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The catalytic mechanism of indole-3-glycerol phosphate synthase (IGPS) investigated by electrospray ionization (tandem) mass spectrometry

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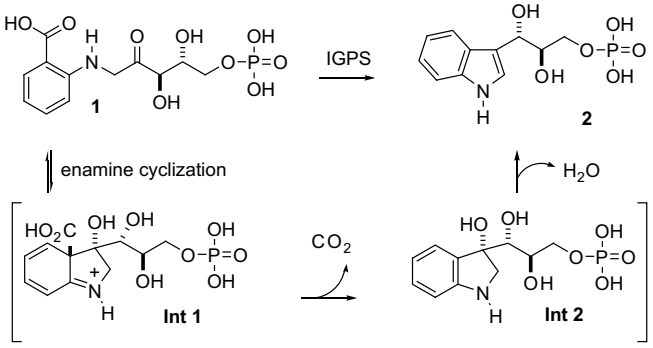
ABSTRACT

An enzymatic reaction has been monitored by on-line direct infusion electrospray ionization (tandem) mass spectrometry. Using this fast and sensitive technique, a key and transient intermediate of *Mycobacterium tuberculosis* indole-3-glycerol phosphate synthase (IGPS)-catalyzed reaction has been trapped. The reaction catalyzed by indole-3-glycerol phosphate *synthase* is part of the tryptophan biosynthetic pathway, and is not present in mammals, including humans. This peculiarity renders this enzyme a potential target for the development of biospecific agents with potential anti-TB activity. The present results indicate the presence of two intermediates in the mechanism of this enzymatic reaction.

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is the most lethal disease caused by a bacterium, being a major threat to human health worldwide.[1] Actually, this bacillus is the most lethal pathogen among all bacteria,[2] and leads to near two million deaths and over eight million cases of *tuberculosis* per year.[3,4] The only current administered vaccine, BCG, is unable to reduce the transmission of TB, and the protection conferred is in many cases ineffective.[5] TB chemotherapy is also long-term and has several side-effects.[6] The understanding of *M. tuberculosis* key metabolism by assessing the kinetic and chemical mechanism of specific enzymatic reactions is therefore important to decipher the mechanisms of virulence and resistance of TB, and to develop new rational strategies to kill or halt the bacillus.[7] Enzymes involved in the biosynthetic pathways of the bacillus, those not used by mammals and whose function is relevant for the pathogen's life or for their ability to cause disease, may be excellent targets to new anti-TB agents.[8] The tryptophan biosynthetic pathway was shown to be essential to the virulence of the pathogen.[3] The current work investigates the mechanism of the reaction catalyzed by indole-3-glycerol phosphate *synthase* (IGPS), the fifth committed step in the biosynthesis of tryptophan in *M. tuberculosis*. IGPS is absent in mammals, including humans; hence, this enzyme constitutes a potential target to new agents against *M. tuberculosis*.

In the biotransformation, it is rationalized that the ring closure promoted by IGPS involves an intramolecular enamine-like nucleophilic addition to the keto form of 1 (Scheme 1) leading to the IGP product 2.

Parry[9] proposed that the reaction proceeds via two intermediates (**Int1** and **Int2**). Bruice et al. studied this biotransformation using molecular dynamics calculations.[10] Fersht and co-workers used the knowledge available for the biotransformation to mimic evolution strategy using the most common fold in enzymes to evolve phosphoribosylanthranilate isomerase activity from the scaffold of IGPS.[11] The cost to form **Int1** is loss of aromaticity, which is further restored when **Int2** is formed by CO_2 loss. All reactants, products, and proposed intermediates are considerably polar, which renders this reaction suitable for electrospray



Scheme 1. Proposed biotransformation catalyzed by IGPS enzyme during indole ring formation.

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ionization (tandem) mass spectrometry investigation. Although ESI-MS(/MS) has been successfully used to intercept and characterize key intermediates of many major chemical reactions,[12] its use to study enzymatic reaction mechanism and their transient intermediates has not yet been demonstrated.[13,14] Therefore, the current manuscript describes the first use of ESI-MS(/MS) to monitor a major enzymatic biotransformation.

First, the biotransformation of reactant **1** to the IGP product **2** (indole-3-glycerol-phosphate) was followed by UV–vis (Fig. 1).

Figure 1A shows that the absorption band at 335 nm decreases during the enzymatic experiment. This band corresponds to the lowest energy absorption of **1** and was assigned to n-π^* (and/or π–π^*) transition from their molar extinction coefficient ($\varepsilon = 3.28$). Concomitant with the reduction of the 335 nm band, a new band at 280 nm is detected at increasing intensity. This 280 nm band has been assigned to **2**.[15] After the appropriated deconvolution of both Gaussians bands at 335 nm and 280 nm, the IGPS-catalyzed reaction evolution profile was obtained (Fig. 1B). In the first minute (Fig. 1B, top), a single exponential decay of the enzyme substrate **1** is observed (rate: $A = A0 + e^{(-x/t)}$), but no absorbance owing to **2** is noted (Fig. 1B, bottom). After the first minute, however, a single exponential growth is noted for the absorbance of **2**. The lack of absorbance owing to **2** and the decrease of absorbance owing to **1** points to the participation of a transient reaction intermediate (or intermediates) connecting **1** to **2**. Since **1** losses its aromaticity to form **Int1**, its formation could hardly be studied by UV–vis, especially because the absorption bands would suffer blue shift as a consequence of lack of conjugation in the aromatic ring. Diode array analysis would be the natural course, but compound **1** is not light sensitive. In fact, **1** is a very unstable substrate[15] and must be used freshly synthesized to allow trustable analysis.

To overcome all drawbacks associated with both substrate and enzyme, ESI-MS was our hope, especially because UV analysis could just provide us with the idea of intermediate(s) participation(s). Using ESI(−)/MS, no ionic species directly related to the proposed reaction path could be detected. To guide ESI-MS(+) monitoring, we first characterized the protonated enzyme substrate **1** (pure and freshly synthesized compound)[15] via ESI-MS/MS from an aqueous HCl solution at pH 5 (Fig. 2).

The tandem mass spectrum of $[\mathbf{1} + H]^+$ shows that the gaseous protonated molecule dissociates by sequential losses of H_2O (*m/z* 332), H_3PO_4 (*m/z* 234), H_2O (*m/z* 216), and a second H_2O (*m/z* 198) whereas the fragment of *m/z* 216 loses in turn CO_2 to form the ion of *m/z* 172. A fragment of *m/z* 150 attributed to $[HO_2C\text{-}Ph\text{-}NHCH_2]^+$ is also formed, which loses H_2O to form the fragment of *m/z* 132.

ESI(+)-MS monitoring of the chemically mimicked intramolecular cyclization process of **1**–**2** was then performed (Fig. 3).

The online monitoring shows that the reaction occurs rapidly at pH 1 (HCl addition), allowing us to try to intercept and characterize by ESI(+)-MS the chemically promoted **Int1** (Fig. 3B), despite severe degradation that was observed at this drastic condition. Above pH 1, the acid-promoted reaction fails to occur or was too slow to be monitored on-line by ESI-MS.

As labeled, the ESI-MS of the chemically mimicked reaction displays ions attributable to all four major reaction species. Note that **Int2** (Fig. 3C) was detected as a very minor ion of *m/z* 306, and its ESI-MS/MS could therefore not be acquired, but such intermediate has been studied and characterized using other techniques.[16] Note that the ESI-MS/MS of **1** (Fig. 3A) display the same fragmentation pattern as previously obtained at pH 5. Plus, additional ions are detected and attributed to Int1, which is an isomer of the same *m/z* 350.

In the presence of IGPS catalyst, nevertheless, the biotransformation proceeds fairly rapid at pH 6 and, during the first minute of reaction, the ESI-MS/MS of the ion of *m/z* 350 was collected (Fig. 4).

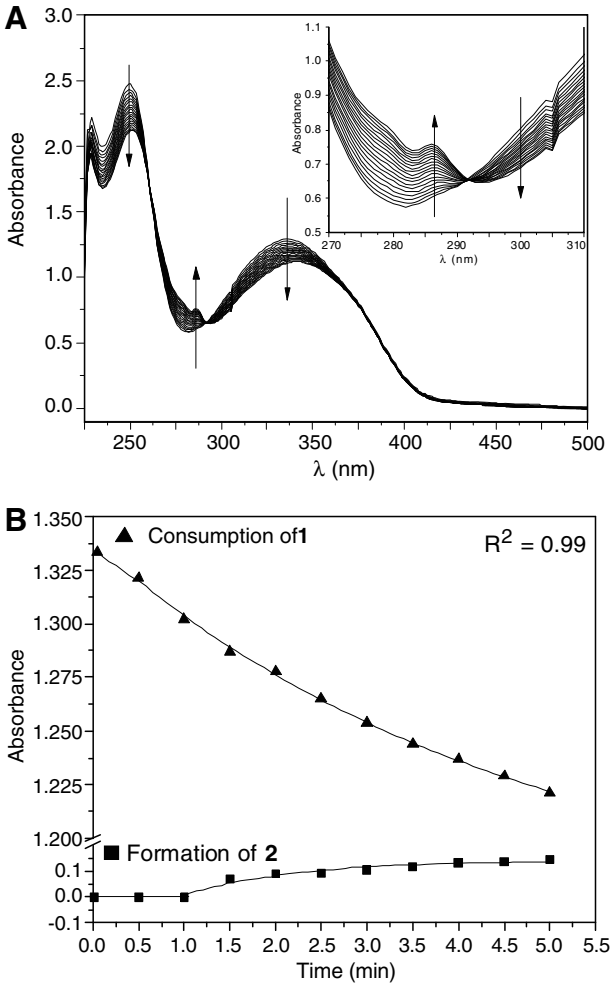


Figure 1. (A) UV–vis of the reaction of **1** leading to **2** catalyzed by IGPS enzyme over a period of 20 min. (B) Reaction rate for the formation of **2** (bottom) and consumption of **1** (top).

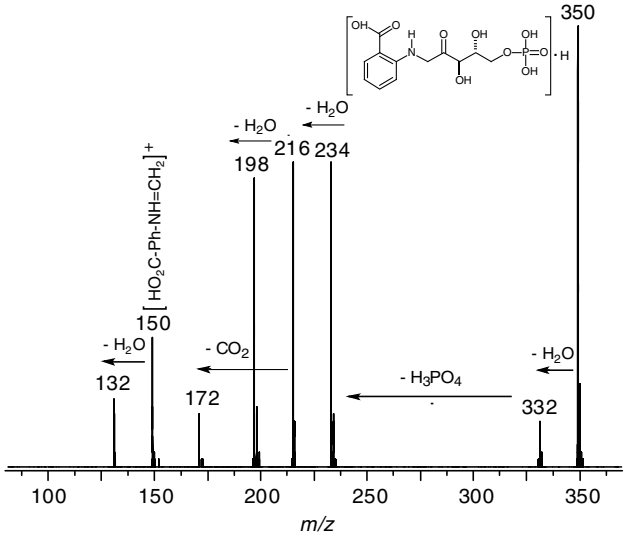


Figure 2. ESI(+)-MS/MS of the protonated and pure freshly synthesized IGPS substrate ion $[\mathbf{1}+H]^+$ f *m/z* 350.

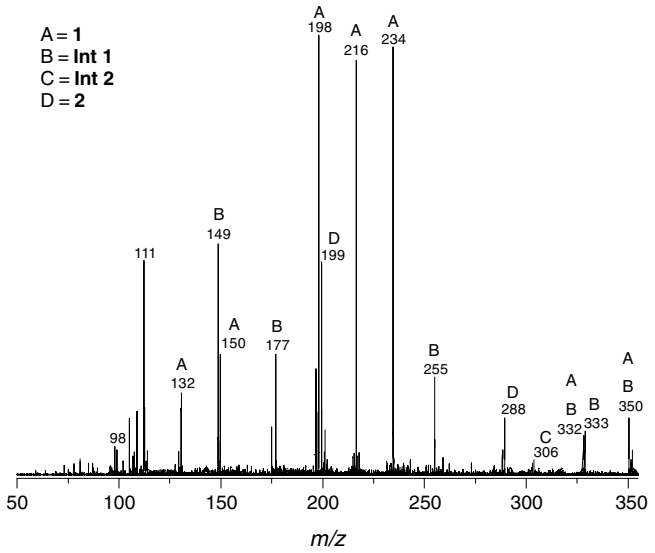


Figure 3. ESI(+)-MS of the chemically mimicked intramolecular cyclization process.

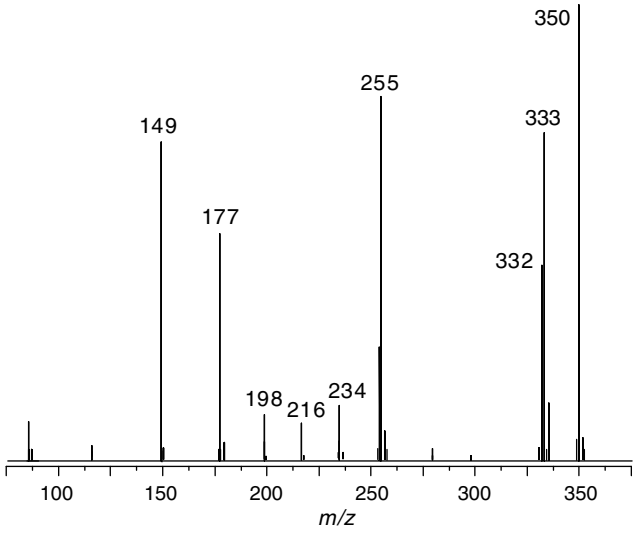
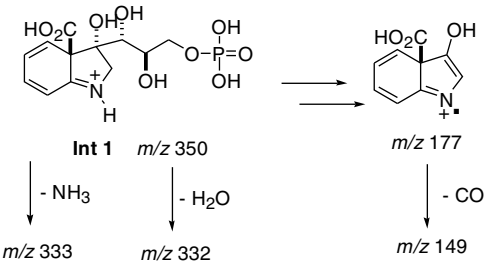


Figure 4. ESI(+)-MS/MS of **Int1** during the enzymatic biotransformation promoted by IGPS enzyme at pH 6. Note that at this pH value no chemically mimicked intramolecular cyclization process take place.

This ion could be either [**1**+H]$^+$ or the protonated **Int1**, or a mixture of both, since they are isomers of the same *m/z* 350. We knew from the chemically promoted reaction, however, that their different structures would lead to distinct dissociation behaviors. The difference observed in the chemical simulation and the biotransformation was the relative abundance of the ions, but the observed fragmentation pattern is quite similar. As pointed out before, **Int1** formation could hardly be studied by UV–vis. Nevertheless, its cationic nature would allow for an on-line characterization by ESI-MS/MS to probe that in fact **Int1** is formed during the enzymatic biotransformation.

The ESI-MS/MS of Figure 4 shows the same set of fragments detected for [**1**+H]$^+$, that is, those of *m/z* 332, 234, 216, and 198 (as seen in Fig. 2), but new fragment ions now predominate, mainly those of *m/z* 333, 332, 255, 177, and 149. The chemically mimicked reaction showed a ESI-MS/MS with the same predominant ions. This drastic change shows that a new isomeric species is now present in the reaction solution; that is, that **1** has been biotransformed, in the presence of IGPS enzyme (at pH 6), to an isomeric



Scheme 2. Major fragments of the trapped isomeric specie of *m/z* 350 during the biotransformation promoted by IGPS. Note the presence of the indole ring fragment ion of *m/z* 177 and 149 in Figure 4.

intermediate, most likely **Int1**. Other important aspect is the detection of substrate **1** now at pH 6, which indicates that the enzyme environment allows easy protonation and further detection.

The very contrasting set of fragment ions shows, therefore, that a new isomeric species has been trapped. A lot of effort trying to rationalize main fragments, particularly that of *m/z* 255 was done. Still, we could not find a reasonable explanation for this intractable ion. It might be a component of the enzyme preparation or reaction mixture component; however, it remains elusive. A complete rationalization of the fragmentation chemistry of these species was not straightforward, but some key and very important fragment ions such as those of *m/z* 177 and *m/z* 149 show the presence of the new formed indole ring (Scheme 2).

The presence of the indole ring is a consistent evidence of **Int1** participation during the biotransformation. The ion of *m/z* 333 is not detected in the ESI-MS/MS of compound **1** (Fig. 2), likewise NH_3 loss occurs likely from **Int1**.

In summary, the drastic change in fragmentation pattern observed for the protonated molecules of *m/z* 350 in the enzymatic experiment[17] points to the on-line interception of the putative enzymatic intermediate **Int1** or an unknown isomeric species that contains an indole ring. This evidence indicates that the enzymatic biotransformation occurs indeed via a two-step mechanism as delineated in Scheme 1. For the first time, therefore, a key information for the mechanism of a major enzymatic bioreaction has been provided by on-line ESI-MS(/MS) monitoring. As exemplified herein, for anti-TB agents, the use of this fast and sensitive technique opens up a new avenue for investigating the (biochemical) mechanism of enzymes biotransformations and hence for the more rational design of new agents with higher biospecificity. Actually, the current manuscript provides a baseline methodology for determining and characterizing enzymatic intermediates while pushing the limits of state-of-the-art instrumental sensitivity. Further work, based on our experience in mass spectrometry[18] and biological systems,[19] will contribute to the elucidation of the kinetic and mechanism of the IGPS biocatalysis.

Acknowledgements

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