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ORIGINAL PAPER

Increasing the catalytic performance of a whole cell biocatalyst harboring a cytochrome P450cam system by stabilization of an electron transfer component

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Abstract Catalytic activity of a recombinant *Escherichia coli* whole cell biocatalyst harboring a cytochrome P450cam monooxygenase system from *Pseudomonas putida* coupled with enzymatic co-factor regeneration was investigated. About 0.7 μmol camphor was hydroxylated per mg dry cells at 4°C in 50 mM Tris/HCl buffer (pH 7.4) when utilizing a stable putidaredoxin (Pdx) mutant, C73S/C85S-Pdx (Cys73Ser, Cys85Ser double mutant), instead of wild-type Pdx, which was about two-fold improvement in the substrate conversion. Ten-micromole camphor was completely hydroxylated at 20°C in 6 h by 15 mg dry cell weight of whole cell biocatalyst including C73S/C85S-Pdx. Thus, modulation of protein-protein interaction in multicomponent enzymatic catalysis in whole cells is important.

Keywords Cytochrome P450 · Electron transfer protein · NADH regeneration · Whole cell biocatalyst

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Introduction

Biocatalysts, such as enzymes or microorganisms, are widely used in industry. They are environmentally friendly alternatives for conventional chemical catalysts in organic synthesis (Schmid et al. 2001). In particular, oxidoreductases, which include oxygenases and dehydrogenases, are potentially useful for industrial applications because they can catalyze selective oxidoreductions to unactivated hydrocarbons under mild conditions, which is important for green chemistry (Hummel 1999). The cytochrome P450 superfamily enzymes are oxygenases that catalyze the addition of O_2 to non-activated hydrocarbons at physiological temperature; a reaction that requires high temperatures to proceed in the absence of a catalyst (Schlichting et al. 2000). P450 requires electron-donating cofactors such as NADH or NADPH and an electron transfer system between redox pairs. When P450s are utilized as isolated and purified enzymes, the high cost of such cofactors and the difficulty in reconstituting an efficient electron transfer system limit the practical application of P450 catalysts.

In terms of regeneration of cofactors, whole cell systems are often favored because the construction of a cofactor regeneration system is generally easier and less expensive in cells than in vitro (Schmid et al. 2001). In fact, recent

advances in DNA technology have enabled the expression of various proteins in *Escherichia coli* as a host organism, and even of multi-component enzymes by co-expression of all protein components in a single strain (Schneider et al. 1999; Schmid et al. 2001; Wilms et al. 2001; Cherry and Fidantsef 2003; Ernst et al. 2005). Considering the utilization of recombinant *E. coli* as whole cell biocatalysts for P450 conversion, co-expression of all protein components required for target biotransformations in a cell should be the most practical and efficient.

The cytochrome P450cam monooxygenase (P450cam) system from the soil bacterium, *Pseudomonas putida*, requires NADH and two proteins, putidaredoxin reductase (PdR) and putidaredoxin (Pdx), as cofactors to activate O_2 for the regio- and stereo-specific hydroxylation of (1*R*)(+)-camphor to 5-exo-hydroxycamphor (Gunsalus and Wagner 1978; Poulos et al. 1987; Nelson et al. 1993). Our previous report described a novel approach to setting up a recombinant oxidation/reduction cycle in *E. coli* for a native P450cam system coupling with glycerol dehydrogenase (GLD) to regenerate NADH (Mouri et al. 2006). GLD efficiently regenerates NADH from the intracellular NAD^+ pool by oxidizing glycerol, whereby camphor hydroxylation was significantly promoted by an NADH-dependent P450cam monooxygenase system. However, catalytic activity reached a plateau after several hours even in the presence of excess glycerol, implying inactivation of a protein component. Since inactivation of one of protein component is critical in the multicomponent system, we focused on an electron transfer component, Pdx, that plays an important role in the P450cam monooxygenase system and is known to be an unstable protein. Sevrioukova et al. (2003) reported the mutation of non-ligating cysteine residues, Cys73 and Cys85, to Ser for elevation of stability of the [2Fe–2S]-containing putidaredoxin. Here we report on how the double mutant, C73S/C85S-Pdx, affects the catalytic performance of an *E. coli* whole cell biocatalyst harboring a P450cam system.

Materials and methods

Materials

(1*R*)-(+)-Camphor, ampicillin and chloramphenicol were obtained from Wako Pure Chemical (Osaka, Japan). δ-Aminolevulinic acid was purchased from Cosmo Bio (Tokyo, Japan).

Preparation of plasmid vectors for the co-expression of four protein components

A typical procedure for the construction of plasmid vectors employed in this study was as follows. The genes encoding P450cam, Pdx, PdR and GLD were obtained as reported elsewhere (Ichinose et al. 2004, 2005). The plasmids, pETDuet-P450cam/Pdx and pACYCDuet-PdR/GLD, were employed for the construction of recombinant *E. coli* harboring P450cam, Pdx, PdR and GLD (Mouri et al. 2006). A gene fragment encoding C73S/C85S-Pdx was amplified by PCR using pHSG-Pdx-C73S/C85S kindly provided by Prof. Nagamune of the Univ. of Tokyo. The amplified gene fragment was digested with NdeI/XhoI and inserted into the same restriction sites of pETDuet-P450cam, resulting in the pETDuet-P450cam/Pdx(C73S/C85S). The protein-coexpression vectors were transformed into *E. coli* strain BL21(DE3) (Novagen, USA). The transformant for the native P450cam system plus GLD was abbreviated as BL21(P450cam/Pdx/PdR/GLD) (Mouri et al. 2006). The same *E. coli* strain transformed with both pETDuet-P450cam/Pdx(C73S/C85S) and pACYCDuet-PdR/GLD plasmid vectors, in which wild-type Pdx was replaced with C73S/C85S-Pdx, was abbreviated as BL21(P450cam/Pdx(C73S/C85S)/PdR/GLD). A schematic illustration of gene mapping of the plasmids is shown in Fig. 1.

Preparation of the resting recombinant *E. coli* cells

Two different transformants, BL21(P450cam/Pdx/PdR/GLD) and BL21(P450cam/Pdx(C73S/C85S)/PdR/GLD), were grown in 1 l LB medium

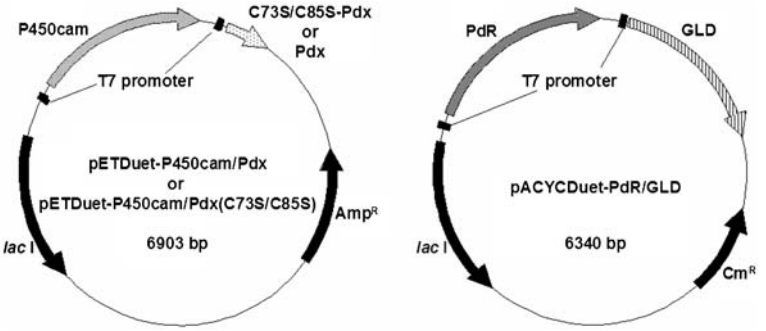

Fig. 1 Schematic illustration of recombinant plasmids, pETDuet-P450cam/Pdx or pETDuet-P450cam/Pdx(C73S/C85S) and pACYCDuet-PdR/GLD. Abbreviations: Amp^R, ampicillin resistance gene; Cm^R, chloramphenicol resistance gene

supplemented with ampicillin (100 mg/l), chloramphenicol (50 mg/l) and δ-aminolevulinic acid (0.5 mM). The cells were grown at 37°C to an optical density (OD_{600}) of 0.6, the temperature was then lowered to 27°C and IPTG was added to give of 0.5 mM. The culture was grown another 24 h. The cells were harvested by centrifugation and washed with 50 mM Tris/HCl buffer (pH 7.4) three times. The washed cells were re-collected by centrifugation, frozen in liquid N_2 and then freeze-dried. The yield was 1.5 g dry cells per litre of LB medium. Resting cells obtained were employed as the whole cell biocatalysts and were stored at –80°C prior to use.

Camphor hydroxylation reaction and product analysis

The resting cells of BL21(P450cam/Pdx(C73S/C85S)/PdR/GLD) were suspended in different buffers containing 100 mM KCl and 5% (v/v) glycerol. The hydroxylation reaction was initiated by adding camphor (2 mM) dissolved in ethanol with agitation at 20 °C. The reaction mixture was sampled, sonicated and centrifuged to obtain the supernatant. To extract the residual substrate and reaction products, the same volume of ethyl acetate containing 0.5 mM decane (internal standard) was added to the aqueous supernatant and vortexed. The ethyl acetate phase was recovered and dehydrated using magnesium sulfate. Catalytic conversion of camphor was determined from the decrease in the substrate or the increase in the product (5-exo-hydroxycamphor) as monitored by GC-MS analysis of the samples recovered. The GC-MS analysis was performed at 70 eV in a Shimadzu GCMS-QP5050A system equipped with a 30 m fused silica column (HP-5; 30 m × 0.53 mm; Agilent Technologies, Palo Alto, California, USA). The oven temperature was programmed to ramp from 80°C to 300°C at a rate of 8°C/min.

Results and discussion

The highest conversion of camphor by the whole cells was at pH 7.4 and was independent of the type of buffer (Fig. 2). Previously, we had found that by in vitro P450cam system created in water-in-oil emulsion, use of phosphate buffer decreased the substrate conversion approximately 3-fold compared with Tris/HCl buffer, which could be attributed to the change in stability of Pdx in different buffers (Michizoe et al. 2005). It is reasonable to assume that direct interaction of protein components with buffer components is minimized in whole cell systems, and therefore buffers simply contributed to adjusting the cellular pH. On the basis of these results, subsequent experiments were conducted with pH 7.4 Tris/HCl buffer.

The conversion of camphor reached a plateau after incubation at 37°C for several hours (open squares in Fig. 3C) (Mouri et al. 2006). Therefore, we investigated the effect of reaction temperature on the camphor hydroxylation catalyzed by BL21(P450cam/Pdx/PdR/GLD) harboring wild-type Pdx. As shown in Fig. 3A–C (open squares), catalytic activity of the recombinant whole cells was modulated by changing the reaction temperature. With the native protein components, the highest conversion was obtained at 20°C after 24 h (Fig. 3B). The variation in the conversion

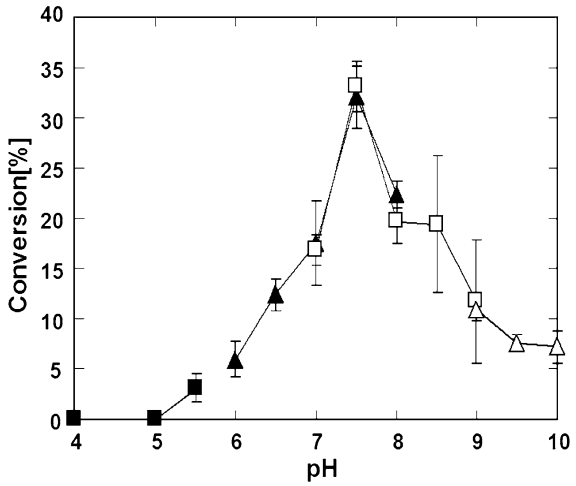

Fig. 2 Effect of pH on the monooxygenase activity. The percent conversion is defined by the hydroxylation of camphor (2 mM). Closed squares, 50 mM succinate/NaOH buffer (pH 4.0, 5.0, 5.5); closed triangles, 50 mM sodium phosphate buffer (pH 6.0, 6.5, 7.0, 7.5, 8.0); open squares, 50 mM Tris/HCl buffer (pH 7.0, 7.5, 8.0, 8.5, 9.0); open triangles, 50 mM borate/NaOH buffer (pH 9.0, 9.5, 10). The reaction mixture was composed of 2 mM camphor, 100 mM KCl, 5% (v/v) glycerol, and 1 mg dry cell wt/ml in each buffer. After incubation at 20°C for 3 h, the samples were analyzed according to the procedure in the Methods section. All the experiments were conducted at least in triplicate. Mean values and standard deviations (error bars) are depicted

between the different reaction temperatures suggests that the catalytic performance relies on the stability of the protein components constituting the P450cam system. Since the low stability of Pdx has been reported, we tested if the replacement of wild-type Pdx with C73S/C85S-Pdx, a stable Pdx mutant (Sevrioukova et al. 2003), improves the catalytic efficiency of the whole cell-based P450cam system. We assumed that the expression levels of wild-type Pdx and C73S/C85S-Pdx were comparable in the host cells because the corresponding genes were on the same plasmid vector and the proteins were expressed with the same promoter (Fig. 1). In addition, we confirmed by

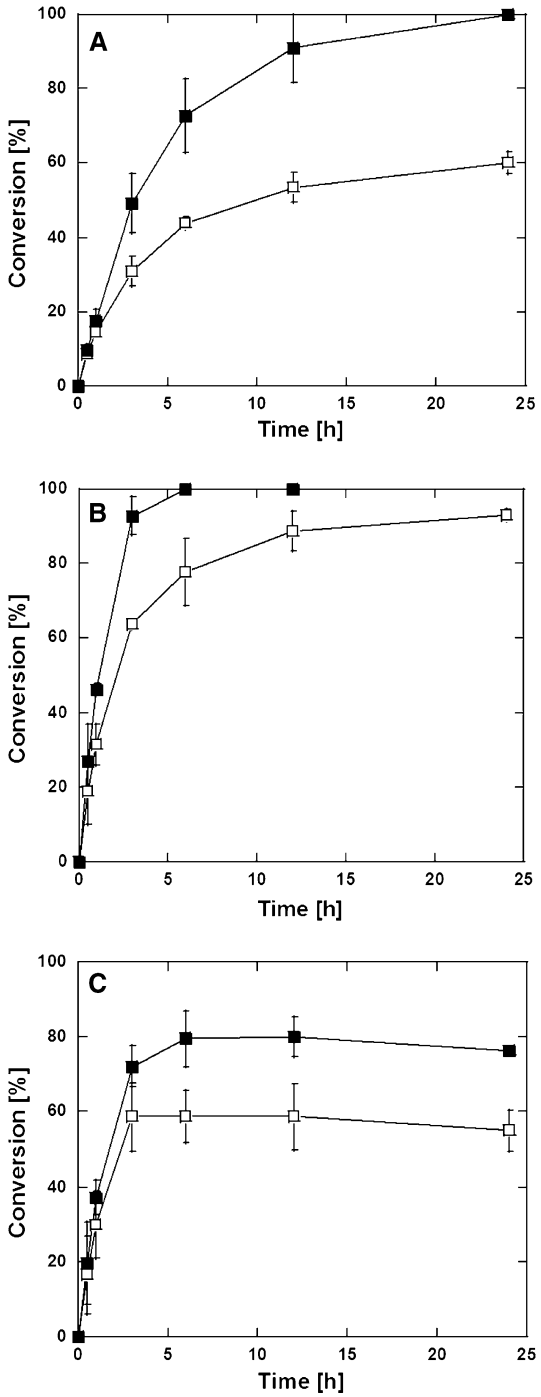

Fig. 3 Time courses of camphor conversion with BL21 (P450cam/Pdx/PdR/GLD) (open squares) or BL21 (P450cam/Pdx(C73S/C85S)/PdR/GLD) (closed squares) at (**A**) 4, (**B**) 20 and (**C**) 37°C. Definition of *y*-axis is the same as in Fig. 2. The reaction mixture was composed of 2 mM camphor, 100 mM KCl, 5% (v/v) glycerol, and 3 mg dry cell wt/ml in 50 mM Tris/HCl buffer (pH 7.4). The reaction mixture was incubated at 4, 20 or 37°C and sampled at predetermined times. The collected samples were analyzed according to the procedure in the Methods section. All the experiments were conducted at least in triplicate. Mean values and standard deviations (error bars) are depicted

spectroscopic analysis of CO-bound P450cam species that the expression levels of P450cam that is on the same plasmid vector of wild-type Pdx or C73S/C85S-Pdx were comparable (data not shown). The conversion of camphor was markedly enhanced at all temperatures tested. Perfect conversion of 2 mM camphor was attained at 20°C in 6 h with BL21(P450cam/Pdx(C73S/C85S)/PdR/GLD) (Fig. 3B). The bioconversion still proceeded at 4°C after 24 h whereas it terminated at 37 °C after 6 h. These results indicate the importance of the stability of all the protein components participating in the catalytic cycle on the construction of stable multicomponent enzymatic systems. This study also suggests that further engineering of each protein component will create practical whole cell biocatalysts with P450 activities.

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