Spontaneous Formation of the Bioactive Form of the Tuberculosis Drug Isoniazid**

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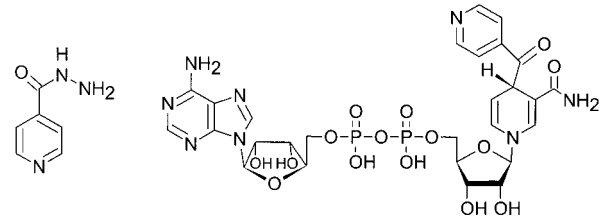
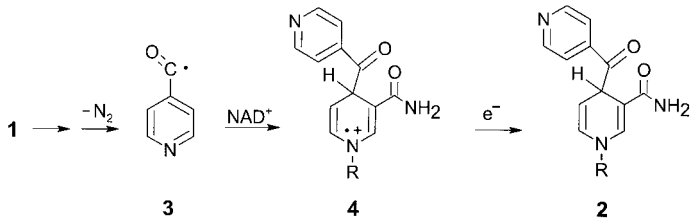
Tuberculosis is one of the leading causes of death worldwide and for 45 years isoniazid (**1**) has been the most important drug for treatment of the disease.[1] Despite the importance of isoniazid, its mechanism of action is still not sufficiently



Isoniazid **1** **2**

understood. It is currently believed that isoniazid is oxidized inside the *Mycobacterium tuberculosis* by the catalase-peroxidase KatG via the isonicotinoyl radical **3** to isonicotinic acid (see Scheme 1).[2] This conversion results in the inhibition of the synthesis of mycolic acids, long-chain fatty acids that are an integral part of the mycobacterial cell wall.[3] To date, two enzymes involved in fatty acid biosynthesis have been identified that can be inhibited by isoniazid: the enoyl–acyl carrier protein (ACP) reductase InhA and the β-ketoacyl-ACP synthase KasA.[4] InhA catalyzes the reduction of ACP-bound α,β-unsaturated fatty acids by NADH.[5] Oxidation of isoniazid in the presence of InhA and its cofactor NADH or NAD^+ leads to inhibition of InhA.[6] The solution of the crystal structure of the resulting InhA-inhibitor complex identified **2** as an inhibitor of InhA.[7] It has been proposed that **2** is formed exclusively within the active site of InhA, most likely by recombination of **3** with a NAD radical generated from NADH.[7] However, in our opinion the direct addition of **3** to NAD^+ and subsequent reduction of the resulting radical cation **4** appears more plausible (Scheme 1).



3 **4** **2**

Scheme 1. R = ADP ribose.

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The rate constants for the addition of acyl radicals to electron-deficient heterocycles such as protonated pyridines (known as the Minisci reaction) is in the order of $10^6\,M^{-1}s^{-1}$.[8] Accordingly, the selective formation of **2** might be explained by the intrinsic affinity of acyl radicals for electron-deficient heterocycles such as NAD^+ and would not depend on specific interactions within the active site of InhA. To test this hypothesis, we first examined if **2** is formed via NADH or NAD^+ and if in both cases actually the same inhibitor is formed. Towards this end, we incubated InhA with isoniazid, peroxidase, Mn^{2+}, and either NADH or NAD^+.[9] After inhibition of InhA, the reaction mixture was dialyzed and analyzed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), which allowed the detection of molecules tightly bound by InhA. In both reaction mixtures a molecule with a molecular mass of 770.1 Da, corresponding to the molecular mass of **2**, was detected. Subsequently, HPLC analysis of the product composition of reaction mixtures typically used in in vitro inactivation experiments revealed that under those conditions, in addition to the oxidation of isoniazid to isonicotinic acid, NADH is rapidly oxidized to NAD^+.[11] For example, a reaction mixture of 100 μM isoniazid, 70 μM NADH, 2 μM KatG, and 0.2 μM $MnCl_2$ at pH 7.5 and 25 °C was shown to contain 18 μM isonicotinic acid and 5.6 μM NAD^+ after incubation for 10 min. Consequently, in the experiments with either NAD^+ or NADH, **2** might be generated in both cases via NAD^+. To investigate whether **2** might also be formed in the absence of InhA, we oxidized isoniazid in the presence of NAD^+ and monitored the reaction by HPLC (Figure 1a). In addition to peaks corresponding to isonicotinic acid and ADP ribose (a NAD^+ degradation product), a number of peaks (A–E) with retention times higher than NAD^+ were detected. Analysis by MALDI mass

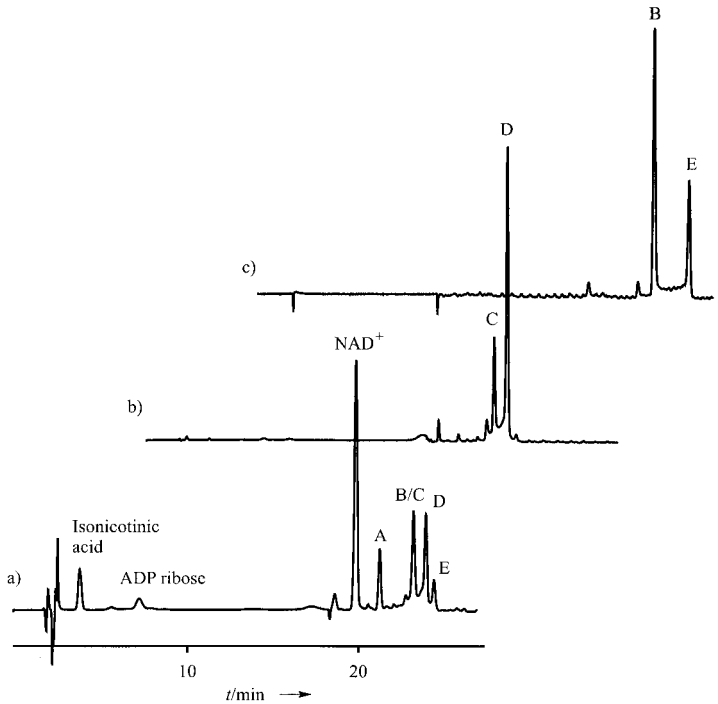

Figure 1. a) HPLC analysis of a reaction mixture containing 100 μM isoniazid, 70 μM NAD^+, 2 μM KatG, and 0.2 μM $MnCl_2$ at pH 7.5 (100 mM Na_2HPO_4) and 25 °C.[11] b) Reinjection of D. c) Reinjection of E.

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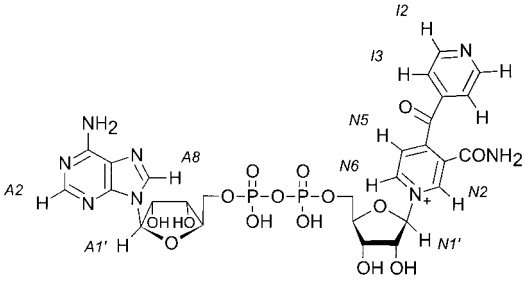
spectrometry and LC/ESI/MS/MS showed that products B – E possess a molecular mass of 770.1 Da and, according to the MS/MS-fragmentation data, are formed by modification of the nicotinamide ring of NAD^+. Furthermore, products B – E display UV spectra characteristic for dihydropyridine derivatives ($\lambda_{max} = 330$ (B), 319 (C), 333 (D), 320 nm (E)).[11] Accordingly, B – E correspond to **2** and, or related isomers. Isolation of B – E revealed that A ($M = 768.1$ DA) is formed in a spontaneous, secondary reaction from B – E. This conversion can be catalyzed by peroxidase. Products B and E as well as C and D are in a dynamic equilibrium with each other. For example, isolation of E by HPLC and subsequent reinjection yielded a mixture of B and E (Figure 1b and c). Isolation of E by HPLC and immediate recording of the absorbance at 330 nm as a function of time revealed that the attainment of the equilibrium between B and E is a first-order process with a rate constant of 0.3 min^{-1}. Using in vitro assays with 2-*trans*-octenoyl-CoA and NADH as substrates, it could be shown that product B/E is a competitive inhibitor of InhA with a K_I of 100 nM ± 50 nM.[12] In contrast, products A and C/D displayed no inhibitory activity. The higher stability of A compared to B/E and C/D allowed the characterization of this compound by NMR spectroscopy. Data obtained from ^1H TOCSY and ROESY spectra are in agreement with A possessing structure **5** (Figure 2). Under these conditions, **5**



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is a mixture of two compounds which are in a dynamic equilibrium with each other. Taking into account the data obtained from mass spectrometry, UV and NMR spectroscopy, the results of the enzymatic assays as well as the

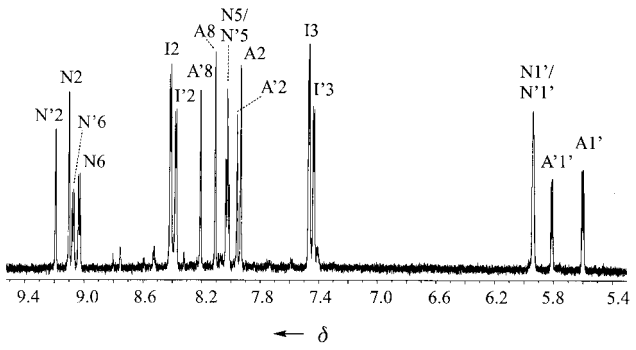
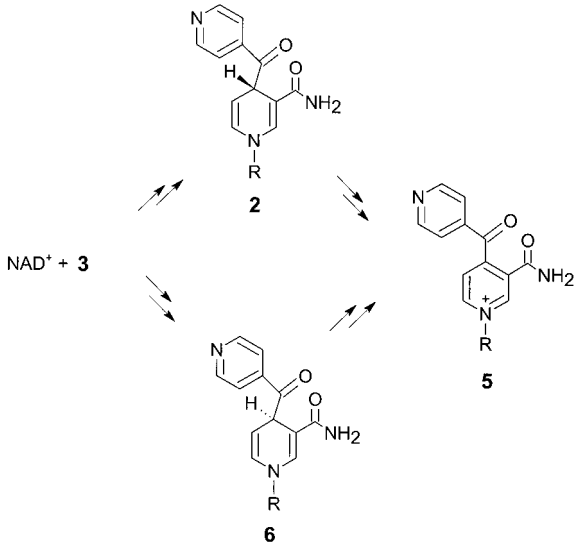


Figure 2. Section of the ^1H NMR spectrum of A (= **5**, ca. 1.5 mM; H_2O/D_2O (9/1), pH 5.5, 25 °C, 600 MHz). The labeling of the peaks is as shown in the structure formula of **5**. The slow chemical exchange between the two equilibrating compounds was demonstrated by observing cross peaks in the corresponding ROESY spectrum.

published crystal structure of the InhA-inhibitor complex, we assign product B/E to structure **2**, C/D to structure **6**, and A to structure **5** (Scheme 2). Consequently, attack of **3** at the C-4 atom of NAD^+ in solution occurs either from the *re* or the *si* face of the nicotinamide ring. Oxidation of **2** and **6** both yield **5**. In our opinion, there are two possible explanations for the



Scheme 2. R = ADP ribose.

observed dynamic equilibrium: One possibility is a hindered rotation around the bond between the isonicotinoyl group and the C-4 atom of the nicotinamide ring. The observed differences in the ^1H NMR chemical shifts of the adenine protons indicate a stacking interaction between the purine residue and the isonicotinoyl – nicotinamide adduct (Figure 2), which might further influence the rotation of the isonicotinoyl group. Another possibility would be that **2**, **5**, and **6** are in equilibrium with the corresponding hydrates. The equilibrium constant for the hydrate formation of pyridine-4-aldehyde is $K_{hydrate} = 2$ and the rate constant for attaining equilibrium at pH 7.2 and 0 °C is 2.5 min^{-1}.[13] Incubation of B/E in $H_2{}^{18}O$ and subsequent analysis by MALDI mass spectrometry showed the incorporation of a single ^{18}O atom in B/E.

To verify the postulated reaction scheme, we analyzed the reaction products of the incubation of isoniazid with deuterated (4-^2H)-NAD^+.[14] After incubation of the products B – E for 24 h at pH 7 and room temperature, no exchange of the deuterium was detected by MALDI mass spectrometry.[14] Accordingly, epimerization at C-4 can be ruled out as an explanation for the observed dynamic equilibrium. However, as predicted by Scheme 2, the deuterium label at C-4 is lost during the oxidation of B/E and C/D to A. Furthermore, epimerization between α- and β-NAD adducts as a possible explanation for the observed equilibrium can be ruled out as well, since starting from either α- or β-NAD^+ led to products with identical molecular mass but different HPLC retention times.

In summary, the obtained data clearly support the proposed reaction mechanism, although further experiments are needed to elucidate the exact nature of the observed dynamic equilibrium.

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The total yield of the NAD adducts A–E based on consumed isoniazid is roughly 50% under the conditions shown in Figure 1 (micromolar concentrations of both isoniazid and NAD^+). The efficient formation of the inhibitor **2** in the absence of InhA is, in our opinion, of great importance for an understanding of the mechanism of action of isoniazid. As the concentration of NAD^+ inside *M. tuberculosis* is also in the micromolar range,[15] we propose that inside the bacterium **2** is formed by the fast addition of acyl radical **3** to electron-deficient heterocycles such as NAD^+ and outside the active site of InhA. Considering the low binding affinity of InhA for NAD^+ ($K_I = 4$ mM) and the resulting low concentration of InhA-bound NAD^+,[5] the also conceivable addition of **3** to NAD^+ within the active site of InhA appears rather unlikely. Furthermore, the catalase-peroxidase KatG does not play an active role in the addition of **3** to NAD^+ (although it is required for oxidation of isoniazid), as the yield of isonicotinoyl-NAD adducts as well as the product composition is about the same after oxidation of isoniazid by KatG or Mn^{3+}. The mechanism of action of isoniazid therefore relies on the efficient formation of the isonicotinoyl–NAD adducts by a Minisci reaction as well as the inhibitory potential of **2** (=B/E), whose K_I value is about 100 nM (see above) and therefore about a factor of 100 below the K_M value of InhA for NADH.[5, 12]

The proposed reaction mechanism also allows one to reinterpret the observations that a number of isoniazid-resistant mycobacteria appear to possess a higher ratio of NADH/NAD^+ as the result of defects in NADH-dehydrogenases,[16a] and that overexpression of NAD^+-binding proteins might contribute to isoniazid-resistance.[16b] A lower intracellular concentration of NAD^+ should, according to our mechanism, directly lead to a diminished rate of formation of **2** and therefore to an increased resistance towards isoniazid.

In summary, the demonstrated spontaneous formation of the bioactive form of isoniazid significantly simplifies the proposed mechanism of action of the drug and should be helpful in obtaining a better understanding of the molecular events leading to isoniazid-resistance.

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A Microporous Lanthanide–Organic Framework**

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The recent upsurge of reports on open metal–organic frameworks has provided compelling evidence for the ability to design and produce structures with unusual pore shape, size, composition, and function.[1] To realize the potential of these materials in host–guest recognition, separation, and catalysis, it is essential that their frameworks exhibit perma-

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