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Thiomorpholine and morpholine oxidation by a cytochrome P450 in *Mycobacterium aurum* MO1. Evidence of the intermediates by *in situ* ^{1}H NMR

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Abstract

Spectrophotometric assays of *Mycobacterium aurum* MO1 cells extracts gave evidence of a soluble cytochrome P450, involved in the degradative pathway of morpholine, a waste product from the chemical industry. In order to get further information, the kinetics of the biodegradation of the sulfur analogue thiomorpholine was monitored by using *in situ* nuclear magnetic resonance (NMR). This technique allowed the identification of two intermediates: the sulfoxide of thiomorpholine resulting from S-oxidation and thiodiglycolic acid owing to ring cleavage. The S-oxidation (S → SO) represents one of the well-known reactions catalyzed by cytochromes P450. The inhibitory effect of metyrapone, a cytochrome P450 inhibitor, on the thiomorpholine and morpholine degradative abilities of *M. aurum* MO1 confirmed the involvement of a cytochrome P450. These results and the decrease of the rate of formation of the first intermediate during the morpholine degradation, 2-(2-aminoethoxy) acetate, proved the key role of the cytochrome P450 in the early events of the biodegradation, i.e, in the C—N bond cleavage.

Introduction

The heterocyclic, secondary amine morpholine, mainly used in the manufacture of rubber additives but with an ever-widening range of applications (catalyst, solvent, antioxidant, …) (Mjos 1978; Anon 1989), is regularly released, via industrial effluents, into the environment. Its presence in water, and even in foods (Singer & Lijinski 1976), is of particular concern owing to its potential sensitivity towards N-nitrosation producing the potent mutagen and carcinogen N-nitrosomorpholine (Anon 1989; Enzmann et al. 1995).

In the literature, many research works have been reported on the biodegradation of morpholine with strains of the genera *Mycobacterium* (Cech et al. 1988; Dmitrenko & Gvozdyak 1988, Knapp et al. 1982, Knapp & Brown 1988; Mazure & Truffaut 1994; Poupin et al. 1998) or *Arthrobacter* (Dmitrenko et al. 1987), and more recently with Gram-negative bacteria (Knapp et al. 1996). The biodegradative pathway was not clearly established because no tool for direct detection of the intermediates was available. However, in a recent work (Combourieu et al. 1998), two metabolites of this pathway in *Mycobacterium aurum* MO1 have been proven by using *in situ* ^{1}H NMR spectroscopy, which allows direct determination and quantification of the intermediary compounds formed. The identification of 2-(2-aminoethoxy) acetate and glycolate as intermediates was in agreement with the pathway suggested by Swain for *M. chelonae* (Swain et al. 1991).

In order to understand the mechanism of this biodegradative pathway in *M. aurum* MO1, we were

thus interested in giving evidences of the type of enzymes involved.

Knapp (Knapp et al. 1982) and Mazure (Mazure & Truffaut 1994) have shown that morpholine degradation was associated with oxygen consumption, indicating the possibility for involvement of a monooxygenase. Monooxygenases are widely distributed enzymes which catalyze dioxygen activation towards the insertion of one oxygen atom from O_2 into a substrate, the other one being reduced into water. A great number of these monooxygenases contain a heme protein called cytochrome P450. These cytochromes P450 are involved in various steps of the biosynthesis of endogenous compounds (steroids, fatty acids...) (Waterman et al. 1986; Jefcoate 1986) and in the oxidative detoxification and elimination of many hydrophobic xenobiotics (pollutants, drugs, pesticides ...) (Wislocki et al. 1980, Guengerich 1990a). Because of their wide distribution in living organisms and their very important role in biochemistry, pharmacology and toxicology, cytochromes P450 have been extensively studied (Guengerich 1990b; Ortiz de Montellano 1986; Ruckpaul & Rein 1984, 1990). Several bacterial cytochromes P450 (from *Pseudomonas putida*, *Streptomyces griseus*, *Bacillus megaterium*) whose cristalline structure was known, were used as models for human cytochromes P450 (Fulco 1991, Asperger & Kleber 1991). Several other bacteria genera also contain cytochromes P450, for example *Rhodococcus*, a genus very close to *Mycobacterium* (Asperger et al. 1990, Karlson et al. 1993, Nagy et al. 1995). Recently, we demonstrated the involvement of a cytochrome P450 in morpholine biodegradation by a strain of *Mycobacterium* (Poupin and Truffaut 1996; Poupin et al. 1998). This prompted us to investigate the possible role of cytochrome P450 in biodegradative abilities of xenobiotics in *Mycobacterium aurum* MO1.

In this work, we bring evidence of the induction of a soluble cytochrome P450 in *M. aurum* MO1 in the presence of morpholine and thiomorpholine by classical spectrophotometric assays. In order to prove its key role in the degradative pathways of xenobiotics by this bacterium, we have studied first, by *in situ* ^{1}H NMR, the biodegradation of thiomorpholine, a sulfur analogue of morpholine. *M. aurum* MO1 do not grow on thiomorpholine (Mazure & Truffaut 1994) but is able to degrade it. Thus, the accumulation of metabolites was expected. Their identification should allow us to have a better understanding of the biodegradative pathway of heterocyclic xenobiotics. Moreover, the sulfur atom of thiomorpholine can be easily oxidized. The ^{1}H NMR technique, performed directly on the incubation medium in H_2O, allowed to prove the formation of an oxidized product and to quantify it, showing its possible involvement in the thiomorpholine degradation. The use of a cytochrome P450 inhibitor, metyrapone, added to the incubation medium containing thiomorpholine or morpholine, showed an inhibitory effect on the degradative properties of *M. aurum* MO1, providing another proof of the key role played by this enzyme in the biotransformations of the two compounds.

Materials and methods

Chemicals. Thiomorpholine, morpholine, metyrapone and *m*-chloroperbenzoïc acid were purchased from Aldrich Chemical (Sigma Aldrich Sarl, St. Quentin Fallavier, France) and tetradeuterated sodium trimethylsilylpropionate ($TSPd_4$) was purchased from EurisoTop (St. Aubin, France).

Growth conditions. *M. aurum* MO1 cultures were grown in 100 ml of Trypcase soy broth (bioMerieux, Marcy l'Etoile, France) in 500-ml Erlenmeyer flasks incubated at 30 °C with agitation at 200 rpm. They were harvested after 48h of culture. For spectrophotometric assays, *M. aurum* MO1 was grown in mineral salts medium (Knapp buffer which contains per liter of deionized water: KH_2PO_4 1 g, K_2HPO_4 1 g, $FeCl_3.6H_2O$ 4 mg and $MgSO_4.7H_2O$ 40 mg, pH 6.6) amended with 10 mM morpholine or 20 mM acetate. When thiomorpholine was used for these experiments, the cells were grown in rich medium, harvested by centrifugation (8.000 $\times$ *g*, 10 min), and washed once with mineral salts medium. They were then resuspended in this medium containing 5 mM thiomorpholine and incubated during 6 h in a gyratory shaker at 30 °C (200 rpm).

Incubation with xenobiotics. Cells were harvested by centrifugation at 9,000 $\times$ *g* for 15 min at 5 °C. The supernatant was eliminated, the pellet was washed twice with Knapp buffer and finally resuspended in this buffer (5 g of wet cells in 50 ml of buffer). The cells were incubated with 10 mM of thiomorpholine or morpholine as the only source of energy in a 500 ml Erlenmeyer at 30 °C with agitation (200 rpm). Metyrapone (5 or 10 mM) was added for the assays of inhibition. Incubation of cells under the same conditions in the absence of substrate constituted a negative control, as did incubation of the substrate in the buffer without cells. Samples (1 ml)

were taken every hour for 12 h and from time to time for 72 h. They were centrifuged at 12,000 × *g* for 5 min. The supernatants were isolated and immediately frozen until NMR analysis.

Spectrophotometric analysis of a cytochrome P450. Mycobacterial cells were harvested by centrifugation (8,000 × *g*, 10 min) at 4 °C, washed and resuspended in 50 mM phosphate buffer (pH 7.4) containing 0.3 mM phenylmethylsulfonyl fluoride. Bacterial walls were broken by three passages through a French pressure cells (SLM-Aminco) at 18,000 psi and centrifuged at 30,000 × *g* at 4 °C for 30 min. The obtained supernatant was reduced by sodium dithionite, divided equally between two optically matched cuvettes and the difference spectra was recorded in the presence of CO (Peterson & Lu 1991). An extinction coefficient of 91 $mM^{-1}.cm^{-1}$ was used to determine the content of cytochrome P450 in crude extracts (Omura & Sato, 1964). All photometric analyses were determined using a Shimadzu UV 160A spectrophotometer. The protein concentration was determined by the Bradford method (Bradford 1976).

^{1}H NMR spectroscopy.

Preparation of NMR samples: The supernatant (540 μl) was supplemented with 60 μl of a 8 mM solution of $TSPd_4$ in D_2O and adjusted to pH 10 with 4N NaOH. pH adjustment avoided changes in chemical shifts. D_2O was used for locking and shimming. $TSPd_4$ constituted a reference for chemical shift (0 ppm) and quantification.

^{1}H NMR spectra: ^{1}H NMR spectra were performed at 300.13 MHz with an Avance 300 DSX or at 400.13 MHz with a 400 AC Bruker spectrometers at 21 °C with 5 mm-diameter tubes containing 500 μl of sample; water was suppressed by saturation with a classical NOE Bruker program. 64 scans were collected. No filter was applied before Fourier transformation but a baseline correction was performed on spectra before integration with Bruker software. Under these conditions, the detection limit was in the range of 0.05 mM.

Quantification of metabolites: The concentration of metabolites was calculated as follows: $[m] = (9\ A_0 \times [TSPd_4])/(b \times A_{ref})$, where [m] is the concentration of metabolite m; A_o is the area of metabolite m resonance in the ^{1}H NMR spectrum; $[TSPd_4]$ is the concentration of the reference; A_{ref} is the area of reference resonance in the ^{1}H NMR spectrum; b is the number of protons of metabolite m in the signal integrated; 9 is the number of protons resonating at 0 ppm.

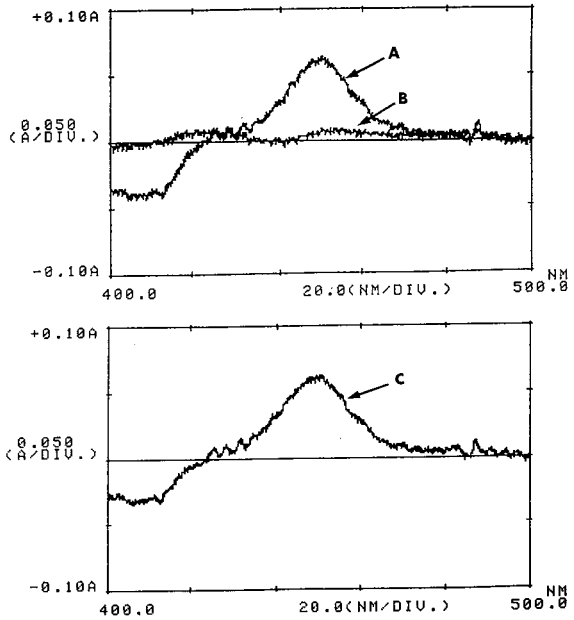

Figure 1. Carbon monoxide difference spectra of cell extracts. Cultures of *M. aurum* were grown with morpholine (spectrum A), acetate (spectrum B), or induced by thiomorpholine (spectrum C); the protein contents were 10, 10, and 11.5 mg/ml, respectively.

Synthesis of the sulfoxide of thiomorpholine (Madesclaire 1986). To a solution of thiomorpholine (500 mg, 4.85 mmol) in dichloromethane (50 ml), stirred and cooled at −15 °C, was added dropwise a solution of *m*-chloroperbenzoïc acid (1.05 eq., 1 g) in dichloromethane (25 ml). The solution was stirred for another 3 h. The solvent was then removed and the crude residue was purified by flash column chromatography (eluent: methanol/ethyl acetate 93/7). The sulfoxide was obtained in 70% yield.

IR: Broad band 1070–1130 cm^{-1}; MS (EI): 119 ($M^{+\cdot}$); 102; 71; 56.

NMR spectroscopy: the assignement of the different signals was done using 1D and 2D (COSY ^{1}H-^{1}H and ^{1}H-^{13}C) experiments. In particular, an heteronuclear correlation ^{1}H-^{13}C allowed us to assign the protons (H_C-H_D) and (H_A-H_B) attached respectively to C-2 and C-3 according to the ^{13}C chemical shifts given by Gallego et al. (1993). NMR ^{1}H (400.13 MHz; CD_3OD) δ: 2.69 ppm (ddd, 2H, H_B or H_A); 2.87 ppm (ddd, 2H, H_C or H_D); 2.96 ppm (ddd, 2H, H_A or H_B); 3.39 ppm (ddd, 2H, H_D or H_C). Coupling constants: J_{AC} = 13.9 Hz, J_{AD} = 2.4 Hz, J_{BC} = 3.1 Hz; J_{BD} = 2.9 Hz. NMR ^{13}C (100.61 MHz; CD_3OD): 39.2 ppm (C-2); 47.8 ppm (C-3).

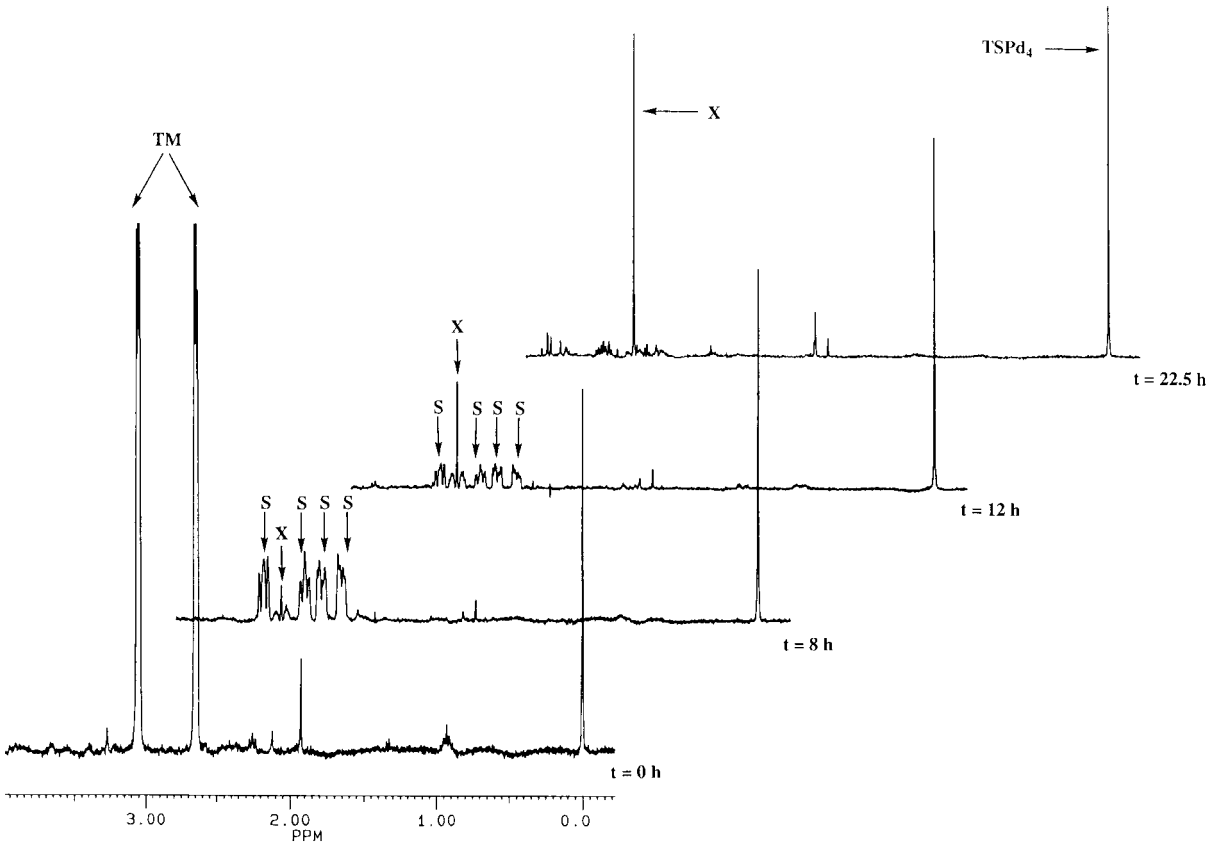

Figure 2. Kinetics of thiomorpholine transformation by *M. aurum* MO1. The ^{1}H NMR spectra were recorded at 400.13 MHz with a 400 AC spectrometer at 21°C with 5-mm-diameter tubes; water was suppressed by saturation with a classical NOE Bruker programm. 64 scans were collected (90° pulse 11.6 μs, relaxation delay 4 s, acquisition time 1.786 s, 16 K data points). TM: thiomorpholine; S: sulfoxide; X: thiodiglycolic acid.

Identification of metabolite X. After 24 h of incubation with thiomorpholine, the supernantant containing only the metabolite X was freeze-dried to be analyzed by mass spectrometry (electronic impact). MS (EI): 150 ($M^{+\cdot}$); 132, 104, 77, 60.

Results

Spectrophotometric evidence of the induction of a cytochrome P450

In a previous work (Poupin & Truffaut 1996; Poupin et al. 1998), we demonstrated the involvement of a soluble cytochrome P450 in the degradation of morpholine by *Mycobacterium* sp. strain RP1. To investigate the presence of such a monooxygenase in *M. aurum* MO1, we measured the carbon monoxide differential spectra of sodium dithionite-reduced soluble fractions of morpholine-induced and uninduced cells (Figure 1). When *M. aurum* MO1 cells were grown on morpholine as sole source of carbon, nitrogen, and energy a peak at 449 nm was present in the spectrum (Figure 1, spectrum A) indicating the presence of a soluble cytochrome P450. Since this peak was not observed in acetate-grown cells (Figure 1, spectrum B), the synthesis of this enzyme was induced by morpholine. Using thiomorpholine as substrate (Figure 1, spectrum C), similar results were obtained. The cytochrome P450 contents were about 70 pmol per mg of protein in morpholine-grown cells extracts and about 60 pmol per mg of protein in thiomorpholine-induced cells. Other structurally related amines, such as pyrrolidine and piperidine, also induced the synthesis of a soluble cytochrome P450 (data not shown). We can reasonably conclude that these cyclic amines induced the synthesis of the same heme-containing monooxygenase.

Degradation of thiomorpholine by M. aurum MO1

The supernatants of *M. aurum* MO1 resting cells (5 g of wet cells in 50 ml of Knapp buffer) were analyzed by ^{1}H NMR spectroscopy as indicated in the Materials and methods section. The spectra were compared with those of the negative controls. Spectra collected at 0, 8, 12 and 22.5 h are presented in Figure 2.

In the spectrum obtained at time zero, three main signals are visible: a singlet at 0 ppm that belongs to the methyl groups of $TSPd_4$, our reference, and two pseudotriplets at 2.66 and 3.08 ppm that correspond to CH_2(a) and CH_2(b) of thiomorpholine (Figure 8). At 8 h, thiomorpholine was completely exhausted and new signals were detected, in particular a group of four signals, resonating at 2.86, 2.99, 3.10 and 3.39 ppm, which seemed to belong to the same molecule (S) because they first increased and then decreased simultaneously. At 22 h 30, the intermediate compound (S) had disappeared. A new signal (X), a singlet resonating at 3.25 ppm, appeared at 8 h and its concentration increased with time.

As the presence of a soluble cytochrome P450 had been previously confirmed by spectrophotometric experiments, a "classical" oxidation of the sulfur atom of thiomorpholine was expected (Oldham 1989). The pattern of the four signals (S) showed that they were coupled. Moreover, their symmetry indicated that the corresponding protons belonged to a ring. So, the intermediate compound (S) could be a sulfoxide. In order to confirm this hypothesis, a chemical reaction of oxidation was conducted with 1 eq. of *m*-chloroperbenzoïc acid (*m*-CPBA) as the oxidant (Madesclaire 1986) at −15 °C. After purification by column chromatography, the structure of the obtained product was confirmed by IR, mass spectrometry, ^{1}H and ^{13}C NMR. After adjustment of the pH to 10 in the buffer, the ^{1}H NMR spectrum of the synthesized sulfoxide was recorded (Figure 3A). The resonances corresponded perfectly to those of the biodegradative product after 8 h of incubation (Figure 3B).

The corresponding sulfone, which could be also observed by the action of a cytochrome P450 (Renwick 1989), was also synthesized from thiomorpholine (addition of 2.5 eq. of *m*-CPBA at room temperature). None of the ^{1}H NMR signals of the synthetic sulfone corresponded to those observed in the spectra obtained during the degradation of thiomorpholine (data not shown).

The complexity of the ^{1}H NMR signals of the sulfoxide was quite surprising. A modelisation of the

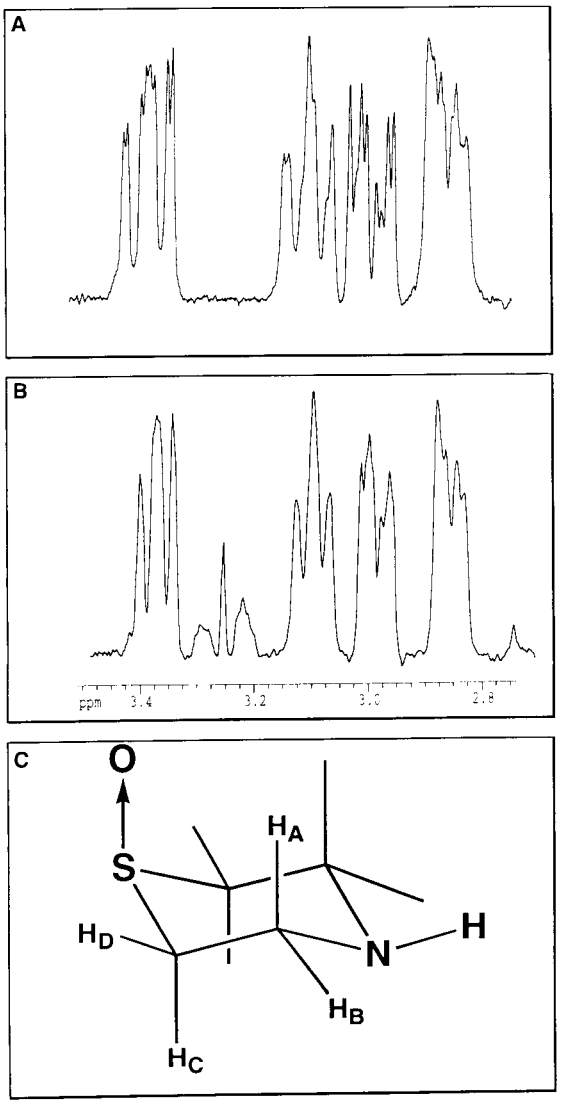

Figure 3. (A) ^{1}H NMR spectrum of synthetic sulfoxide recorded at 300.13 MHz. (B) ^{1}H NMR spectrum of thiomorpholine degradation by *M. aurum* MO1 recorded at 400.13 MHz after 8 h of incubation. (C) Chair conformation of sulfoxide.

product was performed with the programm Batchmin (Monte Carlo procedure). The conformation presented on Figure 3C was found in 407 times out of 1000. In this model, the sulfoxide preferentially takes a chair conformation, where SO is axial. This preferential SO position is in agreement with the conformational analysis obtained by Gallego et al. on different substituted 1,4-thiazanes and their derivatives (Gallego et al. 1993). The protons (A,B) and (C,D) were then non equivalent; that is the reason why the ^{1}H NMR spectrum obtained was complex. The coupling constants

determined by ^{1}H NMR were the following ones: J_{AC} = 13.9 Hz, J_{AD} = 2.4 Hz, J_{BC} = 3.1 Hz, J_{BD} = 2.9 Hz. These values directly measured on the spectrum were consistent with those obtained by calculation.

These results prove the presence in *M. aurum* MO1 of a cytochrome P450 which oxidizes thiomorpholine into its corresponding sulfoxide.

The singlet resonating at δ: 3.25 ppm (metabolite X) was assigned to thiodiglycolic acid as evidenced by NMR and mass spectroscopy. The ^{1}H NMR resonances of this commercial compound and of metabolite X perfectly overlapped. Also the fragmentations obtained from electronic impact spectrum were the same for the two compounds.

The time courses of the concentrations of thiomorpholine, sulfoxide (S) and thiodiglycolic acid (X) are presented in Figures 4A, B and C respectively. While thiomorpholine was degraded, sulfoxide was produced, suggesting that sulfoxide is a primary metabolite. After six hours of incubation the concentration of sulfoxide was decreasing and that of thiodiglycolic acid was increasing in parallel. It seemed that sulfoxide was degraded into metabolite X. To check this hypothesis, the cells were incubated with synthetic sulfoxide; it was effectively metabolized into thiodiglycolic acid (Figure 5).

Inhibition of thiomorpholine transformation by a cytochrome P450 inhibitor: metyrapone

Another way to confirm the key role played by a cytochrome P450 in the transformation of thiomorpholine was to add a selective inhibitor of this enzyme in the incubation medium and to observe its effect on the kinetics of thiomorpholine transformation.

Metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone) was chosen as a specific cytochrome P450 inhibitor (Testa & Jenner 1981). After checking that metyrapone did not affect the viability of *M. aurum* MO1 (the addition of this chemical to the culture medium did not prevent cell growth), experiments were carried out with different concentrations of this compound and analyzed by ^{1}H NMR. To the flasks containing the cells (100 g/l), metyrapone (5 and 10 mM) and thiomorpholine (10 mM) were added. The kinetics of thiomorpholine degradation are shown in Figure 4.

The addition of metyrapone led to an inhibition of the degradative reactions of thiomorpholine (Figure 4A). In the presence of metyrapone (5 mM), the rate of sulfoxide formation decreased and was completely inhibited at a 10 mM concentration (Figure 4B). The same effect was observed for the thiodiglycolic acid formation (Figure 4C). The direct effects of metyrapone on the sulfoxide formation, first intermediate in the thiomorpholine biodegradation, confirmed that a cytochrome P450 was involved in the first step of this degradation.

Inhibition of morpholine degradation by metyrapone

In a previous work (Combourieu et al. 1998), we have shown that morpholine was degraded by *M. aurum* MO1 in 10 h. Two intermediates of the metabolic pathway have been identified: 2-(2-aminoethoxy) acetate and glycolate (Figure 6).

In order to know how the cytochrome P450 was involved in the first steps of the morpholine degradation by *M. aurum* strain MO1, the same kind of experiments as those made with thiomorpholine were carried out, i.e., addition of different concentrations of metyrapone (5 and 10 mM) in the incubation medium containing 10 mM morpholine. The kinetics of the morpholine degradation are reported on Figure 7.

As in the case of thiomorpholine, the addition of metyrapone led to an inhibition of the degradative reactions of morpholine which is concentration dependent. With increasing concentrations of metyrapone, the following effects were observed: (i) the rates of morpholine degradation (Figure 7A) and of the intermediates formation (Figure 7B and C) decreased and (ii) the appearance of the intermediates was delayed. The effects of metyrapone on the formation of the intermediate compounds (especially on 2-(2-aminoethoxy) acetate, which is the first metabolite) indicated that the oxidative ring cleavage takes place in the early events of morpholine degradation. These observations confirm the results obtained with *Mycobacterium* sp. strain RP1 (Combourieu et al. 1998, Poupin et al. 1998).

Discussion

In a previous work (Combourieu et al. 1998), two intermediate compounds were identified, 2-(2-aminoethoxy) acetate and glycolate, during morpholine degradation by *M. aurum* MO1, by using a new approach: ^{1}H NMR spectroscopy performed directly on the incubation medium.

In this paper, we were interested in giving evidences of the enzymes involved in this degradation.

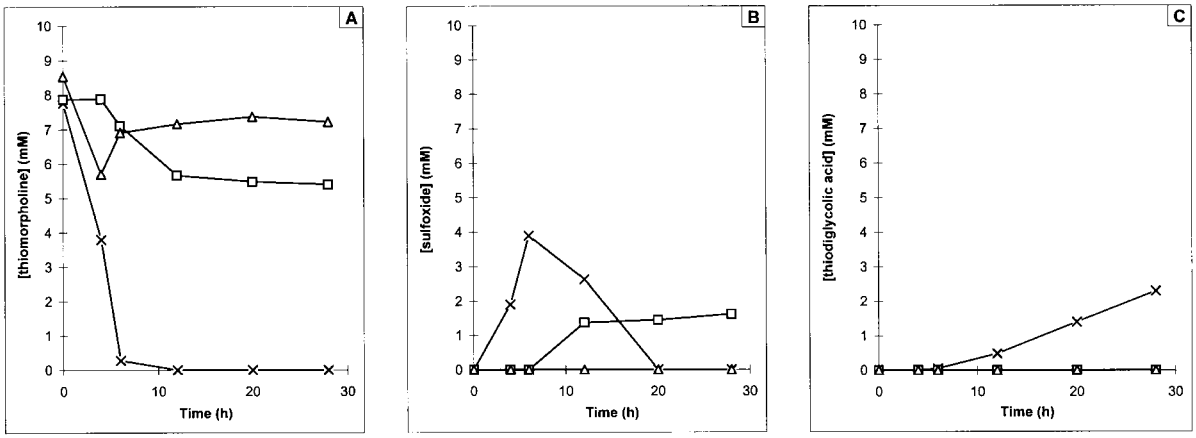

Figure 4. Incubation of *M. aurum* MO1 cells with thiomorpholine (10 mM) in the presence of 5 mM (□) or 10 mM (Δ) metyrapone or in the absence of metyrapone (×). Time courses for the concentrations of thiomorpholine (A), sulfoxide (B) and thiodiglycolic acid (C). Experimental conditions are as in Figure 2.

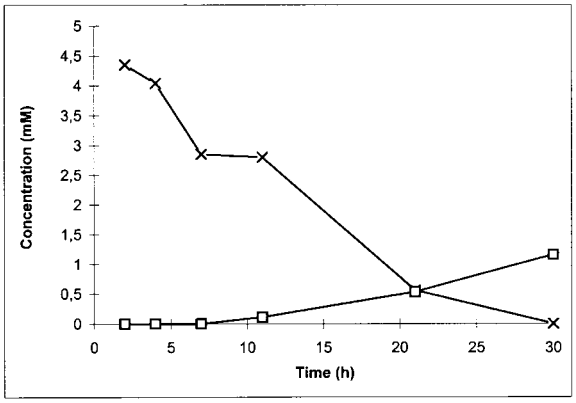

Figure 5. Incubation of *M. aurum* MO1 cells with thiomorpholine-S-oxide (5 mM). Time courses for the concentrations of sulfoxide (×) and thiodiglycolic acid (□).

The presence of a soluble cytochrome P450 was first shown by spectrophotometric assays: the CO-difference spectrum of cell extracts of *M. aurum* MO1 grown on liquid mineral salts medium amended with morpholine exhibited a typical peak at 449 nm. A similar spectrum was observed when *M. aurum* MO1 cells were induced by thiomorpholine but not when they were grown on mineral salts medium amended with acetate. Conclusively, *M. aurum* MO1 contains at least one soluble cytochrome P450 inducible by morpholine and thiomorpholine.

In order to extend studies on the key role played by this cytochrome P450 in the degradation of such xenobiotics, the degradative pathway of thiomorpholine, a sulfur analogue of morpholine, was studied. *M. aurum* MO1 was not able to grow on this compound but degraded it (Mazure & Truffaut 1994). At the same time, the production of a cytochrome P450 was induced. The accumulation of metabolites was then expected. The NMR spectra collected at different incubation times showed that this strain is able to completely transform thiomorpholine which disappears in 8 h, at a rate of about 1.25 mM/h. The ^{1}H NMR technique also allowed us to identify sulfoxide and thiodiglycolic acid as two intermediates of the biodegradative pathway (Figure 8).

The transformation of thiomorpholine into its corresponding sulfoxide, as well as the oxygen consumption associated with morpholine and thiomorpholine degradation (Mazure & Truffaut 1994), showed the presence of an activity due to a monooxygenase. However, if the cytochrome P450, put into evidence by the spectrophotometric assays, can be responsible for this oxidation reaction, many authors have shown in the literature that it could also be catalyzed by flavin-containing monooxygenases (Ziegler 1988). In order to check what kind of monooxygenase was involved in the degradation of thiomorpholine, the influence of a selective cytochrome P450 inhibitor, metyrapone, was studied. The inhibitory effects of metyrapone on the thiomorpholine degradation and on the sulfoxide formation proved the presence of an activity due to a cytochrome P450 in the first step of the biodegradative pathway (Figure 8). In the absence of inhibitory effects, the sulfoxide is in its turn transformed into thiodiglycolic acid which accumulates. The synthesis of this metabolite implies three types of

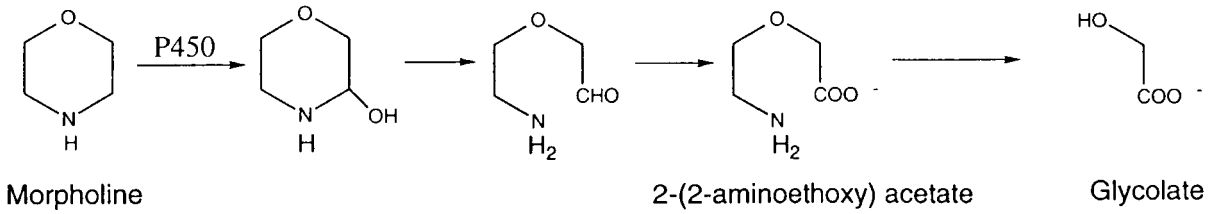

Figure 6. Biodegradative pathway of morpholine by *M. aurum* MO1.

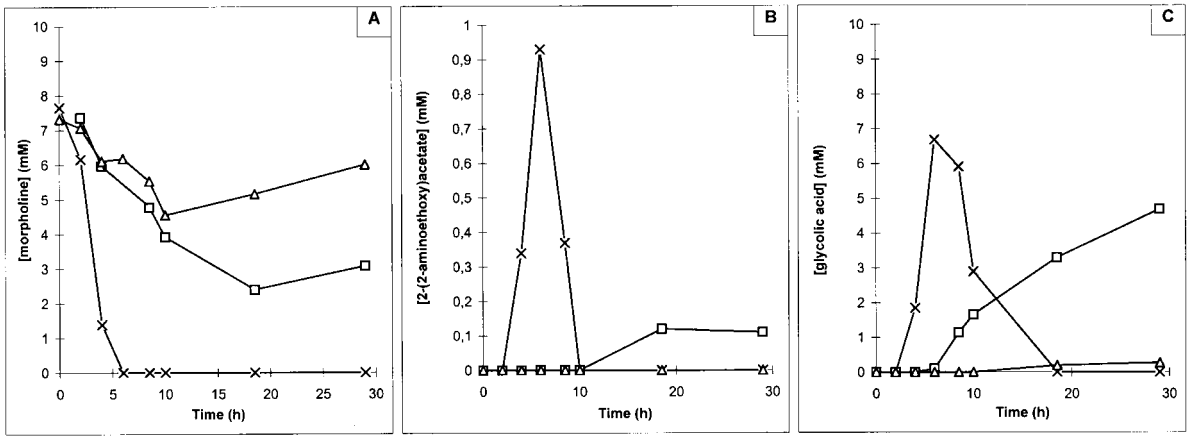

Figure 7. Incubation of *M. aurum* MO1 cells with morpholine (10 mM) in the presence of 5 mM (□) or 10 mM (Δ) metyrapone or in the absence of metyrapone (×). Time courses for the concentrations of morpholine (A), 2-(2-aminoethoxy)acetate (B) and glycolic acid (C). Integrals were measured on ^{1}H NMR spectra recorded at 300.13 MHz with a Avance 300 DSX spectrometer at 21°C with 5-mm-diameter tubes; water was suppressed by saturation with a classical NOE Bruker programm. 64 scans were collected (90° pulse 6.5μs, relaxation delay 5 s, acquisition time 4.561 s, 32 K data points).

reactions: opening of the ring, reduction of the corresponding sulfoxide and deamination. The first step corresponds to the cleavage of the C-N bond leading to 2-(2-aminoethylsulfinyl) acetate. The reduction of this compound could be performed by a sulfoxide reductase, this type of enzyme has been described in procaryotes as well as in eukaryotes cells (Madesclaire 1988). It is not clear wether this reduction takes place before or after deamination.

In order to confirm that the cytochrome P450 was also involved in the oxidative catabolism of morpholine in *M. aurum* strain MO1, metyrapone was added in the incubation medium containing morpholine. The same inhibitory effects were observed on the morpholine biodegradation. The decrease of the intermediates formation rates, in particular 2-(2-aminoethoxy) acetate, evidenced that a cytochrome P450 was also involved and that it took part in the early steps of the degradation. Our results suggest the opening of the ring in morpholine as well as in thiomorpholine. The cytochrome P450 catalyzes an hydroxylation of a C-atom adjacent to the amine group rather than N-oxidation, because protons at the α position are available (Guengerich 1990a). When the cells were incubated with thiomorpholine thiodiglycolate was detected, while glycolate accumulated in the presence of morpholine. However glycolate could result from the cleavage of the ether bond of diglycolate (White et al. 1996). In this case, glycolate would result from deamination of 2-(2-aminoethoxy)acetate and not from its cleavage in ethanolamine and glycolate. The biodegradative pathway of morpholine and thiomorpholine would be similar to that of pyrrolidine mentioned by Swain et al (1991) and would exclude the ethanolamine branch previously proposed for morpholine degradation (Swain et al. 1991). Note that for thiomorpholine the sulfur atom is primary oxidized as its higher reactivity is concerned.

In this work, we showed another example of the detoxification role played by cytochrome P450. The presence of such an enzyme in *Mycobacterium aurum* MO1 is not surprising as it was demonstrated in

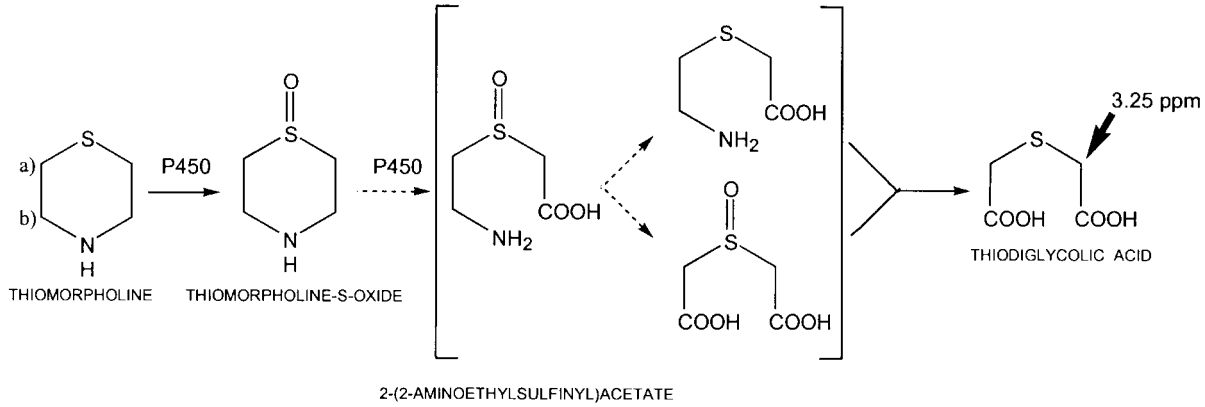

Figure 8. First steps of thiomorpholine biodegradative pathway in *M. aurum* MO1.

very close bacterial genera and involved in cholesterol metabolism of *Streptomyces* sp. (Horii et al. 1990), the sulphonylureas-inducible cytochrome P-450 from *Streptomyces grisoleus* (Omer et al. 1990) or the ethyldipropyl carbamothioate – inducible cytochrome P-450 from *Rhodococcus* sp. strain N186/21 (Nagy et al. 1995).

In conclusion, this work demonstrated the presence of a soluble cytochrome P450 in *M. aurum* MO1 and its key role in the degradation of morpholine and thiomorpholine, its sulfur analogue. The use of *in situ* ^{1}H NMR spectroscopy, performed directly on the incubation medium in H_2O, allowed us to identify unambiguously some of the intermediates formed during the degradation and to quantify them. This technique, both qualitative and quantitative, is easy to apply and could be used to investigate many other biodegradative processes.

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