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Putidaredoxin Reductase-Putidaredoxin-Cytochrome P450_{cam} Triple Fusion Protein

CONSTRUCTION OF A SELF-SUFFICIENT *ESCHERICHIA COLI* CATALYTIC SYSTEM*

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Fusion proteins of cytochrome P450_{cam} with putidaredoxin (Pd) and putidaredoxin reductase (PDR), the two proteins required to transfer electrons from NADH to P450_{cam}, were constructed by fusing cDNAs encoding the three proteins in the expression vector pCWori⁺. Several fusion proteins, in which the order of the three protein domains and the linkers between them were varied, were expressed in *Escherichia coli*, purified, and characterized. The highest activity ($k_{\text{cat}} = 30 \text{ min}^{-1}$) was obtained with a PDR-Pd-P450_{cam} construct in which the peptides TDGTASS and PLEL were used, respectively, to link the PDR to the Pd and the Pd to the P450_{cam} domains. Oxygen and NADH consumption is tightly coupled to substrate oxidation in the fusion proteins. The rate-limiting step in the catalytic turnover of these fusion proteins is electron transfer from Pd to P450_{cam}. This is indicated by high rates of electron transfer from the PDR and Pd domains to exogenous electron acceptors, by an increase in the activity of the P450_{cam} domain upon addition of exogenous Pd, and by the high activity of wild-type P450_{cam} when incubated with a PDR-Pd fusion protein. *E. coli* cells expressing the PDR-Pd-P450_{cam} fusion protein efficiently oxidize camphor to 5-*exo*-hydroxycamphor and 5-oxocamphor. *E. coli* cells expressing the triple fusion protein thus constitute the first heterologous self-sufficient catalytic system for the oxidation of camphor and other substrates by P450_{cam}.

Self-sufficient cytochrome P450 systems are required to harness the catalytic power of these enzymes for practical purposes, including the stereospecific synthesis of fine organic chemicals, the degradation or detoxification of hazardous compounds in the environment, and the development of plants with resistance to specific herbicides. A model for the latter use is provided by the demonstration that heterologous expression of a cytochrome P450-P450 reductase fusion protein in tobacco plants conveys resistance to the herbicide chlortoluron (1). The requirement for practical, self-sufficient catalytic systems is exacerbated by the potential utility of cytochrome P450 enzymes with tailored substrate specificities.

Efforts to construct self-sufficient P450 catalytic systems have focused on the membrane-bound enzymes, for which cy-

tochrome P450 reductase is the most common electron transfer partner. Fusion proteins of mammalian P450 enzymes with P450 reductases have been expressed in *Saccharomyces cerevisiae* and *Escherichia coli* (2–11). Fusion proteins have also been constructed in which mitochondrial cytochrome P450_{scd} is fused to adrenodoxin, an iron-sulfur protein, and adrenodoxin reductase, a flavoprotein (12). Adrenodoxin and adrenodoxin reductase are required to transfer electrons from NADPH to P450_{scd}. These P450_{scd} fusion proteins were not purified or well characterized, however, and were reported to have no activity when expressed outside of mitochondria (13). The P450_{scd} system is the only example so far of a three-component P450 fusion protein.

Cytochrome P450_{cam} (CYP101) (51) was the first P450 for which a crystal structure was obtained (14). The availability of crystal structures of P450_{cam} with a variety of bound ligands and of several mutant forms of the protein make it the best structurally characterized P450 enzyme (15–19). As such, it has served as the model for most of the mechanistic and structural advances made in the P450 field in the past decade. The wealth of information available on the P450_{cam} system makes it an attractive candidate for the development of self-catalytic P450 systems with tailored substrate specificities. The crystal structures of three other soluble bacterial P450 enzymes have recently been determined, and the strategies developed for P450_{cam} should be applicable to the construction of fusion proteins from two of them, P450_{terp} (20) and P450_{eryF} (21). P450_{BM-3}, the other soluble P450 for which a crystal structure is available, is already a fusion protein, in which the heme domain is fused to a domain with high sequence identity to P450 reductase (22).

The function of cytochrome P450_{cam} in *Pseudomonas putida*, the organism from which it is derived, is to catalyze the oxidation of camphor¹ to 5-*exo*-hydroxycamphor. This hydroxylation, the first step in the degradation of camphor, makes it possible for *P. putida* to grow on camphor as its sole carbon and energy source (23). The catalytic turnover of cytochrome P450_{cam} requires two auxiliary proteins, the flavoprotein putidaredoxin reductase (PDR) and the iron-sulfur protein putidaredoxin (Pd) (24). These two proteins mediate the transfer of electrons from NADH to the hemoprotein. All three proteins of the cytochrome P450_{cam} system are soluble and have been cloned and heterologously expressed in *E. coli* (25–27). Although P450_{cam} has

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¹ The abbreviations used are: camphor, (1R)-camphor; 5-*exo*-hydroxycamphor, (1R)-5-*exo*-hydroxycamphor; PDR, putidaredoxin reductase; Pd, putidaredoxin; PCR, polymerase chain reaction; P450_{cam}-PDR-Pd, fusion protein with the three components in the indicated order connected by 7-amino acid linkers; P450_{cam}-(PG)₄-PDR-Pd, P450_{cam}-PDR-Pd with an 8-amino acid extension inserted into the 7-amino acid linker; PDR-Pd-P450_{cam}, fusion protein with the domains in the indicated order.

traditionally been viewed as a camphor-specific enzyme, it metabolizes a diversity of compounds in addition to camphor and camphor analogues. These compounds include styrenes, thioanisoles, ethylbenzene, nicotine, and chlorinated hydrocarbons (28–34), and the reactions catalyzed include carbon hydroxylation, epoxidation, sulfoxidation, and dehalogenation. A computerized search for putative substrates using the crystal structure of P450_{cam} and the docking algorithm DOCK (35) has identified a number of substrates unrelated to camphor (36), and it has been shown that the specificity of the enzyme can be broadened on a rational basis by site-specific mutagenesis.²

The membrane binding properties of the available engineered fusion proteins and the absence of crystallographic data on their active sites limit their potential utility with respect to the rational design of novel catalytic systems. We describe here the expression in *E. coli* of soluble fusion proteins containing P450_{cam}, Pd, and PdR domains. The fusion proteins have been purified and characterized; an analysis has been made of the rate-limiting step in their catalytic turnover; and it has been demonstrated that *E. coli* cells expressing one of the fusion proteins efficiently oxidize camphor.

EXPERIMENTAL PROCEDURES

Materials and Methods—The cDNA clones for P450_{cam}, PdR, and Pd were provided by Julian A. Peterson (University of Texas Southwestern Medical Center, Dallas). The cloning vector pCWori⁺ was provided by Eric Johnson (Scripps Institute) (37). Oligonucleotides were synthesized by the Biomolecular Resource Center of the University of California, San Francisco. A Perkin-Elmer 480 DNA thermal cycler was used for PCR experiments, and the PCRIITM PCR cloning vector (Invitrogen, San Diego, CA) was occasionally used for further manipulations of the PCR product. DEAE-Sepharose and Sepharose S-200 were from Pharmacia Biotech Inc. Cytochrome *c* was from Sigma. UV-visible spectroscopy was performed on an Aminco DW2000 spectrophotometer. A Gilson Oxy5 Oxygraph equipped with a thermostated 1600- μ l glass cuvette was used for measurements of O₂ consumption. Gas chromatography was done on a Hewlett-Packard 5890 instrument equipped with a 30-m DB1 capillary column programmed to run as follows: 130 °C for 3 min; 130–200 °C gradient at 5 °C/min; 200–250 °C gradient at 10 °C/min; and 250 °C for 10 min.

Enzyme Assays—O₂ consumption assays were performed in 100 mM potassium P_i buffer, pH 7.5. Activities were calculated from the camphor-dependent increase in the O₂ consumption over the level observed in the absence of substrate. Cytochrome *c* reduction was followed spectroscopically at 550 nm in 100 mM potassium P_i buffer, pH 7.5, containing 10 μ M cytochrome *c*. The extinction coefficient difference (cytochrome *c*_{red} – cytochrome *c*_{ox} at 550 nm) used to calculate the activity was 21 mM⁻¹ cm⁻¹, as determined by reducing cytochrome *c* with sodium dithionite.

Construction of Fusion Proteins—In contrast to P450_{cam}, relatively little is known about the structures of Pd and PdR except for an NMR-derived structure of Pd (38). The details of the interactions among the three proteins remain obscure, although modeling studies suggest that Pd binds to the P450_{cam} surface at a site immediately behind the iron-coordinated cysteine residue (39, 40). The order of the components in the first fusion construct was based on two considerations: (a) Trp-106 at the C terminus of Pd is required for efficient electron transfer (41, 42); and (b) Arg-112 of P450_{cam}, which is located on the surface of the protein approximately equidistant (~50 Å) from the N and C termini, is within the proposed Pd-binding site and appears to be important for electron transfer (43, 44). These observations suggest the possibility that the C terminus of Pd may interact with Arg-112 of P-450_{cam}. Pd was therefore initially placed at the C terminus of the fusion protein, as this would allow its C terminus the greatest possible degree of freedom. PdR was placed adjacent to Pd to facilitate electron transfer from one to the other, and P450_{cam} was placed at the N terminus.

To construct the initial fusion protein, the P450_{cam} and PdR stop codons were removed, and the codons for a short peptide linker were introduced between the P450_{cam} and PdR domains and between the PdR and Pd domains. This was done by PCR, employing 3'-primers that lacked the stop codon and carried part of the coding sequence for the amino acid linker. The 5'-primers for the adjacent protein carried the

rest of the code for the linker, and both types of primers terminated in unique restriction sites that allowed the genes for the various proteins to be fused as indicated in Fig. 1. *SpeI* and *NheI* were chosen as the restriction sites for use in fusing the genes. The amino acid sequences of the linkers used in the construction of the fusion proteins are shown in Fig. 2. These sites and the sequences of the peptide linkers were the same as those used to fuse the components of the P450_{sec} system (12). Preservation of the wild-type sequences, except for the intended mutations at the termini, was ensured by sequencing (P450_{cam} and Pd) or by a combination of exchange of restriction fragments from the wild-type gene (PdR, *Pf*MI-SacII fragment) and sequencing of the remainder.

Insertion of Linker Extensions—To explore the importance of the link between the fusion protein domains, the link between the P450_{cam} and PdR domains of the fusion protein was varied. A proline- and glycine-rich region close to the N terminus in membrane-bound P450 enzymes has been proposed to function as a flexible hinge between the membrane anchor and the bulk of the newly synthesized P450. The hinge may provide the enzyme with the degrees of freedom required to fold correctly while anchored to the membrane (45). An octa- or dodecapeptide composed of alternating prolines and glycines was therefore inserted into the heptapeptide linkers of the initial fusion protein. The 24- and 36-mer oligonucleotides encoding the (PG)₄ and (PG)₆ polypeptide linkers, compatible with the *SpeI* restriction site in sequence coding for the original heptapeptide linker, were synthesized. As shown below for the 24-mer (Sequence 1), the oligonucleotides self-anneal to form a double-stranded DNA encoding the prolines and glycines in frame with the *SpeI* compatible overhangs.

5' -CTAGGCCCGGGCCCGGGCCCGGGC -3'
3' -CGGGCCCGGGCCCGGGCCCGGATC -5'

SEQUENCE 1

The linkers were inserted by digesting pCWori⁺-P450_{cam}-PdR-Pd with *SpeI*, purifying the linearized plasmid, adding a large excess of the artificial linker, and incubating with T4 ligase. Recircularized plasmid without linker was linearized by cutting with *SpeI* prior to transformation. The insertion of the linker was ascertained by restriction enzyme analysis using *PspAI* and *Asp718*, which generate fragments of 178, 166, and 142 base pairs from the DNA coding for the enzyme with the (PG)₆ linker, the (PG)₄ linker, and the original heptapeptide linker, respectively.

As analysis of the three P450_{cam}-PdR-Pd constructs showed that the PdR-Pd domain and the P450_{cam} domain were individually more active than indicated by the overall activity of the fusion protein, a construct was made in which the PdR-Pd fusion unit was placed in front of the P450_{cam} domain. The PdR-Pd-P450_{cam} construct was made essentially as described for the P450_{cam}-PdR-Pd construct, with the same linker between the PdR and Pd domains as in the original constructs and a 4-amino acid linker between the C terminus of Pd and the N terminus of P450_{cam} (see Figs. 1 and 2).

A PdR-Pd fusion protein was assembled to examine the degree to which this unit retains the ability to transfer electrons. The construction of the PdR-Pd unit made use of the fact that there is an *MluI* restriction site in Pd and a similar site in the pCWori⁺ vector. By cutting both PdR-Pd-P450_{cam} and P450_{cam}-PdR-Pd with *MluI* and ligating the small fragment of PdR-Pd-P450_{cam} with the large fragment of P450_{cam}-PdR-Pd, the PdR-Pd-pCWori⁺ construct was obtained.

Expression—*E. coli* strain DH5 α (Life Technologies, Inc.) was grown in 2.8-liter Fernbach flasks in 2 $\times$ YT medium supplemented with ampicillin (100 mg/liter). Cells were grown to A₆₀₀ = 0.6–0.8 at 37 °C, the temperature was lowered to 20 °C, and isopropyl-1-thio- β -D-galactopyranoside (1 mM) was added. The culture was allowed to grow overnight (18 h) at 20 °C on a rotary shaker (230 rpm). Fusion protein yields of 150–200 nmol/liter of medium are thus obtained, but they can be increased to 600–700 nmol/liter by extending the expression time.

Purification of the Fusion Protein—The cells collected by centrifugation were resuspended in 50 ml of cold (4 °C) buffer containing 50 mM Tris-HCl, 50 mM KCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM β -mercaptoethanol, and 10 mg of lysozyme, pH 7.5. After stirring at 4 °C for 2 h, the suspension was frozen in liquid nitrogen, thawed at 4 °C, and sonicated using a Branson sonicator (medium power output) until the viscous consistency due to the nucleic acids was disrupted (~2 min). The soluble fraction after centrifugation at 100,000 $\times g$ for 25 min was loaded onto an anion exchange column (DEAE Fast Flow) equilibrated in buffer A (50 mM potassium P_i, 50 mM KCl, and 10 mM β -mercaptoethanol, pH 7.5), washed extensively with buffer A, and eluted with a 0–500 mM KCl gradient in buffer A. The

² J. De Voss and P. R. Ortiz de Montellano, unpublished results.

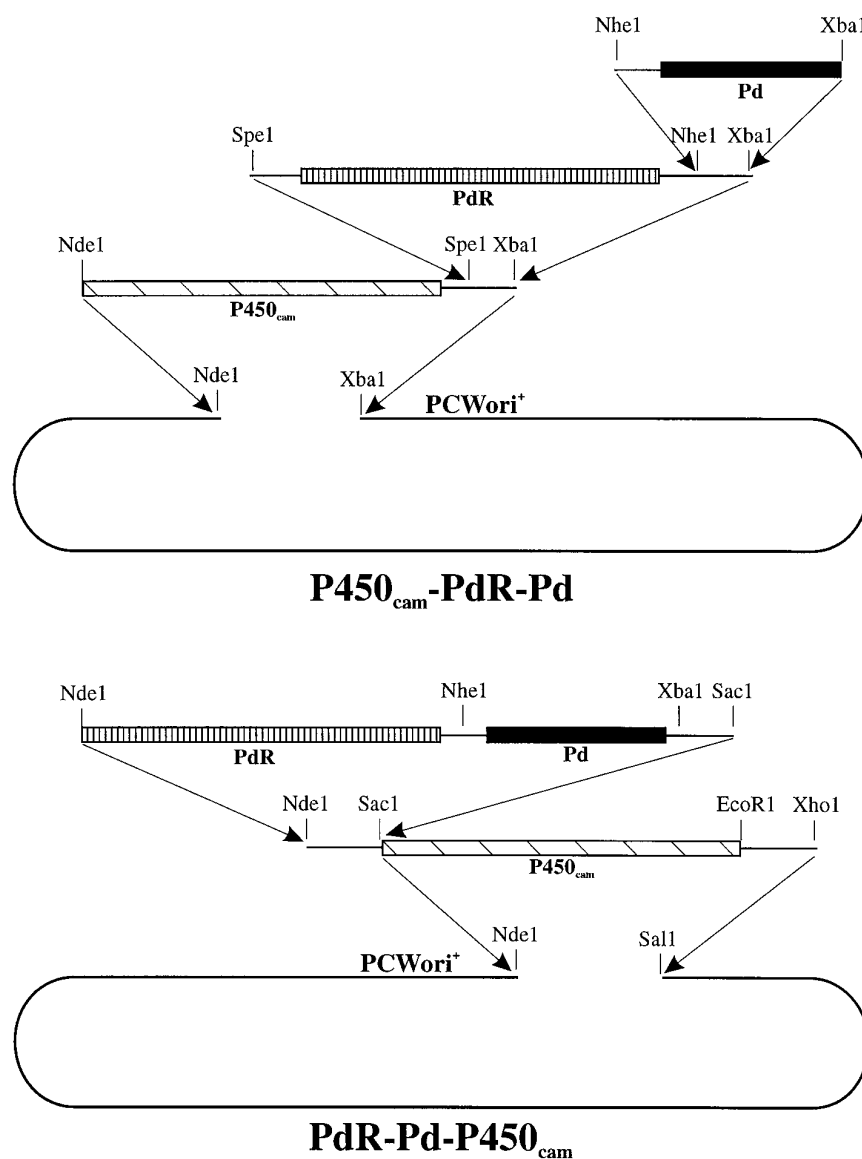


FIG. 1. Assembly of the P450_{cam}-PdR-Pd and PdR-Pd-P450_{cam} fusion proteins. The restriction sites used to fuse the proteins are indicated.

fractions containing the fusion protein, identified by absorption at 418 nm, were combined and concentrated in an Amicon ultrafiltration cell equipped with a YM-30 membrane prior to gel filtration chromatography (Sephacrose S-200) in buffer A + 200 mM KCl. The resulting purified fusion proteins were stored on ice and used for assays within 2 days of purification.

RESULTS

P450_{cam}-PdR-Pd Constructs—Three P450_{cam}-PdR-Pd fusion proteins with different linkers (Fig. 2) between the P450_{cam} and PdR domains have been expressed in *E. coli*. In all three proteins, the linker between the PdR and Pd domains remained the same (Fig. 2). The absorption spectra (300–600 nm) of the three fusion proteins in the ferric, ferrous, and ferrous CO-complexed states (data not shown) are almost identical to those of the corresponding states of the PdR-Pd-P450_{cam} fusion protein (see below).

The activities of the three initial fusion proteins were compared by measuring their O₂ consumption in the presence and absence of camphor. A background O₂ consumption of 6–8 min⁻¹/protein molecule was measured with all the constructs when incubated with NADH in the absence of camphor. This corresponds to the O₂ consumption observed when a mixture of native PdR (2 μM), Pd (8 μM), and NADH (1 mM) is incubated without P450_{cam} or camphor. The catalytic activity of the fu-

sion protein is determined from the increase in O₂ consumption when camphor is added to the fusion protein in the presence of NADH and is thus a minimum value because uncoupled NADH consumption may decrease in the presence of the substrate. As shown by the camphor-inducible O₂ consumption (Fig. 3), increasing the length of the linkers in the P450_{cam}-PdR-Pd construct does not significantly alter the catalytic activity. The activities of the fusion proteins increase linearly with their concentration, as exemplified by P450_{cam}-PdR-Pd (Fig. 3, inset), indicating that electron transfer from NADH via PdR and Pd to P450_{cam} occurs via an intramolecular pathway rather than by intermolecular transfer of electrons from one fusion protein to another. The latter would produce a higher order dependence of rate on the enzyme concentration.

Electron flow through the individual parts of the fusion protein can be monitored with the help of alternative electron acceptors. Experiments show that PdR and Pd are both able to reduce ferricyanide, but only Pd can reduce cytochrome *c*. This difference has made it possible to identify the rate-limiting step in turnover of the fusion proteins. The NADH-dependent rate of cytochrome *c* reduction by increasing amounts of P450_{cam}-(PG)₄-PdR-Pd has been compared with the rate of cytochrome *c* reduction by increasing amounts of Pd in the presence of 4 μM

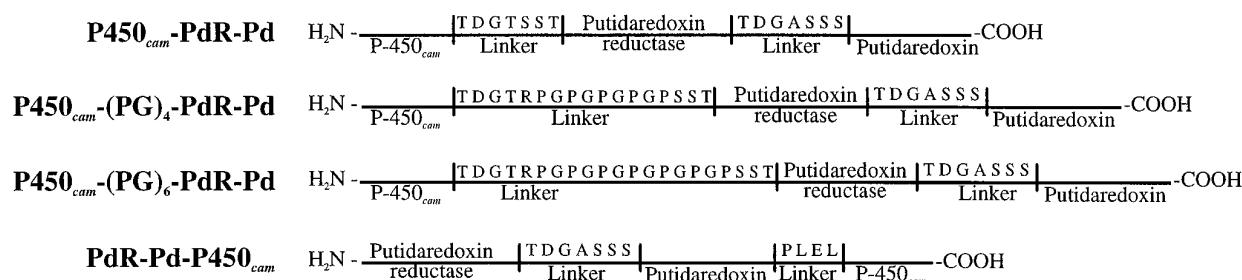


FIG. 2. Schematic diagram showing the linkers between the protein domains in each of the fusion proteins. P450_{cam}-(PG)₆-PdR-Pd, P450_{cam}-PdR-Pd with a 12-amino acid extension inserted into the 7-amino acid linker.

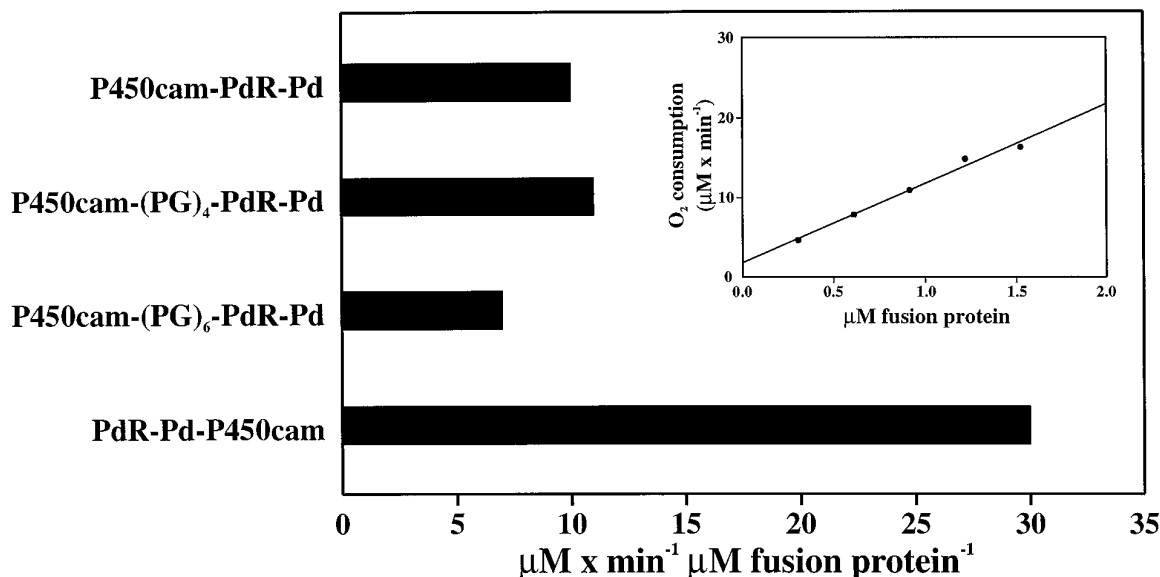


FIG. 3. Catalytic activities of the four fusion proteins as indicated by O₂ consumption measurements. In the inset, the relationship between the catalytic rate and the concentration of the P450_{cam}-PdR-Pd fusion protein is presented. P450_{cam}-(PG)₆-PdR-Pd, P450_{cam}-PdR-Pd with a 12-amino acid extension inserted into the 7-amino acid linker.

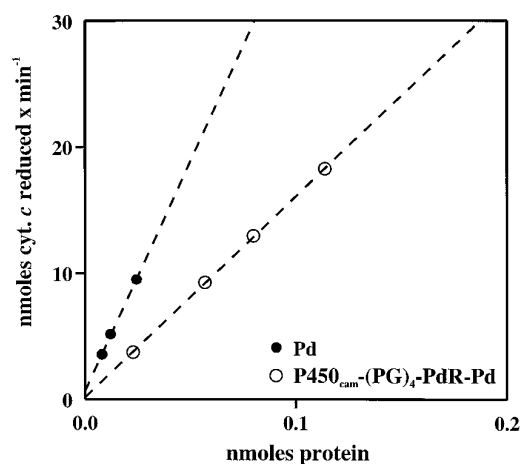


FIG. 4. Comparison of the reduction of 10 μM cytochrome *c* by increasing concentrations of Pd in the presence of 4 μM PdR (●) and by increasing concentrations of the PdR-Pd-P450_{cam} fusion protein (○).

PdR (Fig. 4). Although the rate of cytochrome *c* reduction by the fusion protein is slower than that by native Pd, the fusion protein is still able to deliver electrons at a rate considerably faster than that needed to sustain the observed P450_{cam} activity of 10 turnovers/min. Furthermore, addition of exogenous P450_{cam} to the fusion protein stimulates O₂ consumption above the levels obtainable with the fusion protein alone. The increase in O₂ consumption due to addition of P450_{cam} to the

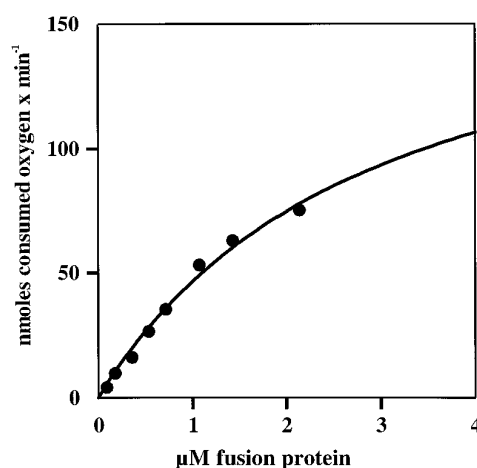
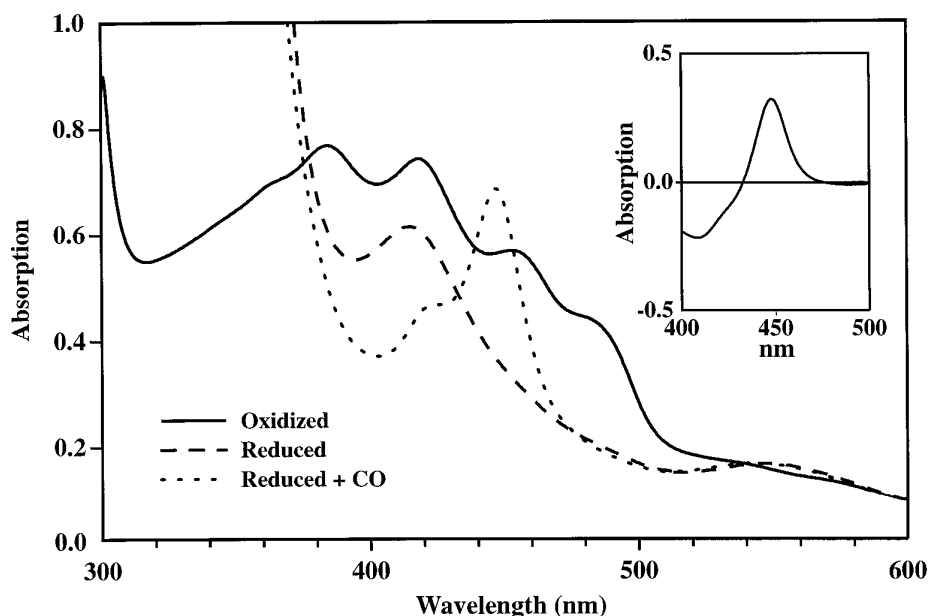


FIG. 5. Catalytic activity as determined by the O₂ consumption of P450_{cam} (0.3 μM) incubated with 1 mM NADH, 1 mM camphor, and increasing concentrations of P450_{cam}-(PG)₄-PdR-Pd. The fusion protein was the sole source of reducing in these incubations.

P450_{cam}-(PG)₄-PdR-Pd fusion protein is shown in Fig. 5. The V_{max} value from the plot is ~600 nmol of O₂/min/nmol of native P450_{cam}, or ~30% of the value for native P450_{cam} supplied with saturating amounts of Pd and PdR. The data clearly show that the P450_{cam}-(PG)₄-PdR-Pd fusion protein has a larger electron donating capacity at the electron-donating site of the Pd domain than is utilized by the hemoprotein domain of the fusion protein.

FIG. 6. Absorption spectra of PdR-Pd-P450_{cam} in the ferric state (—), dithionite-reduced ferrous state (---), and ferrous CO-complexed state (····). The $\pm$ CO difference spectrum is shown in the inset.



The P450_{cam} domain is also capable of faster turnover than indicated by the net activity of the fusion protein, as shown by the finding that the P450_{cam}-(PG)₄-PdR-Pd O₂ consumption increases to 280 nmol of O₂/min/nmol of fusion protein in the presence of 4 μ M native Pd (data not shown). Surplus electrons can thus be donated from the PdR and Pd domains to exogenous Pd and from exogenous Pd to the P450_{cam} domain, indicating that the interaction between the Pd and P450_{cam} domains of the fusion protein is suboptimal.

PdR-Pd-P450_{cam} and PdR-Pd Constructs—As the results with the three P450_{cam}-PdR-Pd constructs indicated that the PdR-Pd unit of the fusion proteins works well, we constructed a binary PdR-Pd fusion protein containing only these two domains and a ternary fusion protein in which the PdR-Pd unit was placed before rather than after the P450_{cam} domain (Figs. 1 and 2). The PdR-Pd-P450_{cam} fusion protein in the presence of 500 μ M camphor exhibits a normal reduced CO difference spectrum (Fig. 6, inset). The spectrum of the ferrous CO-complexed fusion protein indicates that a small fraction of the P450_{cam} domain is in the P420 state. The ferric camphor-bound spectrum indicates that only ~50% of the P450_{cam} domain shifts from the low to the high spin state upon substrate binding despite the high concentration of camphor (Fig. 6). The protein that remains in the low spin state may be partially denatured. Expression of the fusion protein at temperatures between 15 and 30 °C reveals that the low spin component declines from ~95% at 30 °C to 50% at 20 °C (data not shown). Lowering the temperature below 20 °C did not further increase the proportion of the protein that undergoes a low to high spin transition, but markedly decreased the expression rate.

Despite attachment of the C terminus of Pd to the N terminus of P450_{cam}, the PdR-Pd-P450_{cam} construct is three times more active than the best of the P450_{cam}-PdR-Pd fusion proteins (Fig. 3). The activity of PdR-Pd-P450_{cam} increases linearly in proportion to the protein concentration, and its spectroscopic properties are almost identical to those of the reverse-order fusion proteins. The interaction between Pd and P450_{cam} is still clearly suboptimal, however, because addition of native P450_{cam} to the fusion protein again increases O₂ consumption due to increased substrate turnover (data not shown).

The ability of the binary PdR-Pd construct to support the activity of native P450_{cam} is almost as good as that of a 4:1 ratio of the two separate proteins at the concentrations used in our

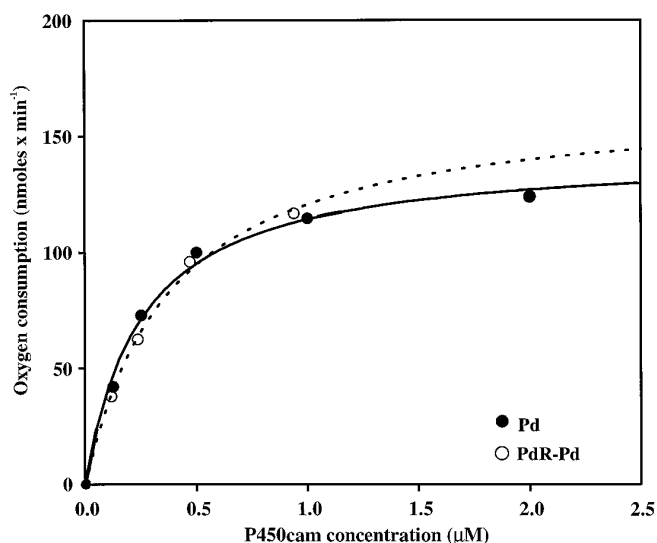


FIG. 7. Catalytic activities of increasing concentrations of P450_{cam} incubated with 2 μ M PdR (3.2 nmol) and either 0.625 μ M Pd (1 nmol) (●) or 0.625 μ M PdR-Pd binary fusion protein (1 nmol) (○).

standard P450_{cam} assay (Fig. 7). Optimal interaction between the Pd and P450_{cam} domains of a ternary fusion protein could therefore have produced an activity of 166 turnovers/min, the k_{cat} of the PdR-Pd construct with native P450_{cam}.

Coupling of O₂ Consumption to Camphor Metabolism—Perturbation of the P450_{cam}-substrate interaction is accompanied by partial uncoupling of O₂ consumption from substrate oxidation (18, 46–48). The O₂ not utilized for substrate oxidation is reduced to H₂O₂ or H₂O (49). The possibility therefore exists that attachment of P450_{cam} to the PdR-Pd domain will result in uncoupled turnover. However, the correlation between O₂ consumption and camphor formation for PdR-Pd-P450_{cam} is similar to that for the wild-type P450_{cam} system (Fig. 8). The data suggest that slightly more than one camphor molecule is hydroxylated per molecule of NADH, but this reflects a small systematic error in the measured NADH consumption introduced by subtracting the background NADH consumption determined in the presence of the P450 inhibitor metyrapone. The catalytic oxidation of camphor appears to be as well coupled to

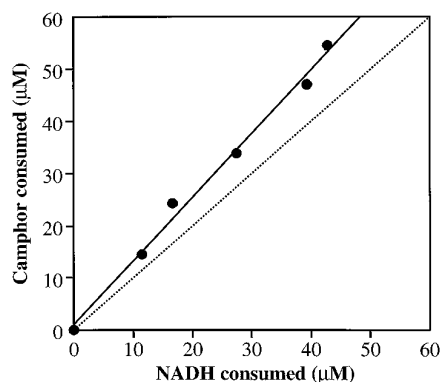


FIG. 8. Comparison of the oxidation of camphor versus the consumption of NADH in incubations of 4 μM PdR-Pd-P450_{cam} fusion protein (●). The dotted line is the correlation expected if each NADH molecule consumed gives rise to a hydroxylated camphor molecule. A similar correlation is observed if O₂ rather than NADH consumption is measured. Incubation of 0.5 μM native P450_{cam} reconstituted with 2 μM PdR and 8 μM Pd gives a line close to the ideal 1:1 correlation.

O₂ consumption in the PdR-Pd-P450_{cam} fusion protein as it is in the native system.

Comparison with the Native System—The activities of PdR-Pd-P450_{cam} and the P450_{cam} system reconstituted with equimolar concentrations of the three components have been compared. As the activity of the fusion protein, in contrast to that of the reconstituted native system, increases linearly with concentration (Fig. 9), we have determined at which concentration PdR-Pd-P450_{cam} and the native 1:1:1 P450_{cam} system have the same activity. This defines a concentration below which the fusion protein is more efficient than the reconstituted system. The activities of the two systems are equal when their respective concentrations are 0.3 μM (Fig. 9). Another way of comparing the two systems is to compare the measured activity of the fusion protein with the theoretical maximum based on the V_{max} of the slowest of the three proteins that make up the native catalytic system. With an excess PdR concentration of 4 μM , the electron flow through a single Pd molecule to cytochrome *c* is 365 min⁻¹ (Fig. 4), which corresponds to a possible P450_{cam} turnover rate of 182 min⁻¹. The fusion protein activity of 30 turnovers/min (Fig. 3) is ~15% of this maximum, assuming that the efficiency of electron transfer from Pd to P450_{cam} and cytochrome *c* is comparable.

Activity of the PdR-Pd-P450_{cam} Fusion Protein in Intact *E. coli*—The oxidation of camphor by *E. coli* cells expressing PdR-Pd-P450_{cam} was examined to determine whether the enzyme is catalytically active within intact *E. coli*. PdR-Pd-P450_{cam} was expressed overnight in *E. coli*; fresh medium and 1 mM camphor were added; and 10-ml samples were incubated in closed vials with a head space equal to five times the volume of the samples. The PdR-Pd-P450_{cam} content of each sample was 1.8 nmol (0.18 μM), as judged by spectroscopic P450 assays. The samples were extracted with CH₂Cl₂ at various time points over a 5-h period, and the camphor and camphor metabolites in the organic extract were quantitated by gas-liquid chromatography (Fig. 10). Cineole was added as an internal standard just before the extraction. Control samples of 1 mM camphor in cell-free medium were incubated in parallel. The loss of camphor by evaporation from these samples was below the detection limit. Because of differences in the extraction efficiency of cineole, camphor, and the camphor metabolites, correction factors were calculated using a set of standards generated by incubating native P450_{cam}, PdR, and Pd with 1 mM camphor and increasing but limiting amounts of NADH. Amounts of *E. coli* cells (as judged by A₆₀₀) and medium equal to those found

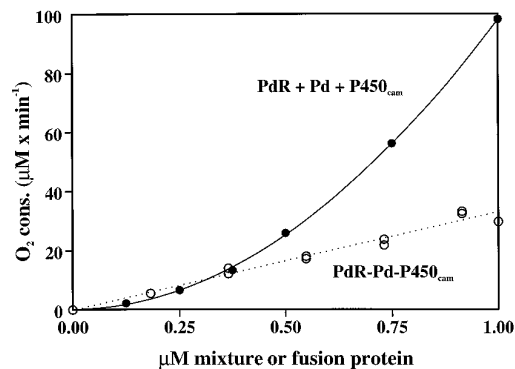


FIG. 9. Catalytic activities as a function of the concentration of the PdR-Pd-P450_{cam} fusion protein (○) or P450_{cam} reconstituted in a 1:1:1 ratio with PdR and Pd (●).

in the experimental cultures were added to the standards prior to extraction, as the cells and medium affect the extraction efficiency. Fig. 10 shows the chromatographic results after incubation for 0, 1, and 4 h, and the inset shows the quantitative data obtained by integrating the peaks at all time points and correcting for extraction efficiency differences. The two products detected are 5-*exo*-hydroxycamphor (retention time = 8.6 min) and 5-oxocamphor (retention time = 6.8 min) (Fig. 10). The identities of these products were established by coelution with authentic samples. Camphor elutes at 5.5 min and cineole at ~4.4 min. The peak at 7.3 min is present even when no camphor is added and is therefore due to a component of the incubation system. A good quantitative correlation exists between the disappearance of camphor and the appearance of the two metabolites (Fig. 10). The production of 5-*exo*-hydroxycamphor in incubations of intact *E. coli* cells expressing PdR-Pd-P450_{cam} increases as a function of the nominal camphor concentration, but much higher concentrations of camphor are required to saturate the system than are required with the isolated, purified fusion protein (Fig. 11). If the *E. coli* cells are sedimented by centrifugation, washed twice with water, and then incubated with camphor, it is possible to convert camphor to 5-*exo*-hydroxycamphor uncontaminated by compounds present in the medium used for bacterial growth. The camphor-oxidizing activity of the *E. coli* system in normal medium declines with time (Fig. 10, inset).

DISCUSSION

Of the four constructs examined, the triple fusion protein PdR-Pd-P450_{cam} is the most active (Fig. 3). Fusion of the three components in the reverse order gives the less efficient construct P450_{cam}-PdR-Pd regardless of whether a hepta-, penta-, or nonadecapeptide is used to link the P450_{cam} and PdR domains. Comparison of the fusion proteins with the reconstituted P450_{cam} system is not straightforward. The reconstituted system in which P450_{cam}, PdR, and Pd are present in a 1:8:20 ratio, respectively, can give product formation rates as high as 2300 nmol/min/nmol of P450_{cam} (48). A more appropriate comparison is one in which P450_{cam}, PdR, and Pd are present in a 1:1:1 ratio, as they are in the fusion protein, but even this comparison is not straightforward because the activity of the fusion protein depends linearly on protein concentration, whereas that of the reconstituted protein exhibits a higher order concentration dependence. The results indicate that the catalytic rate for the fusion protein is slightly higher than that for a 1:1:1 reconstituted P450_{cam} system when the protein concentration is <0.3 μM (Fig. 9). In catalytic terms, the PdR-Pd-P450_{cam} fusion protein is less efficient than the reconstituted wild-type system. Whatever shortcomings the PdR-Pd-P450_{cam} fusion protein has in terms of catalytic efficiency are

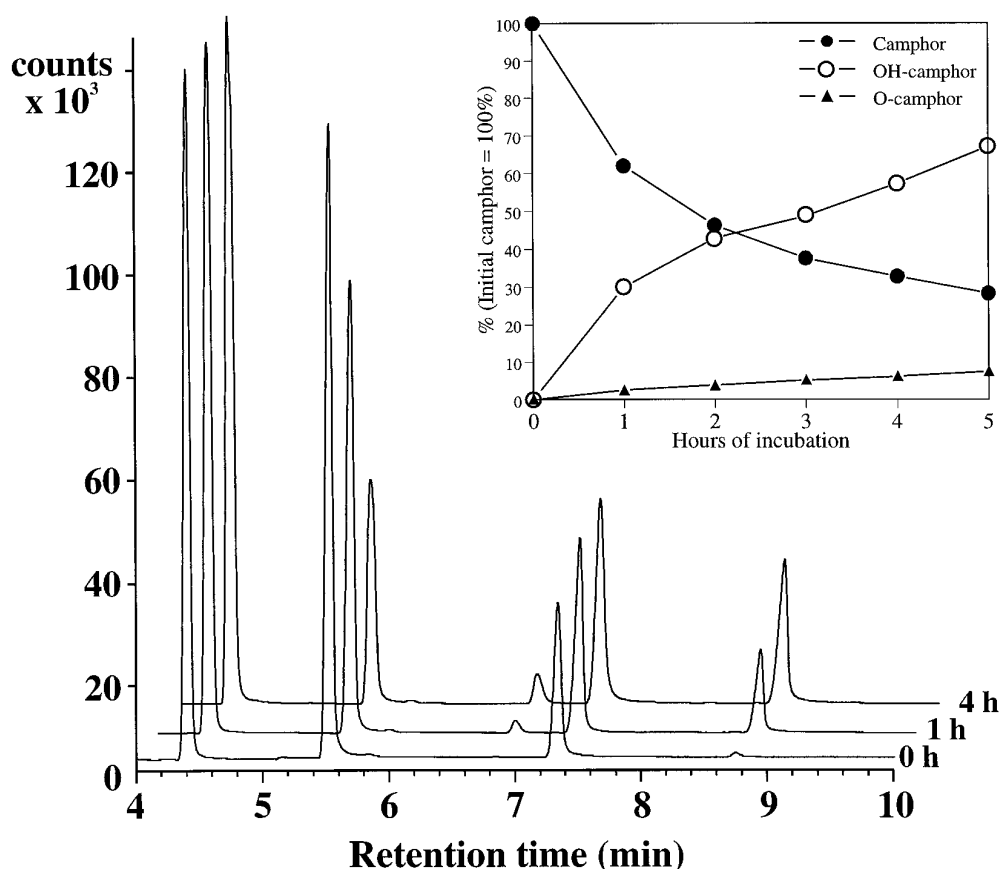


FIG. 10. Gas-liquid chromatograms of CH_2Cl_2 extracts from cultures of *E. coli* cells expressing the PdR-Pd-P450_{cam} fusion protein after 0, 1, and 4 h of incubation. The consumption of camphor and the appearance of metabolites as a function of time are given in the inset. The data have been normalized with respect to an internal standard (cineole). ●, camphor; ○, 5-*exo*-hydroxycamphor; ▲, 5-oxocamphor.

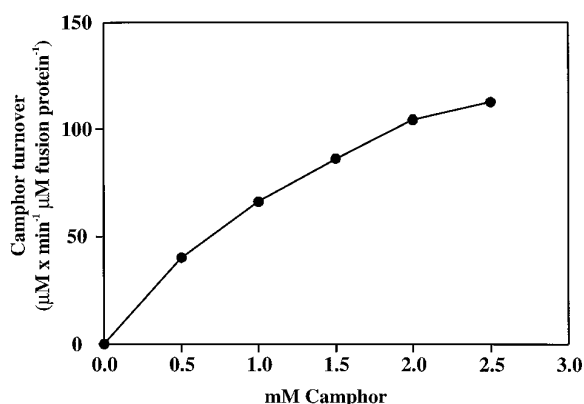


FIG. 11. Camphor turnover as a function of the nominal camphor concentration in incubations with intact *E. coli*. The data are expressed in terms of camphor product formation/P450 molecule in the intact bacteria.

partially compensated for by the fact that P450_{cam} is intrinsically a relatively fast P450 enzyme. Thus, the observed k_{cat} of $\sim 30 \text{ min}^{-1}$ (Fig. 3) is comparable to the k_{cat} values observed with most reconstituted native P450 systems (e.g. Ref. 50).

Two parameters, the order of the components and the length of the linker between the PdR and P450_{cam} domains, have been varied in limited efforts to optimize the P450_{cam} fusion system. The order of the three components appears to be more critical than the length of the linker. Earlier studies of the fusion of P450c17 with yeast P450 reductase suggested that the nature of the linker between the two domains can greatly alter the activity (3, 4), but these studies were done with intact cells rather than with purified enzymes. It is therefore not possible

to determine if linker modification alters electron transport or influences the extent to which correctly folded fusion protein is formed. In agreement with our finding that the order of the components is important, it has been shown that placing P450 reductase at the C rather than the N terminus of a P450c21-P450 reductase fusion protein gives a more active catalytic system (5).

Analysis of the electron flow through the triple fusion proteins shows that suboptimal interaction between the Pd and P450_{cam} domains limits catalytic turnover. The results with the PdR-Pd binary fusion protein establish that electron transfer from PdR to Pd is as effective when the two proteins are fused together as when they are part of a 4:1 Pd/PdR reconstituted native system (Fig. 7). Although electron transfer from PdR to Pd in the binary fusion protein may occur by intra- or intermolecular pathways, the transfer appears to be exclusively intramolecular with the triple fusion proteins (e.g. Fig. 3, inset). Furthermore, the results with the binary fusion protein indicate that docking of PdR-Pd to wild-type P450_{cam} is not impaired. Inefficient turnover, presumably due to inefficient electron transfer from Pd to P450_{cam} in the triple fusion protein, is therefore due to structural constraints in the fusion protein that prevent optimal intramolecular interaction of these two domains. This inference is supported by the finding that exogenous Pd accelerates turnover of the fusion protein, which indicates that wild-type Pd binds normally to the P450_{cam} domain, whereas the Pd domain within the same fusion protein does not. Likewise, the PdR-Pd domain of the triple fusion protein is able to support higher turnover of P450_{cam}, as demonstrated by an increase in substrate turnover when exogenous P450_{cam} is added to the fusion protein. Despite the ability of the P450_{cam} and Pd domains to interact with exogenous wild-type

Pd or P450_{cam}, respectively, the linear dependence of the rate on the concentration of the fusion protein indicates that the Pd domain of one fusion protein does not interact efficiently with the P450_{cam} domain of another (Fig. 3, *inset*).

Expression of PdR-Pd-P450_{cam} in *E. coli* yields a catalytic system that cleanly oxidizes camphor to 5-*exo*-hydroxycamphor and 5-oxocamphor, the normal metabolites produced by P450_{cam} (Fig. 10). These results demonstrate the feasibility of constructing bacterial bioreactors for the defined metabolism of xenobiotics or synthesis of fine chemicals. It is of interest that 5-oxocamphor is formed earlier *in vivo* than expected from the oxidation of camphor by the purified fusion protein or a reconstituted P450_{cam} system. Normally, the secondary oxidation of 5-*exo*-hydroxycamphor to 5-oxocamphor occurs only after virtually all of the camphor has been converted to 5-*exo*-hydroxycamphor (data not shown). The early appearance of 5-oxocamphor suggests that (a) slow diffusion into the cells limits the local camphor concentration and/or (b) slow diffusion out of the cells elevates the local 5-*exo*-hydroxycamphor concentration. Support for the inference that the local concentration of camphor is limited is provided by the observation that much higher nominal concentrations of camphor are required to saturate the enzyme in intact cells than in the isolated, purified state (Fig. 11). The time-dependent decrease in the camphor-oxidizing activity of the bacterial system (Fig. 10) may reflect localized exhaustion of camphor and/or inhibition of the enzyme by intracellular accumulation of 5-hydroxy- and 5-oxocamphor. The possibility of camphor-dependent fusion protein inactivation or impairment of bacterial cell function cannot be excluded, however.

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