Catabolism of isonicotinate by *Mycobacterium* sp. INA1: extended description of the pathway and purification of the molybdoenzyme isonicotinate dehydrogenase

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Catabolism of isonicotinate by *Mycobacterium* sp. INA1 has been shown to proceed via 2-hydroxyisonicotinate, 2,6-dihydroxyisonicotinate (citrazinate), citrazyl-CoA and 2,6-dioxopiperidine-4-carboxyl-CoA. An extended pathway involving propane-1,2,3-tricarboxylate as a further intermediate is presented in this paper. Propane-1,2,3-tricarboxylate was oxidized stepwise to 2-oxoglutarate involving an oxidase, aconitase and isocitrate dehydrogenase. Isonicotinate dehydrogenase catalyses the first step of isonicotinate metabolism in *Mycobacterium* sp. INA1. The enzyme was purified to apparent homogeneity by a three-step procedure. Enrichment was accompanied by partial loss in specific activity. The native enzyme had a molecular mass of either 125 kDa or 250 kDa, when estimated by native gradient PAGE or gel filtration, respectively. SDS-gel electrophoresis revealed three types of subunits with molecular masses of approximately 83, 31 and 19 kDa. N-Terminal amino acid sequences of all three subunits have been determined. Molybdenum, iron, acid-labile sulphur and FAD were present at molar ratios of 1, 4, 4, 1 per protomer (125 kDa). The molybdenum-complexing cofactor was shown to be molybdopterin cytosine dinucleotide. Besides isonicotinate, only quinoline-4-carboxylate was found to be oxidized at appreciable rates.

Introduction

Bacterial metabolism of pyridine was known as early as 1914, when it was tested for its sterilizing activity on soil and found to induce an unexpectedly high increase in bacterial cell number (Buddin, 1914). Whereas pyridine itself is attacked via a reductive step (Watson & Cain, 1975), the bacterial catabolism of many substituted pyridine derivatives was found to involve hydroxylations of the heteroaromatic ring (for review, see Shukla, 1984). This is also true for quinoline (benzopyridine) (Grant & Al-Najjar, 1976), isoquinoline (Aislabie *et al.*, 1989) and some quinoline derivatives (Bauer & Lingens, 1992; Schach *et al.*, 1993). Due to the electron distribution, the pyridine ring permits nucleophilic attack in the *ortho*- and *para*-positions (Sims & O'Loughlin, 1989). There-

fore, in the course of bacterial catabolism of pyridine derivatives, initial hydroxylations usually occur at these positions – with the *ortho*-position being favoured – and are generally catalysed by dehydrogenases. The incorporated hydroxyl group is derived from water (Hunt *et al.*, 1958; Hirschberg & Ensign, 1971; Pereira *et al.*, 1988; Bray, 1988). These dehydrogenases which hydroxylate pyridine derivatives at the *ortho*-position form a structurally very similar group of molybdoenzymes. In contrast, *meta*-hydroxylations (which are not treated in this paper) are catalysed by oxygenases (Block & Lingens, 1992). *Meta*-hydroxylations mostly represent the second (or third) hydroxylation so that the position is especially activated for an electrophilic attack by the electron supplying character of the existing hydroxyl substituent(s).

In the course of our research on molybdenum-dependent bacterial transformations of heterocyclic substrates, we have looked at the degradation of isonicotinate (pyridine-4-carboxylate). The isolation of a mycobacterial species that metabolizes isonicotinate as the sole source of carbon, nitrogen, and energy, and some features of the catabolic pathway have already

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Abbreviations: camMCD, carboxamidomethylmolybdopterin cytosine dinucleotide; DH, dehydrogenase; MCD, molybdopterin cytosine dinucleotide; MGD, molybdopterin guanine dinucleotide.

been described (Kretzer & Andreessen, 1991). We now report on the detection of a propane-1,2,3-tricarboxylate oxidizing enzyme system and the purification and partial characterization of the first catabolic enzyme in the pathway, isonicotinate dehydrogenase. Only preliminary results have been available so far for an 'isonicotinate hydroxylase' that was partly purified from a *Micrococcus* sp. but not structurally characterized and that copurified with 2-hydroxyisonicotinate 'hydroxylase' activity (Gupta & Shukla, 1979b). In *Mycobacterium* sp. INA1, the catabolic enzymes isonicotinate dehydrogenase and 2-hydroxyisonicotinate dehydrogenase are clearly distinct enzymes.

Methods

Organism, growth conditions, preparation of cell extracts, and solubilization of membrane-associated proteins. *Mycobacterium* sp. strain INA1 has been deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, FRG) under the accession number DSM 6387. Conditions by its growth and the preparation of cell extracts have been described previously (Kretzer & Andreessen, 1991). For large-scale cell growth fermenters with different volumes up to 300 l were used with stirring rates of 100 r.p.m. Speeds above 150 r.p.m. inhibited growth. Purification of isonicotinate dehydrogenase was started from extracts which were prepared in a buffer containing 50 mM-sodium citrate and 50 mM-KCl at pH 5.5 (buffer A). Extracts were separated into soluble and membranous fractions by centrifugation at 100 000 g for 2 h. Membrane-associated isonicotinate dehydrogenase was solubilized by stirring the resuspended membranes (1 mg protein per ml buffer A) for 30 min at 0 °C in the presence of 1% CHAPS. After a second ultracentrifugation, isonicotinate dehydrogenase activity was found in the supernatant.

Enzyme assays. All enzyme assays were performed at 30 °C. One unit (U) is defined as the activity that converts 1 µmol of substrate min⁻¹. Isonicotinate dehydrogenase (Kretzer & Andreessen, 1991), aconitase (Fansler & Lowenstein, 1969) and isocitrate dehydrogenase (Cleland *et al.*, 1969) were measured as described. Oxygen consumption in the presence of propane-1,2,3-tricarboxylate was measured using a Clark oxygen electrode (Rank). The reaction mixture contained 50 mM-Tris/HCl (pH 8.0), 1 mM-propane-1,2,3-tricarboxylate, and various amounts of enzyme solution. Reduction of NADP⁺ secondarily coupled to the oxidation of propane-1,2,3-tricarboxylate was measured by the increase of A_{365} in 50 mM-Tris/HCl (pH 7.0), 0.5 mM-MnCl₂, 5 mM-propane-1,2,3-tricarboxylate and 0.5 mM-NADP⁺. Aconitase and NADP⁺-dependent isocitrate dehydrogenase activities were responsible for the coupling between oxidation of propane-1,2,3-tricarboxylate and reduction of NADP⁺. These activities were either present in extracts of *Mycobacterium* sp. INA1 or were added to the reaction mixture [pig heart aconitase (about 20 U g⁻¹) and isocitrate dehydrogenase (about 1 U mg⁻¹), both from Sigma; added at 1.5 mg ml⁻¹].

Separation and purification of proteins

All the following steps were performed at 8 °C; chromatography media were obtained from Pharmacia.

Separation of the propane-1,2,3-tricarboxylate oxidizing system. This was achieved by gel filtration of soluble cell proteins on Sephacryl S-300 HR (1.6 × 95 cm) as described by Kretzer & Andreessen (1991).

Purification of isonicotinate dehydrogenase. This was performed using 50 mM-sodium citrate with 50 mM-KCl, pH 5.5 (buffer A) as principal buffer. Enrichment from either soluble cell proteins or solubilized membrane proteins followed the same procedure. CHAPS present in the membrane-derived protein fraction was almost completely removed during the first chromatographic step, and there was no need to supplement the subsequent elution buffers with a detergent. The starting protein solution was applied to Q-Sepharose fast flow equilibrated with buffer A at a ratio of up to 50 mg protein per ml of gel. The column was washed with 10 bed vols of 50 mM-citrate buffer containing 150 mM-KCl at pH 5.5. Bound protein was further eluted by a 150–600 mM-KCl gradient that had about six times the volume of the anion exchange material. Isonicotinate dehydrogenase activity that eluted between 300 and 500 mM-KCl was pooled and directly applied to Phenyl-Sepharose CL-4B equilibrated with 50 mM-citrate buffer containing 300 mM-KCl at pH 5.5. Protein was kept as low as 1 mg per ml of gel material. The column was washed with 20% (v/v) ethylene glycol in buffer A. When the A_{280} of the eluate had become constant, the ethylene glycol concentration was linearly raised from 20% to 60% (v/v), where it was kept, until isonicotinate dehydrogenase activity was completely eluted. The gradient had approximately 7.5 times the volume of the hydrophobic interaction material. Isonicotinate dehydrogenase eluted late in the gradient and was pooled and concentrated before gel permeation chromatography by binding to a small volume of well packed Q-Sepharose fast flow equilibrated with 40% ethylene glycol in buffer A. After washing with buffer A, the KCl concentration was raised stepwise from 50–500 mM so that the enzyme was eluted in a concentrated manner. Immediately afterwards it was subjected to gel filtration chromatography on either Sephacryl S-200 HR (2.6 × 90 cm) or Superdex 200 prep grade (1.6 × 60 cm), both run with buffer A at 0.5 ml min⁻¹. The former material was preferred for preparative purposes and the latter for molecular mass determinations.

Protein quantification. This was done by the method of Bradford (1976). Whenever stoichiometries were to be established, the method of Scopes (1974) was used in addition. Whereas quantifications of isonicotinate dehydrogenase by these two methods were in good agreement, the method of Elliot & Brewer (1978) yielded lower values and was not used further. Standardization was done with bovine serum albumin.

Characterization of isonicotinate dehydrogenase

Inhibition by cyanide and methanol. This was tested according to Massey & Edmondson (1970) and Coughlan *et al.* (1969).

Electrophoretic methods. For native gradient PAGE, the method of Blaschke *et al.* (1991) was principally followed, but a different electrode buffer was used (12.1 g Tris l⁻¹ and 7.5 g glycine l⁻¹, no pH adjustment). In the case of isonicotinate dehydrogenase, however, it was found that either running times had to be prolonged or polyacrylamide concentrations had to be raised, or both, to bring the enzyme into electrophoretic equilibrium, a prerequisite for molecular mass determinations on the basis of the molecular sieve effect described by Margolis & Kenrick (1967). Appropriate conditions were electrophoresis across a gradient of 15–27.5% polyacrylamide for 24 h in the presence of suitable marker proteins. SDS-PAGE was performed by the system of Laemmli (1970) using 14% (w/v) polyacrylamide gels, SDS gradient PAGE (5–20% (w/v) polyacrylamide) was performed as described by Hames (1981) (8.5 × 7.0 × 0.1 cm gels, run at 25 mA). Visualization of protein bands was achieved by a silver stain (Blum *et al.*, 1987).

Absorption spectra. UV/VIS-spectra of isonicotinate dehydrogenase were recorded with an Uvikon 810 spectrophotometer (Kontron).

Metal and cofactor determinations. **Flavin.** Flavin determinations were performed in the dark as far as possible. Flavin coenzyme was

released from isonicotinate dehydrogenase by boiling for 10 min, and the denatured protein was removed by centrifugation. Differentiation between FAD and FMN was achieved by thin layer chromatography on silica gel 60 plates (Merck) with the following solvent system: 1-butanol:acetone:acetic acid:water (5:2:1:3, by vol.). Spots of flavin were visible under UV light (366 nm) down to 0.25 nmol. Salts derived from buffer A in which isonicotinate dehydrogenase was usually prepared disturbed the chromatographic system. Therefore, isonicotinate dehydrogenase had to be desalted by repeated dilution with water and reconcentration in Centricon microconcentrators (Amicon) before qualitative determination of flavin. For the quantitative determination of FAD after its release from protein, an absorption coefficient of $\epsilon_{450} = 11.3 \text{ mm}^{-1} \text{ cm}^{-1}$ was used (Kozioł, 1971).

Molybdenum, iron and acid-labile sulphur. Molybdenum was determined by induced coupled plasma-MS, iron and acid-labile sulphur by the methods of Van de Bogart & Beinert (1967) and of Beinert (1983), respectively.

Molybdenum cofactor. The presence of a molybdenum-complexing pterin was shown by derivatization to form A as described by Koenig & Andreessen (1990). Molybdopterin cytosine dinucleotide was identified by two methods: (i) detection of CMP after acid hydrolysis of whole protein: isonicotinate dehydrogenase (3 mg) was boiled for 20 min with 3% (w/v) H_2SO_4 , and the precipitated protein was removed by centrifugation. The supernatant was analysed by HPLC on an ET 250/8/4 Nucleosil 120-7 C_{18} column (Macherey & Nagel) using a Waters 991 photodiode array detector. Elution was isocratic with 20 mM-potassium phosphate, pH 3.5, at a flow rate of 0.5 ml min^{-1} . (ii) Isolation of the carboxamidomethyl derivative, performing all the following steps at room temperature: isonicotinate dehydrogenase (6 mg) was incubated overnight under anoxic conditions in the presence of a 3000-fold molar excess of iodoacetamide, 6 M-guanidine/HCl (stock solution at pH 7.2), and 5 mM-sodium dithionite. The carboxamidomethyl derivative was subsequently handled without anoxic precautions. After 5-fold dilution with water, protein was precipitated by the addition of 40% (w/v) ammonium sulphate and removed by centrifugation and filtration over glass wool. The clear solution was then applied to a reversed-phase C_{18} cartridge (0.8 ml bed volume, Waters, Millipore) and after washing with 0.7 ml water, retained substances were eluted with 5 ml methanol. The methanolic solution was concentrated nearly to dryness by rotary evaporation. The residue was bright yellow due to the flavin content that, however, did not interfere with the further chromatographic analysis as it was strongly retained on the reversed phase C_{18} material. The residue was made up to about 300 μl with water and applied in aliquots to the HPLC system described above but using 50 mM-ammonium acetate, pH 6.8, as the eluent (1 ml min^{-1}). Carboxamidomethylmolybdopterin cytosine dinucleotide from carbon monoxide dehydrogenase of *Hydrogenophaga pseudoflava* (formerly *Pseudomonas carboxydoflava*) was prepared under the same conditions.

N-Terminal amino acid sequences. N-Terminal amino acid sequences of protein subunits were determined as described by Hughes *et al.* (1992).

Results

Detection of propane-1,2,3-tricarboxylate-oxidizing activity

Extracts of *Mycobacterium* sp. INA1 catalysed a propane-1,2,3-tricarboxylate-dependent oxygen consumption with an activity of about $40 \text{ U (g protein)}^{-1}$. In addition, a reduction of NADP^+ was also measured in

the presence of propane-1,2,3-tricarboxylate and soluble proteins derived from *Mycobacterium* sp. INA1. In contrast to the oxygen consumption, the NADP^+ -reduction was not linear from the start but became linear after a few minutes, a fact indicating a multi-component system. These components were resolved by gel filtration chromatography (Fig. 1). Two protein fractions, termed I and II in the order of their elution, restored the NADP^+ -reducing activity in the presence of propane-1,2,3-tricarboxylate. Fraction I contained aconitase and NADP^+ -dependent isocitrate dehydrogenase activities, and fraction II the propane-1,2,3-tricarboxylate oxidizing enzyme. Fraction I could be replaced by aconitase and isocitrate dehydrogenase activities from pig heart to restore NADP^+ -reducing activity in the presence of fraction II and propane-1,2,3-tricarboxylate. It was concluded that fraction II converted propane-1,2,3-tricarboxylate to aconitate and that further metabolism of the latter occurred in the presence of fraction I and led to a NADP^+ -reduction via aconitase and isocitrate dehydrogenase. Oxidation of propane-1,2,3-tricarboxylate to aconitate occurred *in vitro* with oxygen as electron acceptor as deduced from the oxygen consumption results and from the fact that NADP^+ -reduction was

Fig. 1. Elution profiles of enzyme activities involved in the oxidation of propane-1,2,3-tricarboxylate, when separated on Sephacryl S-300 HR. Bars indicate the protein pools that were termed fractions I and II. —●—, Isocitrate dehydrogenase activity; —○—, aconitase activity; ●, activity (activities) that complemented fraction II to restore NADP^+ reduction by propane-1,2,3-tricarboxylate; ●, activity that complemented fraction I to restore NADP^+ reduction by propane-1,2,3-tricarboxylate; ◇, activity that was complemented with aconitase and isocitrate dehydrogenase from pig heart to restore NADP^+ reduction by propane-1,2,3-tricarboxylate. The elution volumes corresponded approximately to molecular masses of 250 kDa (isocitrate dehydrogenase), 120 kDa (aconitase) and 40 kDa (fraction II).

Table 1. Purification protocol for isonicotinate dehydrogenase from soluble cell protein of *Mycobacterium* sp. INA1

Purification step	Protein		Activity			Purification factor
	Total (mg)	%†	Total (U)	%†	(U mg ⁻¹)	
Soluble cell protein	308	100	167	100	0.54	—
Q-Sepharose	11.5	4	95	57	8.2	15
Phenyl-Sepharose	1.6	0.5	25	15	15.7	29
Superdex 200*	0.47	0.15	3.5	2	7.5	14

* Only peak fractions were pooled.

† Activity and protein content of the soluble cell protein fraction was taken as 100%.

oxygen-dependent and did not occur under anaerobic conditions. Therefore, an oxygen-dependent propane-1,2,3-tricarboxylate oxidase is responsible for this transformation. The reaction product, aconitate, is in the usual *cis*-conformation, as *Mycobacterium* sp. INA1 only possessed *cis*-aconitase activity.

Purification and characterization of isonicotinate dehydrogenase

Isonicotinate dehydrogenase from *Mycobacterium* sp. INA1 was found to be partly membrane-associated. The degree of membrane association increased from 20–30% to about 70% in the late-exponential growth phase, due to a rapid decrease of specific activity from about 360 to 80 U (g protein)⁻¹ in the soluble fraction when isonicotinate disappeared from the medium. Specific activity, on the other hand, remained quite stable in the membrane fraction (approximately 1000 U g⁻¹). No differences could be observed between the enzymes purified from these two fractions, either in chromatographic behaviour or in catalytic and structural properties.

Purification of isonicotinate dehydrogenase to apparent homogeneity was achieved using anion exchange chromatography, hydrophobic interaction chromatography, and gel permeation chromatography as described in Methods, and a representative purification protocol is given in Table 1. For unknown reasons, isonicotinate dehydrogenase activity was somewhat unstable, and a decrease in specific activity occurred during the late steps of purification, either during hydrophobic interaction chromatography or during gel permeation chromatography. Several modifications have been considered to account for the inactivation of related enzymes, as has been thoroughly discussed for a nicotinate dehydrogenase (Nagel & Andreesen, 1990).

Purified isonicotinate dehydrogenase from *Mycobacterium* sp. INA1 had a pH optimum at 9.5 and a K_m (for isonicotinate) of 1 mM in pyrophosphate buffer at that pH. The enzyme proved to be rather substrate-specific.

Table 2. Catalytic properties of isonicotinate dehydrogenase from *Mycobacterium* sp. INA1

Property	
pH optimum*	9.5
K_m at pH 8.2 (mM) [†]	0.21
K_m at pH 9.5 (mM) [†]	1.03
Optimal assay temperature (°C)	60
Substrate spectrum (activity in %) [‡]	
Isonicotinate	100
Quinoline-4-carboxylate	17
Pyridine-3,4-dicarboxylate	2.6
Pyridine-2,3-dicarboxylate	1.6
N-Methylisonicotinate	1.4

* The optimal pH was determined using the following buffers (40 mM each): Tris/HCl, pH 8.5, 9.0, 9.5; sodium pyrophosphate/KOH, pH 9.5, 10.0; glycine/NaOH, pH 9.0, 9.5, 10.0, 10.5.

† Recorded in Tris/HCl, pH 8.2, and pyrophosphate buffer, pH 9.5, respectively.

‡ The tested substrates were all 5 mM; activity with isonicotinate was set to be 100%; zero activity was found with 2-hydroxyisonicotinate, isoniazid, 4-chloropyridine, nicotinate, picolinate, xanthine and hypoxanthine.

Besides isonicotinate, only quinoline-4-carboxylate (= 'benzoisonicotinate') was found to be oxidized at appreciable rates (Table 2). No activity could be measured with 2-hydroxyisonicotinate. The 2-hydroxyisonicotinate-oxidizing activity present in cell extracts was separated from isonicotinate dehydrogenase during the first enrichment step. Whereas isonicotinate dehydrogenase bound to the anion exchange material under the application conditions described, 2-hydroxyisonicotinate dehydrogenase did not. This is in contrast to isonicotinate- and 2-hydroxyisonicotinate-oxidizing activities of *Micrococcus* sp. (Gupta & Shukla, 1979b) which co-purified. A single enzyme was supposed to be responsible for both reactions, but final proof for this was lacking, since pure preparations were not obtained, nor was the enzyme structurally characterized.

Isonicotinate dehydrogenase of *Mycobacterium* sp.

Fig. 2. Subunits of isonicotinate dehydrogenase as revealed by SDS-PAGE. (a) SDS gradient PAGE; (b) homogeneous SDS-PAGE.

INA1 was estimated to have a molecular mass of either 125 ± 3 kDa (by native gradient PAGE) or 250 ± 10 kDa (by gel filtration chromatography). SDS-PAGE revealed three different types of subunits (see below), and it was assumed that the purified enzyme existed *in vitro* either as a $(\alpha\beta\gamma)$ monomer or as a $(\alpha\beta\gamma)_2$ dimer depending on the experimental conditions. Under the conditions described, both forms exhibited enzymic activity. The native state *in vivo* is not known. Subunit sizes were determined either by SDS gradient PAGE to be about 83, 31 and 19 kDa or by homogeneous SDS-PAGE to be 76, 28 and 18 kDa. Because of the acrylamide gradient, the 18–19 kDa subunit stained weakly in SDS gradient gels. In continuous, non-gradient SDS gels, it ran, for unknown reasons, either in a diffuse manner or as a double band (Fig. 2). The presence of an impurity or a fourth subunit were not considered as possible reasons for this curious running behaviour, as N-terminal amino acid sequencing did not yield a double sequence. Partial degradation, however, cannot be excluded. N-Terminal amino acid sequences of all three subunits of isonicotinate dehydrogenase are shown in Fig. 3.

Earlier studies had shown that expression of active isonicotinate dehydrogenase required molybdenum

Fig. 4. Absorption spectrum of isonicotinate dehydrogenase from *Mycobacterium* sp. INA1.

(Kretzer & Andreesen, 1991). In addition, 10 mM-cyanide and 1.5 M-methanol, both known inhibitors of molybdo-enzymes (Coughlan *et al.*, 1969) were found to inhibit isonicotinate dehydrogenase activity almost completely in the soluble cell protein fraction. Purified isonicotinate dehydrogenase was found to be somewhat less sensitive to inhibition.

The presence of further redox-active components besides molybdenum was indicated by the absorption spectrum of purified isonicotinate dehydrogenase. A maximum around 450 nm and a shoulder around 550 nm pointed to the presence of flavin and iron-sulphur clusters (Fig. 4). The non-covalently-bound flavin was identified as FAD. Molybdenum, iron, acid-labile sulphur, and FAD were determined to be present at ratios

	1	10	20
83 kDa subunit:	M	H D A V D R V T G Q T P F V I N H E I	
31 kDa subunit:	S	G S P A M F H P R T V A D A V E L L D	
19 kDa subunit:	K	? D N I Y R F A E D D (G) R I A V ? L ?	

Fig. 3. N-Terminal amino acid sequences of the three subunits of isonicotinate dehydrogenase.

Fig. 5. Identification of 5'-CMP. HPLC elution profiles of the supernatant of acid-treated isonicotinate dehydrogenase (a) and of the same amount of supernatant after addition of authentic 5'-CMP (b) recorded at 270 nm. The inset shows the absorption spectra of peaks eluting at 7 min without (a) and with addition of authentic 5'-CMP (b).

Fig. 6. Identification of MCD in isonicotinate dehydrogenase. Elution profiles of the carboxamidomethylmolybdopterine derivative obtained from isonicotinate dehydrogenase (a) and purified camMCD from *Hydrogenophaga pseudoflava* carbon monoxide dehydrogenase (b) were recorded at 370 nm. Absorption spectra of the same substances eluting between 6.5 and 6.8 min are given in the inset.

of 0.93, 4.12, 4.09, 0.95 mols per mol of the native 125 kDa protein. The presence of a molybdenum-complexing pterin cofactor was shown by the characteristic fluorescence of the form A derivative (data not shown) (Johnson & Rajagopalan, 1982). For the following reasons it was concluded that molybdopterine cytosine dinucleotide was the organic moiety of the molybdenum cofactor: (i) when isonicotinate dehydrogenase was subjected to acid hydrolysis, a compound was detected in the supernatant by reversed-phase HPLC that exactly coeluted with authentic 5'-CMP and exhibited essentially the same absorption spectrum (Fig. 5); (ii) when the carboxamidomethyl derivative of the molybdenum cofactor was prepared and isolated, it eluted from Nucleosil 120-7 RP18 exactly like carboxamidomethylmolybdopterine cytosine dinucleotide (camMCD) from carbon monoxide dehydrogenase of *Hydrogenophaga pseudoflava*. The camMCDs from both isonicotinate dehydrogenase and carbon monoxide dehydrogenase eluted as double peaks due to the mono- and dicarboxamidomethylated forms. The presence of different molybdopterine dinucleotides in isonicotinate dehydrogenase, as described for formylmethanofuran dehydrogenase from *Methanobacterium thermoautotrophicum* (Börner *et al.*, 1991), was not considered, since the same double peak was obtained with camMCD from carbon monoxide dehydrogenase of *Hydrogenophaga pseudoflava*, an enzyme known to contain only molybdopterine cytosine dinucleotide (Johnson *et al.*, 1990), and both pterin peaks exhibited essentially the same absorption spectra that were identical to those obtained from carbon monoxide dehydrogenase camMCD (Fig. 6).

Discussion

Degradation of isonicotinate by *Mycobacterium* sp. INA1

In an earlier paper we described the isolation of a mycobacterial species that metabolizes isonicotinate as the sole source of carbon, nitrogen, and energy (Kretzer & Andreesen, 1991). Bacterial growth on isonicotinate had been described before for a *Pseudomonas* sp. (Ensign & Rittenberg, 1965), a *Micrococcus* sp. (formerly called *Sarcina* sp.) (Gupta & Shukla, 1979a), and a *Bacillus brevis* strain (Singh & Shukla, 1986). For the latter two organisms, degradation pathways have been proposed that differ from that found in *Mycobacterium* sp. INA1.

Mycobacterium sp. INA1 shares the first two hydroxylation reactions that yield citrazinate (2,6-dihydroxyisonicotinate) with *Pseudomonas* sp. and *Micrococcus* sp. (Kretzer & Andreesen, 1991). However, further metabolism of citrazinate was found to follow

Fig. 7. Proposed pathway of isonicotinate metabolism in *Mycobacterium* sp. INA1. Enzymes: (I) isonicotinate dehydrogenase; (II) 2-hydroxyisonicotinate dehydrogenase; (III) citrazyl-CoA synthetase; (IV) citrazyl-CoA reductase; (V) propane-1,2,3-tricarboxylate oxidase; (VI) *cis*-aconitase; (VII) isocitrate dehydrogenase.

a previously unknown route involving CoA-ester intermediates. In the case of *Pseudomonas* sp., no metabolic steps beyond citrazinate have been resolved at all, but as resting cells of *Pseudomonas* sp. quickly oxidized citrazinate, the latter was thought to be a real intermediate (Ensign & Rittenberg, 1965). In the case of *Micrococcus* sp., direct hydrolytic cleavage of citrazinate to yield *cis*-aconitamide was at first postulated (Gupta & Shukla, 1979a). Failure to measure this reaction, as well as the fact that *Micrococcus* sp. did not metabolize citrazinate, prompted the authors to consider citrazinate as a metabolic dead-end (Sharma & Shukla, 1987). However, following our observations on isonicotinate metabolism by *Mycobacterium* sp. INA1, these facts do not necessarily contradict an involvement of citrazinate as a true intermediate (Kretzer & Andreessen, 1991).

2-Hydroxyisonicotinate and citrazinate were finally also shown to be intermediates in *N*-methylisonicotinate metabolism by the unidentified Gram-positive strain 4C1 (Orpin *et al.*, 1972). A further intermediate was maleamate. Although the links were missing, it seemed very reasonable in that case to postulate an oxygenase reaction that cleaved the aromatic ring between C₂ and C₃ in either citrazinate or 2,6-dihydroxypyridine. A variety of pyridine compounds are metabolized via similar reaction sequences – often referred to as the ‘maleamate pathway’ – that generally do not involve CoA-ester intermediates (Shukla, 1984).

Our previous work had demonstrated that isonicotinate metabolism in *Mycobacterium* sp. INA1 did not follow one of these known routes but involved activation

of citrazinate to its CoA-ester and NADPH + H⁺-dependent reduction of the latter. A physiological demand for this energy-dependent activation of citrazinate is not discernible, but it is proposed that the ester function activates citrazinate for a nucleophilic attack of a hydride ion in the course of the reduction. An analogous catalytic role was proposed for the involvement of CoA-ester intermediates in the bacterial dehalogenation of 4-chlorobenzoate that might succeed via a nucleophilic substitution (Copley & Crooks, 1992; Löffler *et al.*, 1992).

The reduction of citrazyl-CoA yields 2,6-dioxopiperidine-4-carboxyl-CoA and further metabolism has been shown to yield 2-oxoglutarate (Kretzer & Andreessen, 1991). The detection of a *cis*-aconitate-generating propane-1,2,3-oxidizing system as presented in this paper has narrowed the gap of knowledge in isonicotinate metabolism by *Mycobacterium* sp. INA1 (Fig. 7). Only two more reactions remain to be established: CoA-ester hydrolysis or a CoA transferase reaction, and hydrolysis of the cyclic imide function.

Isonicotinate dehydrogenase from Mycobacterium sp. INA1

Hydroxylations in the neighbourhood of the nitrogen – like those converting isonicotinate to citrazinate – are very common in the bacterial metabolism of pyridine derivatives. Some of the responsible dehydrogenases from very different bacteria have been purified and all of them were found to be molybdenum-containing hy-

Table 3. Comparison of selected molybdenum-containing hydroxylases

Abbreviations: DH, dehydrogenase; MoCo, molybdenum cofactor; MPT, molybdopterin; MCD, molybdopterin cytosine; MGD, molybdopterin guanine dinucleotide; ND, not determined.

Enzyme	Organism	Mol. mass (kDa)	Subunits (kDa)			Redox-active centres*				Specification of centres			Reference
						Mo	Fe	S	Flavin	MoCo	FeS	Flavin	
<i>Molybdenum-containing hydroxylases that exhibit strong structural similarities</i>													
Nicotinate DH	<i>Bacillus niacini</i>	300	85	34	20	0.8	4.2	0.8	1.0	MCD†	ND	FAD	Nagel & Andreesen (1990)
6-Hydroxynicotinate DH	<i>Bacillus niacini</i>	120	85	34	15	ND	ND	ND	ND	MGD†	ND	FAD	Nagel & Andreesen (1990)
Nicotine DH	<i>Arthrobacter ureafaciens</i> ‡	120	82	30	15	0.9	4.0	2.0	0.9	ND	ND	FAD	Freudenberg <i>et al.</i> (1988)
Quinoline DH	<i>Pseudomonas putida</i> 86	300	85	30	19	0.8	3.4	4.3	0.8	MCD	ND	FAD	Hettrich <i>et al.</i> (1991)
Quinoline DH	<i>Rhodococcus</i> sp. B1	300	82	32	18	0.6	3.7	3.7	0.9	MCD	ND	FAD	Peschke & Lingens (1991)
Quinoline-4-carboxylate DH	<i>Agrobacterium</i> sp. 1B	320	85	35	21	1.1	4.2	3.6	0.9	MCD	ND	FAD	Bauer & Lingens (1992)
Isonicotinate DH	<i>Mycobacterium</i> sp. INA1	125	83	31	19	0.9	4.1	4.1	1.0	MCD	ND	FAD	This work
Quinaldine DH	<i>Arthrobacter</i> sp. Rü 61a	340	82	35	22	0.8	4.0	3.8	0.9	MCD	ND	FAD	De Beyer & Lingens (1993)
Carbon monoxide DH	Carboxydotrophic bacteria	~ 260	~ 80	~ 30	~ 15	1.0	4.0	4.0	1.0	MCD	Fe ₂ S ₂	FAD	Johnson <i>et al.</i> (1990)
Xanthine DH§	<i>Clostridium barkeri</i>	535	81	30	18	0.6	2.1	ND	ND	ND	Fe ₂ S ₂	FAD	Rienhöfer (1985)
Xanthine DH	<i>Pseudomonas putida</i> 86	550	87	52		1.0	3.6	4.0	1.0	MPT	ND	FAD	Hettrich <i>et al.</i> (1991)
Nicotinate DH	<i>Aspergillus nidulans</i>	300	153			1.0	4.3	ND	1.0	ND	Fe ₂ S ₂	FAD	Coughlan <i>et al.</i> (1984)
Xanthine DH/oxidase	Mammals and birds	~ 300	~ 150			1.0	4.0	4.0	1.0	MPT	Fe ₂ S ₂	FAD	Amaya <i>et al.</i> (1990)
			~ 85	~ 40	~ 20	(domains)							
<i>Molybdenum-containing hydroxylases that differ in their structure and composition from the above listed enzymes</i>													
Xanthine DH	<i>Pseudomonas putida</i> Ful	300	55	25		0.4	1.5	0.6	—¶	MGD§	ND	—	Koenig & Andreesen (1990)
Furoyl-CoA DH	<i>Pseudomonas putida</i> Ful	150	55	25		ND	ND	ND	—¶	MAD§	ND	—	Koenig & Andreesen (1990)

* Values refer to molar ratios per protomer, sulphur being determined as acid-labile sulphur.

† K. Frunzke, personal communication.

‡ Formerly known as strain of *Arthrobacter oxydans*.

§ Enzyme contains additionally 0.3 W and 0.2 Se.

|| Also described as 'purine hydroxylase II'.

¶ Instead of FAD, cytochrome *b* is present; for xanthine dehydrogenase, the ratio was determined to be 0.2 cytochrome *b* per protomer in the final preparation.

droxylases with striking similarities (Table 3). Isonicotinate dehydrogenase from *Mycobacterium* sp. INA1 (this work) fits into that group whose general features are: ($\alpha\beta\gamma$) or ($\alpha\beta\gamma$)₂ structure with subunit sizes of about 85, 30 and 20 kDa and presence of 1 atom of molybdenum, 4 atoms of iron, 4 atoms of acid-labile sulphur and 1 mol FAD per protomer. In nearly all cases, MCD was shown to be the molybdenum-complexing cofactor of those enzymes specifically acting on pyridine derivatives. Only 6-hydroxynicotinate DH from *Bacillus niacini*, an organism that contains two similar molybdoenzymes, both

involved in the catabolism of nicotinate, was found to contain MGD.

Some other molybdenum-containing hydroxylases share most of the structural properties with the above mentioned enzymes, e.g. carbon monoxide dehydrogenases from carboxydophilic bacteria and some bacterial xanthine dehydrogenases (Table 3). Corresponding eukaryotic molybdoenzymes also exhibit great similarities in their content of redox-active components though having a dissimilar α_2 homodimeric structure (Coughlan, 1980). That is also true for a rather substrate-

unspecific enzyme called 'purine hydroxylase II' from *Aspergillus nidulans*. This enzyme is actually a eukaryotic nicotinate dehydrogenase (Lewis *et al.*, 1978; Coughlan *et al.*, 1984). The best characterized eukaryotic hydroxylases are the xanthine oxidases/dehydrogenases from mammalian and avian sources. In these cases the homodimers were shown by partial proteolytic cleavage to be composed of $(\alpha_1\alpha_{II}\alpha_{III})_2$ domains connected by hinge peptides (Coughlan, 1980). In the case of xanthine dehydrogenase from rat liver, results indicate that the molybdenum cofactor, the Fe_2S_2 clusters, and the FAD are located on the 85, 20 and 40 kDa domains, respectively (Amaya *et al.*, 1990). Domain structure and location of the redox-active centres has been confirmed by DNA homology studies (Wootton *et al.*, 1991). The eukaryotic domains might be functional analogues of the bacterial subunits.

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