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Functional expression of mammalian NADPH–cytochrome P450 oxidoreductase on the cell surface of *Escherichia coli*

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Abstract

To develop a whole-cell oxidoreductase system without the practical limitation of substrate/product transport, easy preparation, stability of enzymes, and low expression levels, we here report the development of a whole cell biocatalyst displaying rat NADPH–cytochrome P450 oxidoreductase (CPR, 77-kDa) on the surface of *Escherichia coli* by using ice-nucleation protein from *Pseudomonas syringae*. Surface localization and functionality of the CPR were verified by flow cytometry, electron microscopy, and measurements of enzyme activities. The results of this study comprise the first report of microbial cell-surface display of diflavin-containing mammalian enzymes. This system will allow us to select and develop oxidoreductases, containing bulky and complex prosthetic groups of FAD and FMN, into practically useful whole-cell biocatalysts for broad biological and biotechnological applications including the selective synthesis of new chemicals and pharmaceuticals, bioconversion, bioremediation, and bio-chip development.

Keywords: Diflavin-containing mammalian enzyme; Oxidoreductase; Reduction; Surface display; Whole-cell biocatalyst

Enzymes have found numerous bioprocess applications,and the use of enzymes in biocatalysis is expected to grow significantly [1,2]. Especially, the whole-cell biocatalysts, which have several advantages over purified enzymes, have been widely applied for industrial synthetic processes [1,2]. Although the redox enzyme is one of the most prominent categories among biocatalysts, diflavin-containing oxidoreductase has rarely been developed as an industrial enzyme. Mammalian NADPH–cytochrome P450 oxidoreductase (CPR; NADPH–hemoprotein reductase, EC 1.6.2.4) is a microsomal dual flavin redox protein [3]. The CPR is one of the only four mammalian enzymes known to contain both FAD and FMN in a single polypeptide chain [4,5]. The isoforms of nitric oxide synthase, methionine synthase reductase, and protein NR1 are the only remaining members of the family. Bacterial P450 BM3 and sulfite reductase also contain both FAD and FMN in a single polypeptide chain [4,5]. The main function of the CPR is the transfer of electrons from NADPH, via FAD and FMN cofactors, to cytochrome P450s (P450s; unspecific monooxygenase, EC 1.14.14.1) and heme oxygenase (EC 1.14.99.3). It also plays an important role in the metabolism of a wide range of chemicals and drugs, such as flunitrazepam, doxorubicin, and mitomycin C [6–12]. Recently, clinical significance of the CPR has been noticed. It was found that CPR-deficient individuals have a broad range of disorders, from infants with congenital malformations to women with the polycystic ovary syndrome [13–15].

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The display of heterologous proteins on the surface of bacteria [16,17], viruses [18], and yeast [19], can be used in a variety of biotechnological applications. Most cell-surface display systems are limited in the size of foreign proteins that can be expressed. Proteins smaller than 60 kDa have been displayed on bacterial cell surfaces [17]. A display of bovine adrenodoxin on the surface of *Escherichia coli* has recently been reported [20,21]. Dimeric adrenodoxin molecules, which form spontaneously on the bacterial surface, could be activated to show adrenodoxin-dependent steroid conversion strictly through chemical incorporation of [2Fe–2S]. However, the display of bulky, complex prosthetic groups, such as FAD and FMN, on the bacterial surface has not yet been reported.

To develop a whole-cell oxidoreductase system without the practical limitation of substrate/product transport, easy preparation, stability of enzymes, and low expression levels, we here report the development of a whole cell biocatalyst displaying rat CPR (77-kDa) on the surface of *E. coli* by using ice-nucleation protein (Inp)[1] from *Pseudomonas syringae*. Surface localization and functionality of the CPR were verified by flow cytometry, electron microscopy, and measurements of enzyme activities. The results of this study comprise the first report of microbial cell-surface display of diflavin-containing mammalian enzymes. This system will allow us to select and develop oxidoreductases, containing bulky and complex prosthetic groups of FAD and FMN, into practically useful whole-cell biocatalysts for broad biotechnological applications including the selective synthesis of new chemicals and pharmaceuticals, bioconversion, bioremediation, and bio-chip development.

Materials and methods

Bacterial strains and plasmids

Escherichia coli DH5α F^- ϕ80d*lacZ*ΔM15 Δ(*lac*ZYA-argF) U169 *deoR recA*1 *endA*1 *hsdR*17 *phoA supE*44 λ^- *thi-1 gyrA*96 *relA*1 and JM109*recA1 endA*1 *gyrA*96*thi-1 hsdR* 17 *supE*44 *relA*1 Δ(*lac-proAB*)/ F′[*traD*36 *proAB*$^+$*lac* I^q*lacZ*ΔM15] were used as the recombinant and the expression host, respectively. The original plasmid, pSD, which contains N-terminal and C-terminal regions of the whole Inp gene (*inaK*, Genbank Accession No. AF013159) without central repeating domain (CRD), was used to construct a surface expression vector [22,23]. The pSD, referred to as the null vector, was used as a control for all of our experiments. DNA sequence encoding the rat CPR was amplified from the pIN4OR plasmid, which was kindly provided by Prof. Todd D. Porter (University of Kentucky, USA).

Oligonucleotides were obtained from Genotech (Daejeon, Korea). Restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs (Beverly, MA, USA).

[1] *Abbreviations used:* Inp, ice-nucleation protein; CRD, central repeating domain; IPTG, isopropyl β-D-thiogalactopyranoside.

Construction of the expression vectors

For construction of gene fusion between InpNC (consisting of only N-terminal and C-terminal domains of Inp) and rat CPR, PCR amplification of the rat CPR gene fragment was performed using pIN4OR as a template and two primers, with 5′-AAG**CCCGGG**ATGGGGGACTCTCA CGAAG-3′ and 5′-TGC**AAGCTT**CTAGCTCCACACA TCTAGTGAG-3′. To generate prCPR, the amplified 2.0 kb rat CPR gene was digested with *Xma*I and *Nhe*I and inserted into pSD digested with the same enzymes. Finally, the amplified 2.0 kb rat CPR gene was digested with *Nhe*I and *Hin*dIII and inserted into prCPR, digested with the same enzymes, which generated pSDCPR (Fig. 1). pSD-CPR was used for the expression of CPR on the cell surface throughout this work. pSDCPR directs the expression of fusion proteins which consist the N- and C-terminal regions of Inp open reading frame and target gene (rat CPR) with the initiator, methionine. Expression of the plasmids was under the control of *tac* promoter.

To generate pCWCPR for expressing CPR in the cytoplasm, the amplified 2.0 kb rat CPR gene was digested with *Nde*I and *Hin*dIII and inserted into pCW1A2 digested with the same enzymes [24].PCR amplification of the rat CPR gene fragment was performed using pIN4OR as a template and two primers, with 5′-ACG**CATATG**GCTGACTCTC ACGAAG-3′ and 5′-TGC**AAGCTT**CTAGCTCCACAC ATCTAGTGAG-3′.

The JM109 cells, which express CPR in the cytoplasm (pCWCPR), were used as negative controls, in addition to the JM109 cells containing the null vector pSD, throughout the experiments for activity assays and flow cytometric analysis.

Culture conditions

The expression plasmids and the null vector were used to transform *E. coli* JM109 by electroporation, as described previously [25]. Single colonies of *E. coli* JM109 containing pSDCPR were streaked on LB/ampicillin (100 μg/ml) cultures. To express the fusion proteins, starter cultures were incubated overnight at 25 °C with shaking at 180 rpm, and then diluted 1:100 into LB/ampicillin (100 μg/ml) medium containing additives (1 mM thiamine and 25 μl/ml trace elements). Expression was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) when the culture reached A_{600} of 0.4. The *E. coli* cells harboring each expression plasmid were grown overnight with shaking at 180 rpm at 25 °C, and the expression of hybrid proteins was induced with IPTG. Induced cells were viable during prolonged incubation of over 24 h in the stationary phase.

SDS–PAGE and Western blotting

After the *E. coli* cells expressing the InpNC or InpNC-CPR fusion protein were harvested, fractions which contain proteins from the cytoplasm (with periplasm), inner membranes, and outer membranes, respectively, were