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A new cytochrome P450 belonging to the 107L subfamily is responsible for the efficient hydroxylation of the drug terfenadine by *Streptomyces platensis*

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ABSTRACT

Fexofenadine, an antihistamine drug used in allergic rhinitis treatment, can be produced by oxidative biotransformation of terfenadine by *Streptomyces platensis*, which involves three consecutive oxidation reactions. We report here the purification and identification of the enzyme responsible for the first step, a cytochrome P450 (P450)-dependent monooxygenase. The corresponding P450, designated $P450_{terf}$, was found to catalyze the hydroxylation of the *t*-butyl group of terfenadine and exhibited UV–Vis characteristics of a P450. Its interaction with terfenadine led to a shift of its Soret peak from 418 to 390 nm, as expected for the formation of a P450–substrate complex. In combination with spinach ferredoxin:NADP(+) oxidoreductase and ferredoxin, and in the presence of NADPH, it catalyzed the hydroxylation of terfenadine and some of its analogues, such as terfenadone and ebastine, with k_m values at the μM level, and k_{cat} values around 30 min^{-1}. Sequencing of the $p450_{terf}$ gene led to a 1206 bp sequence, encoding for a 402 aminoacid polypeptide exhibiting 56–65% identity with the P450s from the 107L family. These results confirmed that P450s from *Streptomyces* species are interesting tools for the biotechnological production of secondary metabolites, such as antibiotics or antitumor compounds, and in the oxidative biotransformation of xenobiotics, such as drugs.

Introduction

The availability of drug metabolites is crucial for drug development and the current methods for producing these metabolites are often slow and expensive. They rely upon the use of liver microsomes or recombinant enzymes, in particular cytochromes P450[1] (P450s), that are half-life limited and costly, or upon chemical synthesis that may also be expensive. Many efforts have been made recently in the field of microbial transformations that produce metabolites of xenobiotics [1].

Fexofenadine, the pharmacologically active metabolite of terfenadine, is a H_1 receptor antagonist and a second-generation antihistamine drug prescribed in allergic inflammations [2]. In man, terfenadine undergoes extensive first-pass metabolism due to cytochrome P450-dependent enzymatic activities (Fig. 1). Two oxidation reactions are involved, *i.e.*, an oxidative *N*-dealkylation leading to azacyclonol, that is mainly catalyzed by CYP3A4, and a hydroxylation of the *t*-butyl group leading to hydroxyterfenadine, which is mainly catalyzed by CYP2J2 but also by CYP4F12, CYP3A4 and CYP2D6 [3–8]. Hydroxyterfenadine undergoes subsequent CYP2J2-dependent oxidation into the corresponding carboxylic acid, fexofenadine, the active metabolite. The prodrug terfenadine was superseded by fexofenadine several years ago, because of the cardiotoxicity of terfenadine at high doses [9]. However, despite structural similarities of these two compounds, the synthetic route used to prepare terfenadine was found to be poorly efficient for fexofenadine synthesis and gave very low yields (<10%) [10–12]. Moreover, oxidation of terfenadine by chemical methods mainly led to *N*-oxidation-derived products, such as azacyclonol, a molecule formerly used as an ataractive drug. Therefore, an efficient method of direct transformation of terfenadine into fexofenadine would be of particular interest for pharmaceutical industry.

We have previously demonstrated that *Streptomyces platensis* NRRL 2364 efficiently biotransforms terfenadine into fexofenadine [13,14]. This bioconversion is the result of three consecutive oxidation reactions: (i) a hydroxylation of a methyl group from the *t*-butyl moiety of terfenadine to give the primary alcohol, hydroxyterfenadine, (ii) an oxidation of the alcohol function into the corresponding aldehyde, and (iii) an oxidation of the aldehyde to the corresponding carboxylic acid, fexofenadine.

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[1] *Abbreviations used:* CYP or P450, cytochrome P450; Fd, ferredoxin; FdR, ferredoxin:NADP(+) oxidoreductase; HPLC-ESI-MS, high pressure liquid chromatography electrospray ionization mass spectrometry; UV–Vis, UV–Visible spectroscopy; PVDF, polyvinylidene difluoride; PMF, peptide mass fingerprint.

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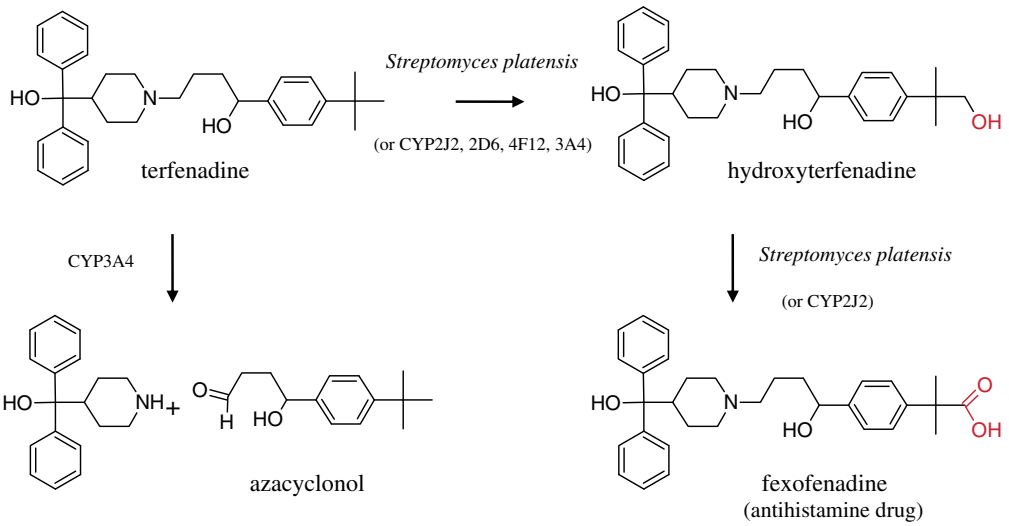

Fig. 1. Oxidation of terfenadine to fexofenadine by *Streptomyces platensis* and metabolism of terfenadine by human liver P450s.

In vivo terfenadine bioconversion studies with *S. platensis* whole cells have shown that: (i) the three successive oxidation reactions required molecular oxygen O_2, (ii) terfenadine and hydroxyterfenadine biotransformation under $^{18}O_2$-enriched atmosphere led to ^{18}O-labelled fexofenadine, (iii) addition of usual P450 inhibitors, such as clotrimazole and fluconazole, inhibited terfenadine oxidation, and (iv) addition of soybean peptones enhanced fexofenadine formation [15,16]. In that regard, it is noteworthy that genistein, an isoflavonoid component of soybean flours, was previously shown to induce the expression of a cytochrome P450, CYP105D1, also named $P450_{soy}$, in *Streptomyces griseus* [17,18]. The above results strongly suggested that the oxidation of terfenadine into fexofenadine should involve one or several P450-dependent monooxygenase(s).

To date, about 600 CYP genes from some 80 *Streptomyces* filamentous bacteria have been reported in all searchable databases [19]. Some of the corresponding P450s are involved in the biosynthesis of secondary metabolites, such as antibiotics, by the bacteria; however, for most of them, no enzymatic activity has been reported so far. Moreover, very few data are presently available on the ability of those P450s to act as biocatalysts for the oxidation of xenobiotics such as drugs, *e.g.*, the oxidation of 7-ethoxycoumarin, precocene II, benzo[a]pyrene and warfarin by CYP105D1 from *S. griseus* [18,20].

In an effort to find P450s from filamentous bacteria that would be new biotechnological tools for the oxidative bioconversion of xenobiotics, it was interesting to characterize the enzyme responsible for the efficient and regioselective oxidation of terfenadine by *S. platensis*. This article reports the isolation, purification and characterization of the P450, called $P450_{terf}$, that is responsible for this reaction. Determination of its amino acid sequence showed that it belongs to the 107L subfamily.

Materials and methods

Biochemicals and chemicals

Yeast extract, malt extract, glucose and agar were purchased from Difco (Detroit, Mich., USA). Soybean peptone was purchased from Organotechnie (La Courneuve, France). NADPH, cytochrome *c*, glucose 6-phosphate dehydrogenase, leupeptin, chicken egg lysozyme, terfenadine, spinach ferredoxin:NADP+ oxidoreductase and spinach ferredoxin were purchased from Sigma–Aldrich (St Quentin Fallavier, France). Deoxyribonuclease I (DNase I), aprotinin and pepstatin were purchased from Euromedex (Souffelweyersheim, France). Ebastine was provided by Pharmapharm (Paris, France). Terfenadone was synthesized as previously described [21]. Kod DNA polymerase from *Thermococcus kodakaraensis*, and detergent-based Bug Buster were from Novagen (Merck Chemicals Ltd., Nottingham, UK).

Bacterial strain and growth conditions

Stock cultures were maintained on 2% malt extract agar and stored at 4 °C. *S. platensis* cells (20 L) were aerobically grown at 30 °C, in YM (yeast extract 4 g/L, malt extract 10 g/L) or YMS medium (Yeast extract 4 g/L, Malt extract 10 g/L, Soybean peptone 5 g/L) in the presence of glucose (16 g/L), in a 25-L incubator (Biostat® C, B. Braun Biotech International, Melsungen, Germany), with vigorous stirring (600 rpm). After 48 h culture, cells were harvested and collected by continuous centrifugation (7000 rpm, 4 °C) and stored at −80 °C.

Cytosolic and membrane extracts preparation

Cells (400 g) were washed in 200 mL cold 50 mM Tris–HCl buffer, pH 7.6, containing 1 mM EDTA and 10% glycerol (TEG buffer), and then resuspended in 3% of the original culture volume in the same buffer containing chicken egg lysozyme (4 g) and DNase I (1 mg). After 2 h incubation at 30 °C, cells were disrupted by addition of detergent-based Bug Buster reagent (0.5×), in the presence of protease inhibitors (0.5 mg leupeptin, 1 mg aprotinin, and 7 mg pepstatin). All the following steps were performed at 4 °C. Cells were sonicated (10 × 10 s, amplitude 40%, Vibracell 75115, Fisher Bioblock Scientific, Illkirch, France) and centrifuged at 6000*g* for 30 min to remove unbroken cells and debris. The cloudy supernatant, which contained both cytosolic and membrane fractions, was then fractionated by centrifugation at 100,000*g* for 1 h (Beckman Ti50.2 rotor, Beckman ultracentrifuge). The supernatant containing soluble proteins was collected and used for further purification. The pellet containing membrane proteins was resuspended in TEG buffer. Protein concentration of both cytosolic and membrane fractions was estimated using the Bradford method [22].

Cytochrome P450 purification

Fractional precipitation of the proteins with ammonium sulfate was performed at 40% and 80% (w/v). The 80% ammonium sulfate