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A recombinant *Escherichia coli* whole cell biocatalyst harboring a cytochrome P450cam monooxygenase system coupled with enzymatic cofactor regeneration

Received: 22 September 2005 / Revised: 1 December 2005 / Accepted: 4 December 2005 / Published online: 19 January 2006
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Abstract A cytochrome P450cam monooxygenase (P450cam) system from the soil bacterium *Pseudomonas putida* requires electron transfer among three different proteins and a cofactor, nicotinamide adenine dinucleotide (NADH), for oxygenation of its natural substrate, camphor. Herein, we report a facile way to significantly enhance the catalytic efficiency of the P450cam system by the coupling of its native electron transfer system with enzymatic NADH regeneration catalyzed by glycerol dehydrogenase (GLD) in *Escherichia coli* whole cell biocatalysts. Recombinant *E. coli* harboring the P450cam system, but lacking GLD, exhibited little activity for camphor hydroxylation. In contrast, coexpression of GLD with the proteinaceous electron transfer components of P450cam resulted in about tenfold improvement in the substrate conversion, implying that the whole cell biocatalyst utilized molecular oxygen, endogenous NADH, and glycerol in the cell for catalysis. The addition of glycerol to the reaction media further promoted camphor hydroxylation, suggesting that exogenous glycerol is also available for GLD in the host cell and actively participates in the catalytic cycle. These results clearly show the utility of GLD towards functional reconstruction of the native P450cam system. The present approach may also be useful for *E. coli* whole cell biocatalysts with the other NADH-dependent oxygenases and oxidoreductases.

Introduction

Recently, the use of biocatalysts such as enzymes and microorganisms was studied for industrial applications (Panke et al. 2002; Cherry and Fidantsef 2003). Hydrolase and isomerase have been isolated and purified for use in bioconversion. On the other hand, one limitation to the practical application of oxidoreductases and oxygenases is their requirement for expensive electron-donating cofactors such as NAD(P)H. In terms of the regeneration of cofactors, whole cells 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). Advances in modern recombinant DNA technology enable the expression of various proteins in *Escherichia coli* as a host organism. There are several ways to adapt various enzymatic activities to different substrates (Schneider et al. 1999; Wilms et al. 2001; Jose and von Schwichow 2004; Ernst et al. 2005). Considering the utilization of recombinant *E. coli* as whole cell biocatalysts for oxidoreductive conversion, coexpression of all of the protein components required for target biotransformations in a cell should be the most practical and efficient method.

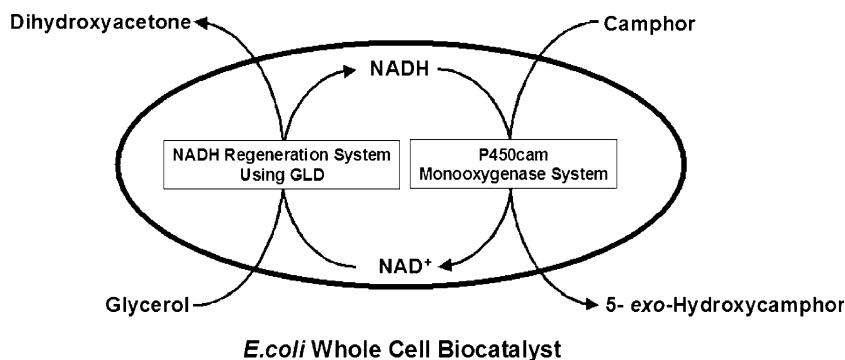
Oxygenase catalyzes selective oxygenation of unactivated C–H bonds under mild conditions and is of great industrial importance. In terms of P450 catalysis, a bottleneck for the use of this biocatalyst in industrial applications is the difficulty in the reconstitution of an efficient electron transfer system between redox pairs. The reconstitution of a native cytochrome P450 monooxygenase system for hydroxylation has been explored using *E. coli* cells transformed with tricistronic plasmids (Bell et al. 2001). Another way to overcome this limitation was seen in the study by Joo and coworkers, who reported that cytochrome P450 mutants efficiently utilized hydrogen peroxide in lieu of molecular oxygen (O₂) and nicotinamide adenine dinucleotide (NADH) (Joo et al. 1999). In the present study, we focused on the coupling of different enzymatic activities towards the monooxygenation of one substrate.

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Fig. 1 *Escherichiacoli* whole-cell monooxygenation system with P450cam incorporating the enzymatic NADH-regeneration system



Our approach was based on the utilization of a recombinant *E. coli* whole cell biocatalyst harboring a P450cam system and a NADH regeneration system. The cytochrome P450cam monooxygenase (P450cam) system from the soil bacterium *Pseudomonas putida* requires the cofactor NADH and two proteinaceous cofactors, putidaredoxin reductase (PdR) and putidaredoxin (Pdx), to activate O_2 for the oxidation of its natural substrate, camphor (Gunsalus and Wagner 1978; Poulos et al. 1987; Nelson et al. 1993). To elucidate the importance of NADH regeneration on the substrate conversion, we incorporated a glycerol dehydrogenase (GLD)-mediated NADH regeneration system. The present substrate oxygenation process was thus composed of four exogenous protein components in the host cell (Fig. 1). The feasibility of the whole-cell P450cam biocatalyst employing inexpensive glycerol as the sacrificial substrate was demonstrated both in an aqueous system and in an aqueous-organic biphasic system.

Materials and methods

Construction of plasmid vectors for coexpression of the four protein components

The nucleotide sequences of the polymerase chain reaction (PCR) primers used are summarized in Table 1. The genes encoding wild type P450cam, PdR, Pdx, and GLD were amplified by PCR using pET22-wild type P450cam, pET22-PdR, pET22-Pdx, and pET22-GLD as the DNA templates (Ichinose et al. 2004). The gene fragment encoding P450cam was digested with *NcoI/EcoRI* and inserted into pETDuet cut with the same restriction enzymes.

The gene fragment encoding Pdx was digested with *NdeI/XhoI* and inserted into the same restriction sites of pETDuet-P450cam, and then the protein-coexpression vector, pETDuet-P450cam/Pdx, was prepared. The pACYCDuet-PdR plasmid was obtained by cleaving pACYCDuet with *NcoI/EcoRI* and insertion of the gene fragment encoding PdR that had been cut with the same restriction enzymes. The gene fragment encoding GLD was phosphorylated at the 3'-end with T4 polynucleotide kinase, digested at the 5'-end with *NdeI*, and then inserted into the *NdeI/EcoRV* restriction sites of pACYCDuet-PdR, (pACYCDuet-PdR/GLD). The two protein-coexpression vectors were transformed into *E. coli* strain BL21(DE3) (Novagen). The transformant was abbreviated as BL21 (P450cam/Pdx/PdR/GLD). The same *E. coli* strain transformed with both the pETDuet-P450cam/Pdx and the pACYCDuet-PdR plasmid vectors, in which three native P450cam components but not GLD were present, was abbreviated as BL21(P450cam/Pdx/PdR).

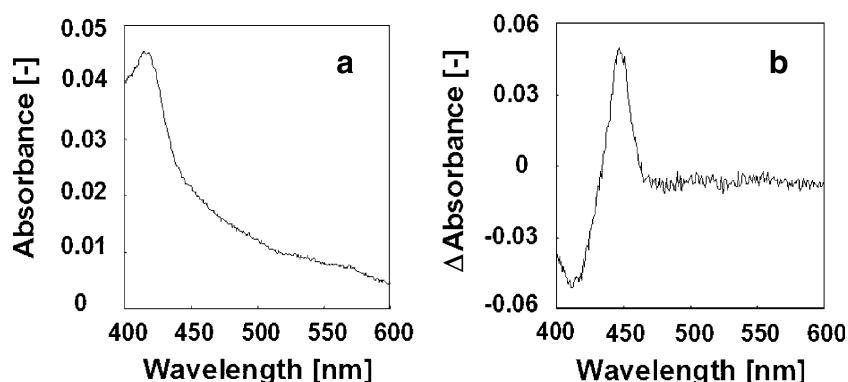
Culturing of recombinant *E. coli* cells and preparation of the resting cells

Three different transformants were grown in 40 mL of LB medium supplemented with ampicillin (100 mg/L) and chloramphenicol (50 mg/L) at 37°C. The overnight culture was used to inoculate 1 L of LB medium supplemented with ampicillin (100 mg/L), chloramphenicol (50 mg/L), and 5-aminolevulinic acid (0.5 mM). The cells were grown at 37°C to an optical density (OD_{600}) of 0.6. When the OD_{600} reached this value, the temperature was lowered to 27°C, IPTG was added to the medium to a final concen-

Table 1 Nucleotide sequences of the primers for PCR

Primers	Nucleotide sequence
Primer-1(N-terminus of P450cam)	5'-ACATGCCATGGGAACGACTGAAACCATACAAAGCAACG-3'
Primer-2(C-terminus of P450cam)	5'-CCGGAATTCTTATACCGCTTTGGTAGTCGCCGGA-3'
Primer-3(N-terminus of Pdx)	5'-CCGCTCGAGTTACCATTCGCTATCGGGAACATCG-3'
Primer-4(C-terminus of Pdx)	5'-ATGCATGCCATATGATGTCTAAAGTAGTGTATGTGTAC-3'
Primer-5(N-terminus of PdR)	5'-ACATGCCATGGGAACGCAAACGACAACGTGGTCATCG-3'
Primer-6(N-terminus of PdR)	5'-CCGGAATTCTCAGGCACTACTCAGTTCAGCTTTG-3'
Primer-7(N-terminus of GLD)	5'-ATGCATGCCATATGATGGACCGCATTATTCAATCACCGG-3'
Primer-8(N-terminus of GLD)	5'-GCATGCATGATATCTTATCCCACTCTTGCAGGAAACGC-3'

Fig. 2 Spectroscopic analysis of the crude cell extracts. **a** Ultraviolet-visible spectrum of the cell lysates; **b** CO difference spectrum of the cell lysates



tration of 0.5 mM, and the culture was grown for 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 recollected by centrifugation, frozen in liquid nitrogen, and lyophilized in a freeze-drying apparatus (FD-5N, EYELA, Tokyo) for 24 h. Resting cells obtained were employed as the whole cell biocatalysts and were stored at -80°C prior to use.

Spectroscopic measurement of CO-bound cytochrome P450cam in cell lysates

BL21(P450cam/Pdx/PdR/GLD) resting cells were resuspended at a concentration of 1 mg of dry cell/mL and disrupted by sonication in 50 mM Tris-HCl buffer, pH 7.4, containing 0.1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 10 mM 2-mercaptoethanol. The lysates were centrifuged to remove insoluble fractions. The supernatant was employed for spectroscopic measurement using a ultraviolet-visible spectrophotometer (Ubest-570, JASCO, Japan). The crude cell extract solutions were reduced by the addition of a small amount of sodium dithionite and the baseline spectrum was recorded in the range of 400–600 nm. The solution was then infused with carbon monoxide and the difference spectrum was immediately

recorded in the same spectral range. The content of P450cam in the aqueous solution was determined by the CO difference spectrum with a molar extinction difference of $91\text{ mM}^{-1}\text{ cm}^{-1}$ between 450 and 490 nm (Omura and Sato 1964).

Camphor hydroxylation reaction in an aqueous system

A typical experimental procedure was as follows: Resting cells (30 mg of dry cell/mL) were suspended in 50 mM Tris-HCl (pH 7.4) containing 100 mM KCl, in the absence or presence of 10 vol% glycerol. The hydroxylation reaction was initiated by adding the substrate (camphor) dissolved in ethanol to give a final concentration of 2 mM. The reaction mixture was incubated at 37°C with agitating. After 24 h of reaction, the catalytic conversion of camphor was determined by gas chromatography (GC)–mass spectrometry (MS) analysis. 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. Control experiments with resting cells were carried out in the same manner. All the experiments were conducted at least in triplicate.

Fig. 3 Camphor hydroxylation reaction catalyzed by the resting cells in an aqueous system (50 mM Tris-HCl buffer, pH 7.4). *Line A* native BL21 with 10 vol% glycerol; *line B* BL21(P450cam/Pdx/PdR); *line C* BL21(P450cam/Pdx/PdR) with 10 vol% glycerol; *line D* BL21(P450cam/Pdx/PdR/GLD); *line E*, BL21(P450cam/Pdx/PdR/GLD) with 10 vol% glycerol

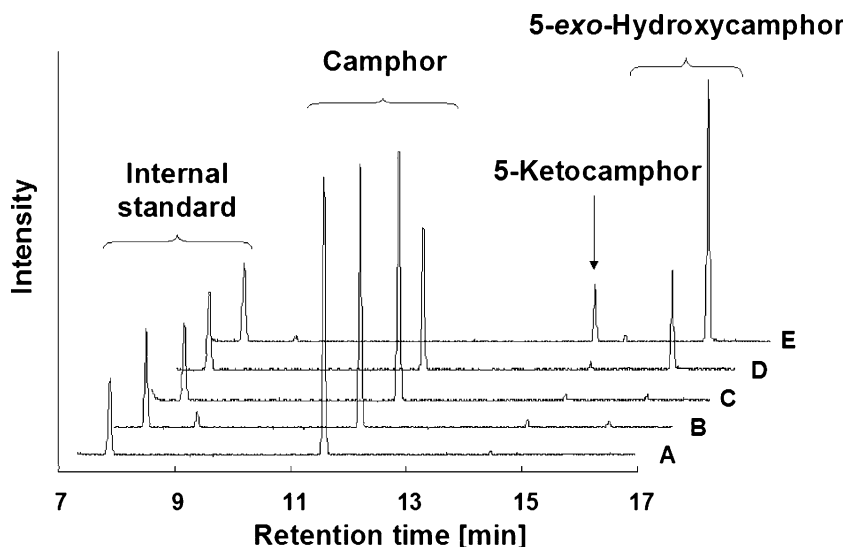


Table 2 Hydroxylation rates of camphor in different systems

	Hydroxylation rate [%]
Aqueous systems	
A: BL21 + 10 vol% glycerol	N. D. ^a
B: BL21(P450cam/Pd/PdR)	4±1
C: BL21(P450cam/Pd/PdR) + 10 vol% glycerol	4±1
D: BL21(P450cam/Pd/PdR/GLD)	37
E: BL21(P450cam/Pd/PdR/GLD) + 10 vol% glycerol	100
Aqueous-organic biphasic systems	
F: BL21 + 10 vol% glycerol	N. D. ^a
G: BL21(P450cam/Pd/PdR) + 10 vol% glycerol	3±1
H: BL21(P450cam/Pd/PdR/GLD) + 10 vol% glycerol	30±5

^aN. D. Not determined because GC signal of 5-*exo*-hydroxycamphor was below the detection limit

Effect of glycerol on camphor hydroxylation in an aqueous system

BL21(P450cam/Pdx/PdR/GLD) (3 mg of dry cell/mL) was suspended in 50 mM Tris-HCl (pH 7.4) containing 100 mM KCl and different amounts of glycerol (0, 0.1, 1.0, 5.0, or 10.0 vol%). The hydroxylation reaction was initiated by adding the substrate (2 mM) dissolved in ethanol and agitating at 37°C. The reaction mixture containing the cells was sampled at predetermined times (0.5, 1, 3, and 6 h), sonicated, and centrifuged to obtain the supernatant. The samples were analyzed by GC-MS ac-

cording to the procedure in the preceding section. All the experiments were conducted at least in triplicate.

Camphor hydroxylation in an aqueous-organic biphasic system

An aqueous-organic biphasic system was constructed with isooctane (800 µL) and 50 mM Tris-HCl buffer (pH 7.4, 4 mL) containing 10 vol% glycerol and 100 mM KCl. In this case, the substrate (camphor) was dissolved in isooctane to give a final concentration of 2 mM. The reaction solutions were incubated at 37°C with stirring of the aqueous phase. After 24 h of reaction, the reaction mixture was sonicated and centrifuged to remove the cells. After phase separation, the aqueous phase was batched off. An equal amount of an ethyl acetate solution containing 0.5 mM decane (internal standard) was added to the aqueous phase to extract the substrate and reaction products for GC-MS analysis. All the experiments were conducted at least in triplicate.

GC-MS analysis

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. The GC-MS analysis was performed at 70 eV in a Shimadzu a 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

Expression of P450cam in the recombinant *E. coli* cells

To check the expression of active P450cam in the recombinant *E. coli* cells, we spectroscopically analyzed crude cell lysates of BL21(P450cam/Pdx/PdR/GLD). A characteristic spectrum of hemoproteins in the Soret region was observed for the lysate (Fig. 2a). The CO difference spectrum showed a maximum absorbance at 450 nm (Fig. 2b). Based on the data of the aqueous extract recovered from dry cell suspension, the content of P450cam was estimated to be about 50 pM in 1 mg of dry cell weight/mL.

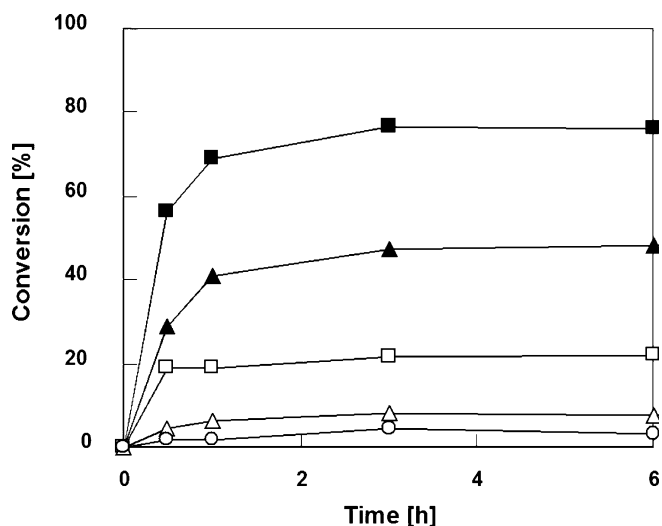
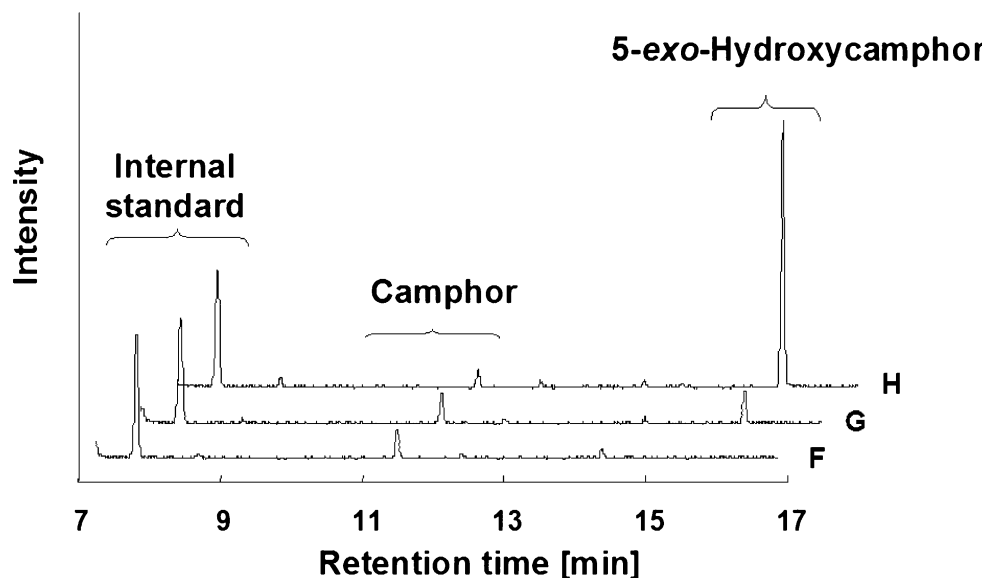


Fig. 4 Time courses of camphor conversion with BL21(P450cam/Pdx/PdR/GLD) in the presence of different amounts of glycerol. BL21(P450cam/Pdx/PdR/GLD) without glycerol (○) or with 0.1 vol% (△), 1 vol% (□), 5 vol% (▲), and 10 vol% (■) glycerol

Camphor hydroxylation in an aqueous system

The hydroxylation reaction of camphor catalyzed by the whole cell biocatalysts, BL21, BL21(P450cam/Pdx/PdR), and BL21(P450cam/Pdx/PdR/GLD) was investigated under

Fig. 5 Hydroxylation of camphor catalyzed by the resting cells in an aqueous-organic biphasic system containing 10 vol % glycerol in the aqueous phase. Line F native BL21; line G BL21(P450cam/Pdx/PdR); line H BL21(P450cam/Pdx/PdR/GLD)



different reaction conditions. Results are summarized in Fig. 3 and Table 2. Little oxidation resulted when the host *E. coli* BL21 cells harbored neither P450cam, nor GLD (Fig. 3, line A). The BL21(P450cam/Pdx/PdR) resting cells slightly catalyzed the reaction in 50 mM Tris-HCl buffer (Fig. 3, line B and C). A substrate conversion of 4% was obtained and was independent of the presence of 10 vol% glycerol. On the other hand, 37% of the camphor was hydroxylated by BL21 (P450cam/Pdx/PdR/GLD) resting cells in the same buffer even in the absence of glycerol (Fig. 3, line D). Addition of 10 vol% glycerol to the reaction medium resulted in complete conversion of 2 mM camphor with BL21 (P450cam/Pdx/PdR/GLD) resting cells (Fig. 3, line E). It is worth mentioning that a significant amount of 5-ketocamphor was detected in the final system.

Effect of glycerol on camphor hydroxylation in an aqueous system

The role of GLD in the present system was further evaluated. To reduce the influence of intrinsic glycerol on the analysis, the quantity of resting cells was simply lowered from 30 to 3 mg of dry cell weight/mL. Figure 4 shows the effect of the addition of exogenous glycerol on camphor hydroxylation with BL21(P450cam/Pdx/PdR/GLD) resting cells. As shown in the figure, it was found that the higher the glycerol content, the higher the camphor hydroxylation rate.

Camphor hydroxylation in an aqueous-organic biphasic system

To test the applicability of the whole-cell P450cam biocatalyst to an aqueous-organic biphasic system, the camphor hydroxylation reaction was investigated in an aqueous/isooctane biphasic system. In the present case, the isooctane

phase formed a gel upon sonication of the reaction mixture to extract substrates and products from the cells, which prevented complete recovery of the product. However, as can be seen in Fig. 5 and Table 2, it was shown that BL21 (P450cam/Pdx/PdR/GLD) resting cells allowed the reaction to proceed in the biphasic system. Camphor conversion based on the amount of hydroxylated product recovered in the aqueous phase was about 30% (line H in Fig. 5).

Discussion

The $\text{Fe}_2^+ - \text{CO}$ complex that gives a characteristic maximum absorbance around 450 nm could be derived from cytochrome P450cam (Fig. 2). It is known that inactive P420 species give a maximum absorbance at 420 nm. Because the P420 form was not clearly detected from the differential spectrum of crude cell extracts, P450cam expressed in the recombinant *E. coli* cells was mainly in the catalytically active form.

NADH is an essential reducing agent in the P450cam monooxygenase system. Because the P450cam-catalyzed monooxygenation is initiated by electron transfer from NADH to PdR, catalytic camphor hydroxylation will be terminated when NADH in the reaction system is completely consumed. Incorporation of a cofactor regeneration system in *E. coli* whole cell biocatalysts was demonstrated to be a powerful strategy for enhancing the catalytic efficiency of oxidoreductases (Kataoka et al. 2003; Ernst et al. 2005). Alcohol dehydrogenases (ADHs) have been used for the construction of a cofactor regeneration system in whole cell systems. In terms of ADHs, the high concentration of alcohols is usually required to shift the equilibrium towards desired products; however, alcohols and the oxidative product, aldehydes, can inactivate the constituents of host cells including enzymes of interest. On the other hand, glycerol is widely employed to stabilize proteins and cells. We thus selected glycerol as a reducing

agent and GLD as a coupling partner of the P450cam system.

In an aqueous system, although NADH was not supplemented, camphor was hydroxylated in all the systems in which three proteinaceous components (P450cam, Pdx, and PdR) of P450cam were incorporated (Table 2). This suggests that BL21(P450cam/Pdx/PdR) utilized endogenous NADH for the reaction. P450cam catalyzes the oxygenative reaction of a substrate using O₂ and two electrons from NADH. NADH consumed for camphor hydroxylation in BL21(P450cam/Pdx/PdR) was thus estimated to be about 0.1 mM based on the results of systems B and D (Fig. 3) where the hydroxylation rate was 4%. Assuming that the amount of available endogenous NADH in the resting cells (30 mg of dry cell weight/mL) for P450cam catalysis was about 0.1 mM, it was calculated that more than 1.9 mM NADH was regenerated by GLD in BL21(P450cam/Pdx/PdR/GLD) in system E (Fig. 3).

GLD is capable of regenerating NADH from NAD⁺ with an inexpensive sacrificial substrate, glycerol. In the system D (Table 2 and Fig. 3), we first found that endogenous glycerol was involved in the regeneration of NADH. Consequently, exogenous glycerol was shown to be available for enhancing the P450cam monooxygenase system in BL21(P450cam/Pdx/PdR/GLD) (system E in Table 2 and Fig. 3). These results demonstrated the successful construction of a P450cam system coupled with GLD-catalyzed NADH regeneration in one *E. coli* cell. It is noted that significant amounts of 5-ketocamphor were detected in system E, implying accumulation of the first product, 5-*exo*-hydroxycamphor, in the cells, owing to the high efficiency of the substrate conversion.

We further investigated the effect of an exogenous addition of glycerol on the catalytic conversion of camphor by varying the glycerol concentration. Figure 4 depicts the marked promotion of camphor hydroxylation concomitant with an increase in the glycerol concentration. The results show that exogenous glycerol is available for GLD in the cells, which implies that glycerol efficiently passes through the membrane of BL21 *E. coli* cells. It has been reported that *E. coli* cells have two types of transport systems for exogenous glycerol: an active transport system and a passive transport system (Sanno et al. 1968). Because the catalytic conversion of camphor was increased with an increase in the glycerol concentration, it is likely that glycerol passively diffused into the cells and was preferentially used for NADH regeneration because of the high local concentration of overexpressed GLD in the present system.

Whole cell biocatalysts have also been involved in potentially useful biotransformations targeting hydrophobic substrates, such as aliphatic, aromatic, and heterocyclic compounds. Because these compounds and organic solvents are often toxic to the cells, a number of whole cell systems were investigated to determine their feasibility for organic solvent-based bioconversion (Marconi et al. 1997; León et al. 1998; Panke et al. 2002; Na et al. 2005). Application of an aqueous-organic biphasic system offers an alternative technical solution to this problem (Favre-Bulle and Witholt 1992;

Kim and Rhee 1993; Wubbolts et al. 1994, 1996; Schmid et al. 1998; Simpson et al. 2001; Cruz et al. 2002, 2004; Sello et al. 2004). Therefore, we investigated the applicability of BL21(P450cam/Pdx/PdR/GLD) to an aqueous-organic biphasic system. In the present system, the resting cells catalyzed hydroxylation of camphor distributed from the isooctane phase to the aqueous phase. Although the hydroxylation rate was decreased in comparison to that in the aqueous system, most camphor distributed to the aqueous phase was oxygenated by the resting cells (system H in Table 2 and Fig. 5). In addition, 5-ketocamphor observed in the aqueous system E in Fig. 3 was not detected, suggesting that camphor was supplied gradually to the aqueous phase from the organic phase, which in turn increased the product selectivity.

In summary, we have constructed a recombinant *E. coli* whole cell biocatalyst that affords cytochrome P450cam-catalyzed monooxygenation with O₂ and endogenous NADH in the cells. NADH regeneration coupled with the coexpression of GLD was highly efficient, which resulted in a marked increase in the efficacy of the monooxygenative reaction. The present system clearly demonstrated that it is of great importance to provide sufficient reducing power for the P450 system, which can easily be achieved by the incorporation of a cofactor regeneration system. The feasibility of GLD-catalyzed cofactor regeneration in the *E. coli* resting cells can be further extended to different types of P450 enzymes, as well as oxidoreductase which requires NADH for its catalysis.

Acknowledgement This research was supported by the 21st Century Center of Excellence Program "Functional Innovation of Molecular Informatics" from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to M.G.).

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