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Design of an *Escherichia coli* system for whole cell mediated steroid synthesis and molecular evolution of steroid hydroxylases

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Abstract

The 15β-hydroxylase (CYP106A2) from *Bacillus megaterium,* one of the few bacterial steroid hydroxylases, which has been isolated and characterized so far, catalyses the 15β-hydroxylation of a variety of steroids. The enzyme can be supported in its activity with adrenodoxin (Adx) and adrenodoxin reductase (AdR) from bovine adrenals, supplying this enzyme with the reducing equivalents necessary for steroid hydroxylation activity. This three-component electron transfer chain was implemented in *Escherichia coli* by coexpression of the corresponding coding sequences from two plasmids, containing different selection markers and compatible origins of replication. The cDNAs of AdR and Adx on the first plasmid were separated by a ribosome binding sequence, with the reductase preceding the ferredoxin. The second plasmid for CYP106A2 expression was constructed with all features necessary for a molecular evolution approach. The transformed bacteria show the inducible ability to efficiently convert 11-deoxycorticosterone (DOC) to 15β-DOC at an average rate of 1 mM/d in culture volumes of 300 ml. The steroid conversion system was downscaled to the microtiter plate format and a robot set-up was developed for a fluorescence-based conversion assay as well as a CO difference spectroscopy assay, which enables the screening for enzyme variants with higher activity and stability.

Keywords: Adrenodoxin; Adrenodoxin reductase; CYP106A2; Whole cell biocatalyst; Steroid synthesis

1. Introduction

The cytochrome P450 superfamily is composed of a large group of related enzymes responsible for the metabolism of a wide variety of hydrophobic compounds (Nelson et al., 1996). They are found ubiquitously and convert exogenous as well as endogenous compounds, such as various drugs, xenobiotics, fatty acids, bile acids or steroids. In mammals, steroid hormones are mainly being synthesized in the adrenal and sexual glands (Bernhardt, 2000; Miller and Tyrell, 1995). They play an important role as hormones

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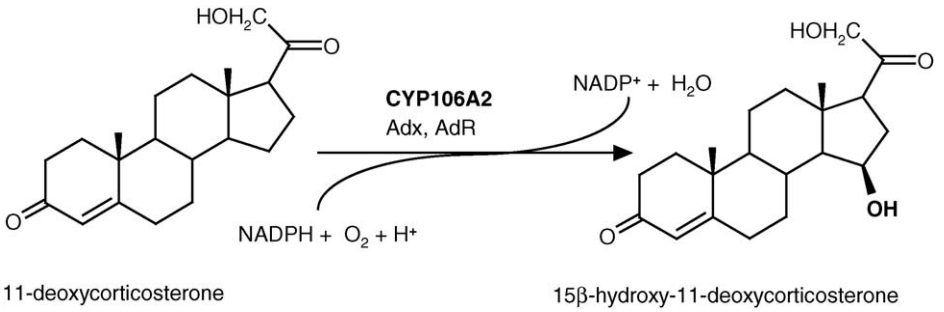

Fig. 1. Schematic drawing of the hydroxylation reaction in the 15β-position of the steroid 11-deoxycorticosterone (DOC) catalyzed by CYP106A2. Redox equivalents are transferred from NADPH via the proteins AdR and Adx to the steroid converting enzyme CYP106A2.

(glucocorticoids, mineralocorticoids and sex hormones) and, in addition, many steroids are interesting pharmaceutical target substances for the production of anti-inflammatory, diuretic, anabolic, contraceptive, antiandrogenic, progestational and antitumor drugs.

Besides the steroid converting systems of mammals, steroid converting enzymes are also found in plants and fungi and a few years ago steroid hormone biosynthesis has also been demonstrated to occur in bacteria (Megges et al., 1990). In the bacterial strain *Bacillus megaterium* ATCC 13368 cytochrome P450 CYP106A2 was identified as steroid hydroxylating enzyme and is so far one of the few bacterial enzymes with this specificity, which have been isolated and at least partially characterized (Berg et al., 1979a,b). CYP106A2 catalyzes as main reaction route the 15β-hydroxylation of several steroids, e.g. 11-deoxycorticosterone, testosterone, progesterone, and corticosterone (Fig. 1 and Table 1). Recently, CYP106A2 has been shown to synthesize besides 15β-OH-steroids the mono-hydroxylated products 9α- and 11α-OH-progesterone (Lisurek et al., 2004). Both functions have been reported to be of pharmaceutical relevance because 9α-OH-steroid derivatives exhibit glucocorticoidal and progestational activity (Masuda et al., 1987) and 11α-OH-steroid derivatives have been discussed to exhibit antiandrogenic activity with minimal estrogenic and progestational side effects (Tamm et al., 1982).

The biological function of CYP106A2 as well as the natural electron transfer partners in *B. megaterium* are unknown but the activity can be reconstituted with the redox partner proteins from bovine adrenals (Berg et al., 1979a,b). The bovine electron transfer system utilizes the ferredoxin adrenodoxin (Adx) to carry electrons from adrenodoxin reductase (AdR) to cytochrome P450scc (CYP11A1), which in bovine adrenals catalyzes the side-chain cleavage of cholesterol, the initial

Table 1
So far known steroid substrates of CYP106A2

Substrate	Product	Source
Testosterone	15β-Hydroxytestosterone	Berg et al. (1976)
4-Androstene-3,17-dione	15β-Hydroxyandrostenedione	Berg et al. (1976)
Progesterone	15β-Hydroxyprogesterone	Berg et al. (1976)
	6β-Hydroxyprogesterone	Berg et al. (1976)
	11α-Hydroxyprogesterone	Kang et al. (2004), Lisurek et al. (2004)
	9α-Hydroxyprogesterone	Kang et al. (2004), Lisurek et al. (2004)
20α-Dihydroprogesterone	15β-Hydroxy-20α-dihydroprogesterone	Berg et al. (1976)
17α-Hydoxyprogesterone	15β,17α-Dihydroxyprogesterone	Berg et al. (1976)
11-Deoxycorticosterone (DOC)	15β-Hydroxy-11-deoxycorticosterone	Berg et al. (1976)
Corticosterone	15β-Hydroxycorticosterone	Berg et al. (1976)
11-Deoxycortisol (RSS)	15β-Hydroxy-11-deoxycortisol	Virus et al. (in preparation)