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Coexpression of redox partners increases the hydrocortisone (cortisol) production efficiency in CYP11B1 expressing fission yeast *Schizosaccharomyces pombe*

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Abstract

Cytochromes P450 play a vital role in the steroid biosynthesis pathway of the adrenal gland. An example of an essential P450 cytochrome is the steroid 11β-hydroxylase CYP11B1, which catalyses the conversion of 11-deoxycorticol to hydrocortisone. However, despite its high biotechnological potential, this enzyme has so far been unsuccessfully employed in present-day biotechnology due to a poor expression yield and inherent protein instability. In this study, CYP11B1 was biotransformed into various strains of the yeast *Schizosaccharomyces pombe*, all of which also expressed the electron transfer proteins adrenodoxin and/or adrenodoxin reductase – central components of the mitochondrial P450 system – in order to maximise hydrocortisone production efficiency in our proposed model system. Site-directed mutagenesis of CYP11B1 at positions 52 and 78 was performed in order to evaluate the impact of altering the amino acids at these sites. It was found that the presence of an isoleucine at position 78 conferred the highest 11β-hydroxylation activity of CYP11B1. Coexpression of adrenodoxin and adrenodoxin reductase appeared to further increase the 11β-hydroxylase activity of the enzyme (3.4 fold). Adrenodoxin mutants which were found to significantly enhance enzyme efficiency in other cytochromes in previous studies were also tested in our system. It was found that, in this case, the wild type adrenodoxin was more efficient. The new fission yeast strain TH75 coexpressing the wild type Adx and AdR displays high hydrocortisone production efficiency at an average of 1 mM hydrocortisone over a period of 72 h, the highest value published to date for this biotransformation. Finally, our research shows that pTH2 is an ideal plasmid for the coexpression of the mitochondrial electron transfer counterparts, adrenodoxin and adrenodoxin reductase, in *Schizosaccharomyces pombe*, and so could serve as a convenient tool for future biotechnological applications.

Keywords: Adrenodoxin; Adrenodoxin reductase; Hydrocortisone; CYP11B1; Fission yeast; *Schizosaccharomyces pombe*; Steroid 11β-hydroxylation

1. Introduction

P450 cytochromes are instrumental to the bioconversion of a variety of different substrates relevant to biotechnical applications. Nevertheless, P450 cytochromes have enjoyed very limited biotechnological use due to the complexity, instability and, low catalytic activity often displayed by this enzyme family. Still, some examples of successful applications of P450 cytochromes in industrial processes have been recently summarized (Bernhardt, 2006). Whole cell systems are now used in preference to partially isolated enzymes for biotechnological processes (Straathof et al., 2002). In this way some of the problems encountered during the protein isolation, e.g. co-factor regeneration, can easily be circumvented using the cell machinery.

Cytochrome P450 enzymes generally perform oxidation reactions on their organic substrates while reducing and splitting molecular oxygen. The electrons needed for the reaction are provided via a short electron transfer chain that consists of one or two additional proteins. In case of the mitochondrial P450 systems, reduction equivalents are transferred from NADPH or an adrenodoxin reductase (AdR; a NADPH dependent flavoprotein) and via adrenodoxin (Adx; a [2Fe-2S] cluster ferredoxin) to the P450 cytochrome which carries out the actual hydroxylation reaction (Fig. 1). A prominent example of a mitochondrial P450 is human CYP11B1 (steroid 11β-hydroxylase), which is expressed in the zona fasciculata/reticularis of the adrenal and converts 11-deoxycorticol (RSS) to hydrocortisone (F), the most

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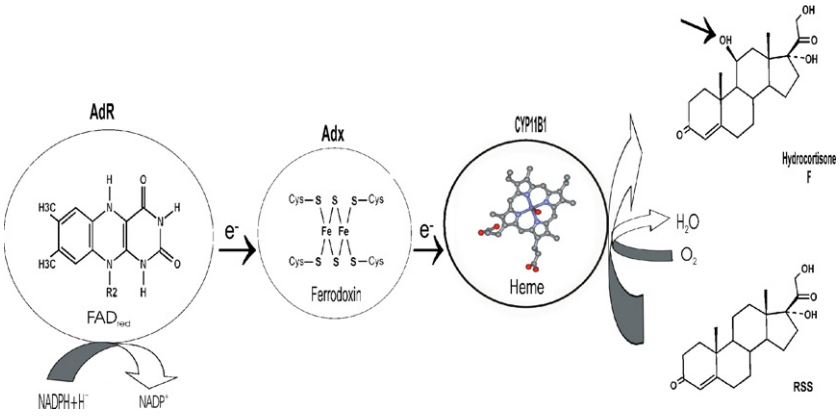

Fig. 1. The mitochondrial steroid hydroxylase CYP11B1 and its electron transfer system.

important glucocorticoid in humans (Bureik et al., 2002a; Hakki and Bernhardt, 2006). Glucocorticoids are used as important anti-inflammatory agents and generally require an 11β-hydroxy group as a functionally essential entity. During the industrial synthesis of these compounds, the microbiological introduction of the 11β-hydroxy group into the steroid scaffold not only represents the most costly synthesis step, but also the step whereby most of the losses occur due to the formation of by-products.

Unfortunately, functional expression of human CYP11B1 in bacteria has thus far not been successfully carried out. While expression of rat CYP11B1 in *E. coli* has occurred (Nonaka et al., 1998), no results have yet been published that demonstrate its biotechnological potential due to a very low expression yield and the instability of the protein. Furthermore, although expression of human CYP11B1 in cell culture was previously used to test potential inhibitors (Denner et al., 1995) and to investigate mutations associated with diseases such as congenital adrenal hyperplasia (CAH) (Hampf et al., 2001; Krone et al., 2006), this system is not suited to biotransformation at a technical scale.

In recent years, we have demonstrated that the fission yeast *Schizosaccharomyces pombe* is a very suitable model system for the investigation of P450 dependent steroid hydroxylases (Bureik et al., 2002a; Bureik et al., 2002b), and during these investigations the construction of recombinant fission yeast strains which functionally express human CYP11B1 was reported. In these strains (named CAD1 (Bureik et al., 2004) and SZ1 (Dragan et al., 2005), respectively), 11β-hydroxylation of RSS was accomplished without the need for coexpression of the other components of the CYP11B1 electron transfer chain (Adx and AdR).

While the hydrocortisone production efficiency using strain SZ1 (Dragan et al., 2005) is considerably higher than the values reported for production by other steroid 11β-hydroxylation systems with recombinant microorganisms (e.g. those from bovine CYP11B1 expressed in bakers yeast *Saccharomyces cerevisiae* (Dumas et al., 1996)), all bioconversion activities published to date appear to still be not competitive enough for the consideration of their use for industrial applications. Therefore, the purpose of our study was to further improve the efficiency of hydrocortisone bioproduction at the laboratory level. To accomplish this goal, we investigated the biotransformation activity in strains which, in addition to CYP11B1, coexpress the corresponding electron transfer proteins Adx and AdR.

2. Materials and methods

2.1. Media and general techniques

Media and genetic methods for studying fission yeast have been described in detail (Moreno et al., 1991; Alfa et al., 1993). Cells were cultivated at 30 °C in Edinburgh minimal media (EMM) with supplements as required (see Table 1); cell densities were determined using a haemocytometer. General DNA manipulation methods were performed using standard techniques (Sambrook et al., 1989). Disruption of the *ura4*

Table 1
Fission yeast strains created in this study

Name	Parental strain	Expression construct(s)	Expressed protein(s)	Required supplement(s)
SZ52	NCYC 2036	pCAD1-hCYP11B1^{L52V78}	hCYP11B1^{L52V78}	Leucine
SZ78	NCYC 2036	pCAD1-hCYP11B1^{M52I78}	hCYP11B1^{M52I78}	Leucine
TH1	SZ1	pAT539	hCYP11B1^{L52I78}	Leucine, uracil
TH2	TH1	pNMT1-Adx^{WT}	hCYP11B1^{L52I78} + Adx^{WT}	Uracil
TH3	TH1	pTH1	hCYP11B1^{L52I78} + AdR^{WT}	Leucine
TH4	TH1	pNMT1-Adx^{WT} + pTH1	hCYP11B1^{L52I78} + Adx^{WT} + AdR^{WT}	None
TH6	TH1	pNMT1-Adx^{S112W} + pTH1	hCYP11B1^{L52I78} + Adx^{S112W} + AdR^{WT}	None
TH7	TH1	pNMT1-Adx^{D113Y} + pTH1	hCYP11B1^{L52I78} + Adx^{D113Y} + AdR^{WT}	None
TH75	SZ1	pTH2	hCYP11B1^{L52I78} + Adx^{WT} + AdR^{WT}	None
TH175	1445	pTH2	Adx^{WT} + AdR^{WT}	Adenine, uracil, histidine