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A Novel NADPH-dependent flavoprotein reductase from *Bacillus megaterium* acts as an efficient cytochrome P450 reductase

Mohammed Milhim, Adrian Gerber, Jens Neunzig, Frank Hannemann, Rita Bernhardt*

Institute of Biochemistry, Saarland University, 66123 Saarbrücken, Germany

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ABSTRACT

Cytochromes P450 (P450s) require electron transfer partners to catalyze substrate conversions. With regard to biotechnological approaches, the elucidation of novel electron transfer proteins is of special interest, as they can influence the enzymatic activity and specificity of the P450s. In the current work we present the identification and characterization of a novel soluble NADPH-dependent diflavin reductase from *Bacillus megaterium* with activity towards a bacterial (CYP106A1) and a microsomal (CYP21A2) P450 and, therefore, we referred to it as *B. megaterium* cytochrome P450 **r**eductase (BmCPR). Sequence analysis of the protein revealed besides the conserved FMN-, FAD- and NADPH-binding motifs, the presence of negatively charged cluster, which is thought to represent the interaction domain with P450s and/or cytochrome *c*. BmCPR was expressed and purified to homogeneity in *Escherichia coli*. The purified BmCPR exhibited a characteristic diflavin reductase spectrum, and showed a cytochrome c reducing activity. Furthermore, in an *in vitro* reconstituted system, the BmCPR was able to support the hydroxylation of testosterone and progesterone with CYP106A1 and CYP21A2, respectively. Moreover, in view of the biotechnological application, the BmCPR is very promising, as it could be successfully utilized to establish CYP106A1- and CYP21A2-based whole-cell biotransformation systems, which yielded 0.3 g/L hydroxy-testosterone products within 8 h and 0.16 g/L 21-hydroxyprogesterone within 6 h, respectively. In conclusion, the BmCPR reported herein owns a great potential for further applications and studies and should be taken into consideration for bacterial and/or microsomal CYP-dependent bioconversions.

1. Introduction

Bacillus megaterium is a nonpathogenic Gram-positive rod-shaped bacterium, which was described first in 1884. The complete genome sequence was published in 2011 (Eppinger et al., 2011). It is found in diverse ecological habitats and is able to grow on a wide variety of carbon sources, which allows its cultivation on low-cost simple media. It is one of the biggest known bacteria with a vegetative cell size of 4 μm in length and 1.5 μm in diameter, which is about 100 times larger than *Escherichia coli* (Bunk et al., 2010). *B. megaterium* does not produce endotoxins associated with the outer membrane, nor does it have alkaline proteases, which improves the recovery of recombinant proteins (Vary, 1994). In the last decades *B. megaterium* has been used industrially for the production of several substances and enzymes such as vitamin B_{12} (Raux et al., 1998), oxetanocin, a viral inhibitor of HIV, hepatitis B virus and herpes virus (Morita et al., 1999), α- and β-amylase (Hebeda et al., 1988; Takasaki, 1989), pencillin amidase (Martín et al., 1995), glucose dehydrogenase (Nagao et al., 1992) and HIV antigen (Shivakumar et al., 1998). Some of the most interesting proteins expressed naturally by *B. megaterium* are the cytochromes P450, which have been shown to be very important in many biotechnological and pharmaceutical applications (Brill et al., 2013; Kiss et al., 2015).

Cytochromes P450 (P450s) are heme-thiolate monooxygenases, which catalyze diverse reactions in a regio- and stereoselective manner and are involved in – for example but not limited to – the biosynthesis of hormones, signal molecules, defense-related chemicals and secondary metabolites in addition to their central role in the metabolism of endogenous (steroids and fatty acids) and exogenous (drugs and toxins) substances (Bernhardt, 2006). P450s catalyze a wide range of reactions including hydroxylations, epoxidations, deaminations, desulfurations, dehalogenations, *N*-, *S*-, and *O*-dealkylations, *N*-oxidations, peroxidations and sulfoxidations (Bernhardt and Urlacher, 2014). They are found in all kingdoms of life including mammals, plants, insects, fungi, bacteria and also in viruses (Nelson et al., 1996). P450s require auxiliary redox partners for the activation of the molecular oxygen. These redox partners transfer two electrons in two single steps from the cofactor NAD(P)H to the heme of the P450. Depending on com-

* Corresponding author at: Institute of Biochemistry, Campus B2.2, Saarland University, D-66123 Saarbrücken, Germany.
E-mail address: ritabern@mx.uni-saarland.de (R. Bernhardt).

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ponents of the electron transfer chains, P450s are classified into different classes, of which the most investigated are: class I, which comprises bacterial and mitochondrial P450s, and class II, which contains the microsomal P450s. Mitochondrial P450s are associated with the inner mitochondrial membrane and rely on a likewise membrane associated NADPH-oxidizing ferredoxin reductase for electron supply. The electrons are shuttled to the P450 *via* a soluble ferredoxin. In bacterial class I systems, all three components are soluble. Microsomal P450s are embedded into the membrane of the endoplasmic reticulum and receive electrons through a cytochrome P450 reductase (CPR) (Hannemann et al., 2007). This diflavin reductase from the FNR-like superfamily contains a flavin adenine dinucleotide (FAD) and a flavin mononucleotide (FMN) as prosthetic groups, allowing both the oxidation of NADPH and the shuttling of electrons to the P450 (Munataliev et al., 2004).

The CPR is N-terminally anchored to the membrane of the endoplasmic reticulum by a hydrophobic N-terminus, comprising about 55 amino acids (Pandey and Flück, 2013). So far, there has been only one study reporting the identification of a bacterial class II electron transport system, which involves a P450 and a soluble CPR, both from *Streptomyces carbophilus* (Serizawa and Matsuoka, 1991). In addition, the flavin domain of the self-sufficient CYP102A1 (BM-3) has been utilized to create an artificial, soluble CPR which provides electrons to the microsomal P450s CYP3A4 and CYP2B4 (Davydov et al., 2010; Sadeghi and Gilardi, 2013).

In this paper, we describe a di-flavin electron transfer system: a natural, soluble flavoprotein reductase from *B. megaterium* DSM319, which supports the electron transfer from NADPH to the class I bacterial P450 (CYP106A1) as well as to the eukaryotic, microsomal class II P450 (CYP21A2) with a high efficiency. The properties of the protein are analyzed on a bioinformatic and biochemical level. In addition, we show that the characterized reductase can be efficiently used in an *E. coli* whole-cell based biocatalyst system with both cytochromes P450.

2. Materials and methods

2.1. Strains, expression vectors, enzymes, and chemicals

E. coli TOP10 from Invitrogen (Karlsruhe, Germany) was used for cloning experiments. *E. coli* C43 (DE3) and the expression vector pET17b, both from Novagen (Darmstadt, Germany), were used for recombinant gene expression. 1,2-Dilauroyl-*sn*-glycero-3-phosphocholine (DLPC), kanamycin sulfate, arabinose, HPLC-grade acetonitrile and steroids were from Sigma–Aldrich (Schnelldorf, Germany). delta-Aminolevulinic acid (δ-ALA) and isopropyl-β-D-thiogalactopyranoside (IPTG) were obtained from Carbolution Chemicals GmbH (Saarbrücken, Germany). NADPH was from Gerbu (Wieblingen, Germany). Glucose-6-phosphate and glucose-6-phosphate dehydrogenase (G6P-DH) were purchased from Roche (Mannheim, Germany).

2.2. Cloning of the gene encoding BmCPR *from* B. megaterium, *bovine* CPR *and bovine* CYP21A2

For purification purposes, the DNA fragment encoding the full length BmCPR was PCR amplified from the genomic DNA of *B. megaterium* strain DSM319 using the forward primer (CGCGCCATATG CAACTTAAGGTAGTAAACAGCC) and reverse primer (TATATGGTACCTCA*GTGGTGGTGGTGGTGGTG ACCACC-ACC* GTATACATCACGCTGATAACG) and cloned in the expression vector pET17b with the *Nde*I/*Kpn*I restriction sites. The start codon was changed from TTG to ATG, to enhance *E. coli* expression (Supplementary material Fig. S1). For purification with Immobilized Metal Ion Affinity Chromatography (IMAC), the 3′end of the gene was extended with a sequence coding for three glycines and six histidines. Bovine CPR was cloned in pET17b as mentioned elsewhere (Neunzig et al., 2014). The cDNA of the bovine *CYP21A2* was cloned *via* the restriction sites *Nde*I and *Bam*HI into the pET17b vector. To facilitate the expression of the bovine CYP21A2, the sequence coding for the membrane anchor (from amino acids 1–29) of this P450 was replaced with a sequence that codes for MAKKTSSKGK as obtained from CYP2C3 (Arase et al., 2006) and the C-terminus was extended with six histidines for purification purposes. All plasmids were verified by sequencing.

2.3. Heterologous gene expression and purification

For heterologous gene expression, pET17b vector derivatives harboring the corresponding genes were transformed into *E. coli* C43 (DE3). For BmCPR, cultures were grown at 37 °C to an optical density of 0.6 in 400 ml TB medium containing the suitable antibiotic. The expression of the protein was induced by adding 1 mM IPTG, and the cells were grown at 30 °C and 120 rpm for 24 h. BmCPR was purified using the following procedure. Cell pellets were sonicated in 50 ml buffer A (50 mM potassium phosphate (pH 7.4), 20% glycerol, 0.1 mM DTE, 0.1 mM EDTA, 500 mM sodium acetate, 1.5% Tween 20, and 0.1 mM PMSF). After centrifugation at 30,000*g* for 30 min at 4 °C, the supernatant was applied on a Ni–NTA agarose column equilibrated with buffer A. The column was washed with 100 ml buffer A supplemented with 40 mM imidazole followed by 20 ml elution buffer (buffer A supplemented with 200 mM imidazole). The eluted protein was dialyzed against buffer A using a SERVAPOR dialysis tubing MWCO 12,000–14,000 (Serva Feinbiochemica, Heidelberg, Germany). The purified protein was concentrated using the centrifugal device Amicon Ultra-4 MWCO 50 kDa (Merck Millipore, Darmstadt, Germany) before being stored at −80 °C.

Bovine CPR was expressed and purified as mentioned previously (Neunzig et al., 2014). Bovine CYP21A2 was co-expressed with pGro12 encoding the chaperones GroEL/GroES. Cell pellets were diluted in lysis buffer, consisting of 50 mM potassium phosphate buffer (pH 7.4), 500 mM sodium acetate, 0.1 mM EDTA, 20% glycerol, 1.5% sodium cholate, 1.5% Tween20, 0.1 mM PMSF and 0.1 mM DTE. Cells were disrupted by sonification and centrifuged with 30,000*g* for 30 min at 4 °C. The supernatant was taken for the subsequent purification with IMAC as mentioned elsewhere (Arase et al., 2006).

Recombinant bovine AdR, Adx_{wt} and the $Adx_{4–108}$ (truncated form of Adx comprising amino acids 4–108) were purified as reported previously (Sagara et al., 1993; Uhlmann et al., 1992). The preparation of the fission yeast protein Arh1 was carried out as reported previously (Bureik et al., 2002; Ewen et al., 2008). Fdx2 was purified as mentioned elsewhere (Brill et al., 2013).

The concentration of recombinant P450 was estimated using the CO-difference spectral assay as described previously with $\varepsilon_{450–490} = 91\ mM^{-1}\ cm^{-1}$ (Omura and Sato, 1964). The concentrations of BmCPR and CPR were quantified by measuring the flavin absorbance at 456 nm with $\varepsilon_{456} = 21\ mM^{-1}\ cm^{-1}$ for the oxidized enzymes (Lee et al., 2014; Vermilion and Coon, 1978). The concentration of the AdR and Arh1 was measured using the extension coefficient $\varepsilon_{450} = 11.3\ mM^{-1}\ cm^{-1}$ (Ewen et al., 2008; Hiwatashi et al., 1976). The concentration of Fdx2 was measured using the extension coefficient $\varepsilon_{390} = 6.671\ mM^{-1}\ cm^{-1}$ (Brill et al., 2013).

2.4. Cytochrome c assay

Different concentrations of cytochrome *c* (1–150 μM) were mixed with 50 pmol reductase (BmCPR) in 50 mM potassium phosphate buffer pH 7.4 in a total volume of 1 ml. The reaction was started by addition of 100 μM NADPH, and the reduction