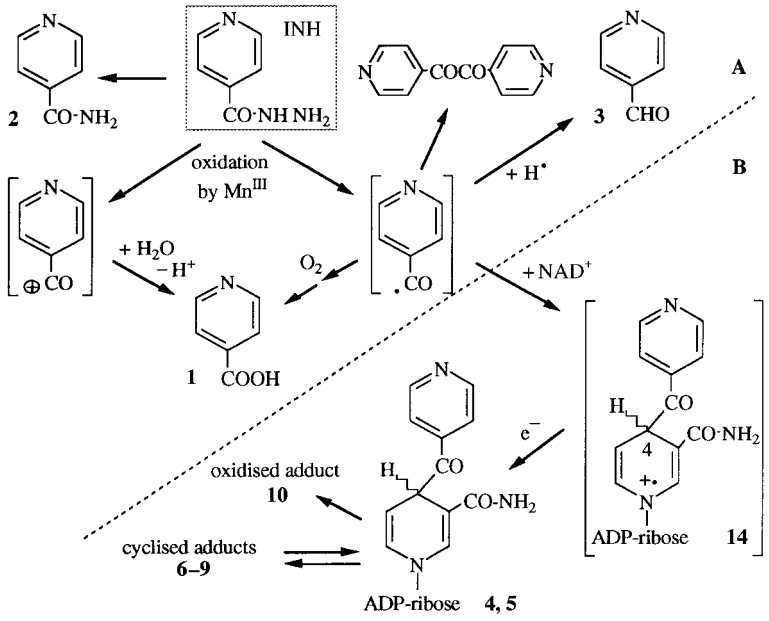
explains in each case the formation of four diastereoisomeric adducts (**6** – **9** and **17** – **20**); the carboxylate group of the $DNAD^+$ residue does not allow this cyclisation process and only two open structures could be shown in each case (**11**, **12** and **21**, **22**).

In addition, some alternative hypotheses to explain the formation of several types of dihydropyridine INH – NAD(H) adducts can easily be discarded: 1) Alkylation of the nicotinamide ring in NAD^+ must only occur in position 4 since we were only able to detect compound **10** with loss of the label at position 4 (run 3, Table 2); 2) isomerisation from the 1,4-dihydro-NAD structure present in **4** or **5** to 1,2- or 1,6-dihydro-NAD structures should remove the label in position 4, but this was not the case (run 3, Table 2; see ref. [23] for such an isomerisation process observed for NADH); 3) an equilibrium of compounds **4** and **5** with the corresponding ketone hydrates as suggested in ref. [7] did not explain either the number of isomers or the dehydration phenomenon observed in their mass spectra.

What are the reactive species involved in adduct formation?

In previous reports, the mechanism for oxidation of isoniazid[4] and for formation of adduct(s) between the activated form(s) of INH and NAD^+ or NADH[6, 7] has been discussed. It has been proposed that the adducts can be formed either by attachment of an isonicotinoyl anion to NAD^+[6] or more likely by direct addition of an isonicotinoyl radical to an $NAD^{\bullet}$ radical[6] or to NAD^+ with subsequent reduction of the radical cation.[7] A proposed mechanism for oxidation of INH by manganese(III) pyrophosphate is shown in Scheme 4. The isonicotinoyl radical is a key intermediate in the formation of isonicotinic acid (**1**), isonicotinaldehyde (**3**) and, in the presence of NAD^+, in the formation of INH – NAD(H) adducts. The di(4-pyridyl)glyoxal formed by recombination of two isonicotinoyl radicals plays the role of a marker and was effectively detected during LC/ESI-MS analyses ($[M+H]^+ = 213$; the RRT was 0.18 under the experimental conditions used to obtain Figure 1). The transient existence of the isonicotinoyl radical was also suggested by INH oxidation experiments performed in $H_2{}^{18}O$ (data not shown) which led to about 20% of **1** without the ^{18}O label; this was indicative of the reaction of the isonicotinoyl radical with dioxygen. In similar experiments performed with the KatG enzyme, this percentage was 33%.[4] So, as proposed by Wilming and Johnsson,[7] addition of the isonicotinoyl radical to NAD^+ should give radical **14** (Scheme 4) with creation of a first new chiral carbon at position 4 of the nicotinamide ring. Its fast one-electron reduction, possibly by INH, gives the open structures **4** and **5**, which are in slow equilibrium with the four cyclised adducts **6** – **9**. The creation of a second new chiral centre explains that these cyclised dihydropyridine derivatives exist in four diastereoisomeric forms. Most likely, the enantiomeric ratio *R*/*S* at position 4 of the isonicotinamide is close to 1:1 (because the isonicotinoyl radical can attack equally the two faces of the nicotinamide ring to give **4** and **5**) but the cyclisation giving rise to the hemiamidal structures is selective enough to produce preferentially the two diastereoisomers **6** and **7** (with opposite configurations at C4) over the two minor ones **8** and **9**. Slow oxidation of these different adducts can give the common oxidised compound **10**. It should be noted that the first generated adduct formed cannot be **10** with subsequent reduction to dihydropyridine adducts **4** – **9** since, in this case, the label of $[^2H]NAD^+$ would not be retained (run 3, Table 2).

In conclusion, these data illustrate the ability of manganese(III) pyrophosphate used in stoichiometric amounts to oxidise INH and, in the presence of the nicotinamide coenzyme, to give INH adducts which are potential inhibitors of the enoyl – acyl carrier protein reductase InhA. When compared to the slow and uncomplete oxidation observed with O_2/Mn^{II} or to the use of the not easily available catalase – peroxidase KatG, manganese(III) pyrophosphate represents an efficient and fast oxidation system in attempts to isolate and characterise labile and reactive intermediates formed during the INH oxidation. In addition, the structural information on the different possible forms of INH – NAD(H) adducts in solution should help us to design new inhibitors of InhA as potential antituberculosis drugs. Presently, preliminary results indicate that a crude mixture of the INH – NAD(H) adducts allow the in vitro inhibition of this target enzyme[24] when, under similar conditions, INH – DNAD(H) adducts do not give any significative inhibitory effect (data not shown). This point underlines the role of the amido group in the nicotinamide moiety, either through its interaction with the active site of the enzyme or by favouring the formation of cyclic hemiamidal structures as possible reactive inhibitors.



Scheme 4. *Proposed mechanism for manganese(III)-mediated oxidation of (A) INH alone and (B) in the presence of NAD^+, which leads to the formation of INH – NADH adducts.*

Experimental Section

Materials: Isoniazid, carboxymethoxylamine hemihydrochloride $[(NH_2OCH_2COOH)_2 \cdot HCl]$, MnO_2, NAD^+

and DNAD$^+$ were obtained from Sigma – Aldrich, sodium pyrophosphate, pyrophosphoric acid and anhydrous sodium sulfite from Fluka, $Mn^{III}(OAc)_3$ from Merck – Schuchardt, $MnCl_2$ and $MnSO_4$ from Janssen Chimica. $KHSO_5$ is the triple salt, $2KHSO_5$, $KHSO_4$ and K_2SO_4, and is available from Alfa – Ventron (Curox). Mn-TMPyP was prepared according to ref. [25]. Labelled water, $H_2{}^{18}O$ (98%), was purchased from Euriso-top.

HPLC analyses: Analyses were performed on a reverse-phase C18 column (nucleosil, 10 μM, 250 × 4.6 mm) with a mixture of methanol and a 5 mM aqueous solution of NH_4OAc (5:95) as the eluent for oxidation experiments and a linear gradient from a 70 mM aqueous solution of NH_4OAc to acetonitrile for INH – NAD$^+$ and INH – DNAD$^+$ adduct analyses (flow rate: 1 mL min^{-1}). The column was coupled to a diode array detector (Kontron) for the detection of products at 260 nm and the monitoring of UV/Vis spectra. Yields were calculated for INH and **1** – **3** by comparaison with authentic sample calibration curves. For INH adducts, the same ε_{260} value as for NAD$^+$ (ε_{260} = 18100) was used. In order to detect **3** as the *O*-carboxymethyloxime derivative, reaction samples were analyzed 5 min after addition of an aqueous solution of carboxymethoxylamine (20 mM final concentration).

LC/ESI-MS analyses: The analyses were performed under the conditions indicated above but with a quaternary pump. Only 4% of the flow eluted from the column was introduced into the electrospray source after automated mixing with a 1% solution of HCOOH with a Harvard Apparatus syringe pump. The ESI-MS spectrometer was a Perkin-Elmer SCIEX API 365 and the analyses were performed in the positive mode.

Preparation of $[Mn^{III}(H_2P_2O_7)_3]^{3-}$: An aqueous solution of sodium pyrophosphate (200 mM), acidified to pH 4.5 (or pH 6.5) with pyrophosphoric acid and containing $Mn^{III}(OAc)_3$ (5.5 mM), produced after stirring for 24 h at RT the red complex $[Mn^{III}(H_2P_2O_7)_3]Na_3$ in quantitative yield (> 90%, yield based on literature data:[21] ε_M = 6200 M^{-1} cm^{-1} at 260 nm and 104 M^{-1} cm^{-1} at 480 nm). This product is stable for several months at 4 °C. Use of sodium pyrophosphate, $MnSO_4$ and MnO_2 (according to ref. [21]) yielded only 15% of $[Mn^{III}(H_2P_2O_7)_3]Na_3$.

Preparation of labelled compounds [^{2}H]NAD$^+$ and [^{18}O]INH: The compound [^{2}H]NAD$^+$ (^{2}H in position 4 of the nicotinamide ring) was prepared according to the method of Charlton et al.[26] In brief, NAD$^+$ and potassium cyanide were dissolved in an ^2H_2O solution of KO^2H adjusted to pH 12 with HCOO^2H and the reaction was left at room temperature for 2 h. The solution was then acidified and the HCN was removed. Cold acetone was added, the solution was left at 4 °C overnight and the white precipitate was collected, dissolved in water and lyophilised to dryness. The ^{1}H NMR spectrum (250 MHz, ^2H_2O) was identical with a spectrum of undeuterated NAD$^+$[27] except for the loss of the resonance signal assigned to the proton at position 4 of the pyridine ring (δ = 8.72) and the simplification of the 5-H signal of the pyridine ring (doublet at δ = 8.19 ppm instead of a double doublet at δ = 8.08 ppm). MS data: m/z = 665.1 $[M+H]^+$. The isotopic purity of [^{2}H]NAD$^+$ was > 98%.

Preparation of [^{18}O]INH was adapted from the method used by Gasson for the synthesis of unlabelled INH.[28] Hydrazine monohydrate (64%; 85.5 μL, 1.125 mmol) and 4-cyanopyridine (104 mg, 1.0 mmol) in $H_2{}^{18}O$ (421 μL, 20 mmol) were heated for 5 h at 100 °C in the presence of alkali (10 M aqueous solution of NaOH 2 μL). The precipitate, after filtration, washing with Et_2O and drying, was crystallised from ethanol to give a white solid (60 mg; 58%). The ^{1}H NMR spectrum (250 MHz, deuterated *N*,*N*-dimethylformamide (DMF)) was identical with a spectrum of authentic isoniazid δ = 4.75 (br s, 2H, NH_2), 7.66 (d, 2H; 3-H, 5-H), 8.59 (d, 2H; 2-H, 6-H), 9.98 (br s, 1H; NH). In the IR spectrum, ^{18}O substitution results in a shift (24 cm^{-1}) of the C=O frequency, consistent with the usual mass effect of ^{18}O atom on $\nu_{C=O}$ ($\nu_{C=O}$ = 1644 cm^{-1} for [^{18}O]INH and 1668 cm^{-1} for [^{16}O]INH). MS data: m/z = 140.2 $[M+H]^+$. The isotopic purity of [^{18}O]INH was 90 – 92%.

Conditions for INH oxidation: (See Table 1.) Runs 1 and 2: Control and oxidation with $MnCl_2$; the reaction medium (1 mL of water) containing phosphate buffer (50 mM; pH 7.5), INH (500 μM) and $MnCl_2$ (500 μM; only present for run 2) was stirred at RT for 50 h. Run 3: Oxidation with Mn-TMPyP/$KHSO_5$; the reaction medium (1 mL of water) containing phosphate buffer (50 mM; pH 7.5), INH (500 μM), $KHSO_5$ (1 mM), and Mn-TMPyP (5 μM; Mn-TMPyP was introduced in five consecutive additions, every 2 min) was stirred at RT for 15 min after the last ingredient addition and HPLC analysis was performed immediately. Run 4: Oxidation with Mn-TMPyP/Na_2SO_3/O_2; the conditions were the same as described for run 3, except that Na_2SO_3 was the last ingredient added; Na_2SO_3 (5 mM final concentration) was introduced in five consecutive additions, every 2 min; control experiments with Mn-TMPyP alone or Na_2SO_3 alone gave no conversion. Runs 5 and 6: Oxidation with manganese(III) pyrophosphate; the reaction medium (1 mL of water) containing phosphate buffer (50 mM; pH 7.5), INH (500 μM) and manganese(III) pyrophosphate (1 mM; prepared at pH 4.5 (run 5) or 6.5 (run 6) and introduced in five consecutive additions, every 2 min) was stirred at RT for 15 min after the last addition of oxidant and then directly analysed by HPLC. In all the above experiments, 2-nitrobenzoic acid (200 μM) was used as an internal standard. Run 7:[4] Oxidation with the KatG protein; the reaction medium (1 mL of water) contained phosphate buffer (15 mM; pH 7.5), INH (200 μM) and 0.3 μM enzyme; incubation was for almost 2.5 h. Run 8:[15] Oxidation with KatG protein/Mn^{II}; the reaction medium (1 mL of water) contained phosphate buffer (50 mM; pH 7.5); after 4 h preincubation of INH (150 μM) with 2 μM Mn^{II} in the reaction medium, the enzyme (3 μM) was added and incubation continued for 2.5 h.

Preparation of the INH-NAD and INH-DNAD adducts: (See Table 2 and the legend for Figure 1.) The reaction mixture was stirred at RT for 20 min after the addition of the last ingredient. HPLC and LC/ESI-MS analyses were directly performed at the end of the reaction.

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