Improving the Cytochrome P450 Enzyme System for Electrode-Driven Biocatalysis of Styrene Epoxidation

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Cytochrome P450 enzymes catalyze a vast array of oxidative and reductive biotransformations that are potentially useful for industrial and pharmaceutical syntheses. Factors such as cofactor utilization and slow reaction rates for nonnatural substrates limit their large-scale usefulness. This paper reports several improvements that make the cytochrome P450cam enzyme system more practical for the epoxidation of styrene. NADH coupling was increased from 14 to 54 mol %, and product turnover rate was increased from 8 to 70 min^{-1} by introducing the Y96F mutation to P450cam. Styrene and styrene oxide mass balance determinations showed different product profiles at low and high styrene conversion levels. For styrene conversion less than about 25 mol %, the stoichiometry between styrene consumption and styrene oxide formation was 1:1. At high styrene conversion, a second doubly oxidized product, α-hydroxyacetophenone, was formed. This was also the exclusive product when Y96F P450cam acted on racemic, commercially available styrene oxide. The α-hydroxyacetophenone product was suppressed in reactions where styrene was present at saturating concentrations. Finally, styrene epoxidation was carried out in an electroenzymatic reactor. In this scheme, the costly NADH cofactor and one of the three proteins (putidaredoxin reductase) are eliminated from the Y96F P450cam enzyme system.

Introduction

Chiral epoxides are used extensively in industry for production of pharmaceuticals and fine chemicals (*1*). The growing appreciation of new products arising from improved or novel properties of optically active compounds has led to a substantial rise in asymmetric synthesis methods, including biocatalytic approaches involving whole organisms or single enzymes (*2*). The epoxides are useful by themselves as synthetic precursors or for generating chiral synthons via a variety of ring-opening chemistries. Epoxidation of styrene at the vinyl position leads to styrene oxide, which can adopt either *S*-(−) or *R*-(+) chirality. Control of the stereospecificity of this epoxidation may offer new opportunities in polymer synthesis and in pharmaceutical and agricultural product development. In addition, the two enantiomers often formed during biological decomposition of styrene give different toxicological responses in mammals (*3*).

Among all biosynthetic routes for oxygenating styrene at the vinyl position (*4*−*8*), monooxygenase biocatalysis, as carried out by whole cells (*4*, *5*) or by isolated cytochrome P450 enzymes (*6*, *8*), has been shown to give the highest enantiomeric excess of single stereoisomers at high turnover rates. Both whole cell and isolated enzyme approaches have practical hurdles limiting their industrial usefulness. Process advantages and limitations for whole cell epoxidation processes have been described by Furuhashi (*2*). An important practical hurdle is the constraint of needing the enzyme's natural source of reducing power, an essential feature for maintaining the stereospecificity of styrene epoxidation via in vitro P450 styrene monooxygenase activity. Peroxidase activity using alternative oxidants (e.g., hydrogen peroxide) leads to styrene oxygenation, but with loss of product optical purity and reduction of turnover rate (*9*).

P450s require reducing power in the form of expensive cofactors such as NADH and NADPH and one or two additional redox proteins to transfer electrons to the P450 active site. At costs such as $8500/mole for NADH in kilogram quantities (Schweizerhall, Inc., Piscataway, NJ), industrial application of reactions involving such cofactors would necessitate efficient, long-lived cofactor regeneration schemes. NAD(P)H regeneration for industrial-scale syntheses has been reviewed (*10*).

In this study, we employ P450cam, a three-protein enzyme system originally isolated from a strain of *Pseudomonas putida* that uses camphor as its sole carbon source (*11*) to develop a practical in vitro enzymatic process for epoxidation of styrene to styrene oxide. P450cam is the single most studied and characterized P450 and serves as the model for P450 biocatalysis. In the native reaction, the hydroxylase activity of cytochrome P450cam (CYP101; EC 1.14.15.1) catalyzes conversion of 1*R*-(+)-camphor to 5-*exo*-hydroxycamphor via a putative iron-oxo species (*12*). Reducing equivalents are passed from NADH to CYP101 through a FAD-containing redox protein, putidaredoxin reductase (PdR), and an iron−sulfur ferredoxin, putidaredoxin (Pdx). Putidaredoxin passes two electrons to the cytochrome in two distinct electron-transfer steps along the catalytic cycle. Other low potential iron−sulfur proteins can perform these individual electron-transfer steps, but to date no

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other protein has been reported that can perform the complete function of Pdx in the catalytic oxygenation cycle. Spinach ferredoxin and bovine adrenodoxin can donate the first electron to CYP101 but not the second electron, whereas reduced rubredoxin and cytochrome b_5, which are incapable of giving the first electron, can provide the second electron yielding the reaction products (*13*).

The coupling of NADH consumption to product formation is close to 100 mol % for camphor hydroxylation. P450cam has been found to oxygenate a large number of substrates, from camphor analogues such as norcamphor to aromatic alkanes such as ethylbenzene and tetralin (*6, 14*). Coupling of NADH utilization to product formation decreases when P450cam converts non-camphorlike substrates. Three putative electron shunt reactions, derived from oxidation of the P450cam active site iron, have been shown to produce superoxide, hydrogen peroxide, or water (*15*). Overall product formation rates are also significantly lower for non-native substrates. There have been previous efforts to engineer the active site of P450cam to accommodate non-native substrates (*16, 17*), including styrene (*8*). Product turnover rates and NADH utilization efficiency can be improved in this way by controlling water access to the heme center of the reduced, substrate-bound P450cam.

In this study, improvements in applying P450cam biocatalysis to styrene oxidation are made in several different ways. Site-directed mutagenesis is employed to generate a P450cam Y96F mutant that is more suited for hydrophobic substrate oxidation. NADH coupling and product formation rates for styrene oxidation are measured for wild type and Y96F P450cam. Mass balance measurements are made so that styrene to styrene oxide stoichiometry under low and high styrene conversion conditions can be determined. Finally, we adapt our previous electroenzymatic reactor for camphor hydroxylation with wild-type P450cam (*18*) to the epoxidation of styrene with the Y96F mutant.

Materials and Methods

Protein Expression and Site-Directed mutagenesis. Plasmids (pIBI125) containing the wild-type cytochrome P450cam gene were purified from *E. coli* DH5α cells. Site-directed mutagenesis (QuikChange, Stratagene) was carried out to substitute phenylalanine for tyrosine at amino acid residue 96. Products of the mutagenesis reaction were transformed into *E.coli* XL1-Blue cells (Stratagene). Individual transformants were grown in 5 mL of YT broth, and the plasmids were purified from cell lysates (Wizard Plus miniprep, Promega). Individual plasmids were sequenced with dye-terminator cycle sequencing chemistry (Applied Biosystems) using a Perkin-Elmer 9600 thermocycler and an Applied Biosystems 373 DNA sequencer. Plasmids containing the mutation were transformed into *E. coli* DH5α cells, and cell lines that showed the best level of expression were selected for subsequent culture.

Protein Purification. Putidaredoxin (Pdx), putidaredoxin reductase (PdR), and cytochrome P450cam were expressed in recombinant *E. coli* and purified as previously described (*19*). Purification of the Y96F mutant P450cam was identical to the wild-type protein. Pdx and PdR proteins were quantified from absorptivity coefficients taken from previous studies (*20, 21*). Wild type and Y96F P450cam were quantified using absorptivity coefficients shown in Table 2. Absorbance ratios and SDS−PAGE were used to measure the purity of Pdx, PdR, and wild-type P450cam. As a result of the different absorbance spectrum of Y96F P450cam, only SDS−PAGE was used to measure purity. All protein preparations exceeded mole fraction purity of 0.97. Prior to experiments, proteins were buffer exchanged with 50 mM Tris−HCl, 0.2 M KCl, pH 7.4 (22 °C) using established protocols (*19*).

Absorptivity Measurements. A solution of approximately 1 mM wild-type P450cam or the Y96F mutant was prepared in Buffer S {50 mM Tris−HCl, 0.2 M KCl, pH 7.4 (22 °C), 1 mM dithiothreitol (DTT, Sigma Chemical, St. Louis, MO) with 1 mM camphor (Sigma Chemical, St. Louis, MO)} and kept at 4 °C until further use. DTT was added to the reference solution so that formation of small amounts of P420 or dimerized P450cam was prevented (*22*). It was determined previously that the addition of 1 mM DTT had no effect on the camphor-bound P450cam spectrum from 350 to 600 nm. A 25 mg portion of reference solution was diluted gravimetrically into 6 g of Buffer S. The diluted reference solution was then placed into a quartz cuvette, and the absorbance spectrum from 350 to 600 nm was measured in a DU-650 spectrophotometer (Beckman-Coulter, Fullerton, CA). The cell holder was thermostated at 22 °C with a circulating water bath (Neslab, Newington, NH). Two dilutions of each reference solution were made, and three scans were recorded for each diluted reference solution. The standard deviation at any point in the spectrum was less than 0.2%.

Concentration of P450cam or the Y96F mutant in the reference solution was determined by quantifying iron content by protein digestion, followed by complexation of iron with phenanthroline (*23*). In short, samples of reference solution were added gravimetrically to 5 mL glass volumetric flasks, after which perchloric acid and hydrogen peroxide were introduced. The samples were digested at 100 °C for 30 min. Hydroxylammonium chloride (Sigma Chemical, St. Louis, MO) was used to reduce the iron, and then 1,10-phenanthroline (Sigma Chemical, St. Louis, MO) was added to the flask, followed by pyridine and water. A reagent blank omitting the protein sample was treated identically. The corrected absorbance at 510 nm was then used to calculate the iron content of the sample. A linear calibration curve for iron vs absorbance was developed using either a standard iron solution or iron(II) ammonium sulfate standards (Sigma Chemical, St. Louis, MO). The calculated extinction coefficient of iron using this method was 11376 M^{-1}. The standard deviation was 0.4%. Six independent digestions and iron determinations were made for the reference solution. Overall relative uncertainty for the measured absorptivity coefficients was calculated to be 3%.

NADH Utilization Measurements. NADH utilization measurements were carried out with the DU-650 spectrophotometer using a cell holder held at 22 °C by a circulating water bath (Neslab, Newington, NH). All reaction solutions contained 2 mM styrene, 10 μM Pdx, 1 μM PdR, 2000 U /mL bovine liver catalase (Boehringer-Manheim, Indianapolis, IN) in Buffer T (50 mM Tris−HCl, 0.2 M KCl, pH 7.4, 22 °C). For measurements with wild-type P450cam, 0.5 μM P450cam was added, while for measurements on the Y96F mutant, 0.1 μM of the cytochrome protein was added. The reaction was initiated by the addition of 250 μM NADH, and its concentration measured by monitoring absorbance at 340 nm (ϵ_{340} = 6.22 mM^{-1} cm^{-1}). Incubations devoid of P450cam were performed to measure the Pdx-dependent NADH oxidation. This value was subtracted from the total NADH

oxidation rate to yield the P450cam-dependent NADH utilization rate.

Extraction and Measurement of Reaction Mixture Analytes. Extraction proceeded in the same microvial in which reaction occurred in order to minimize systematic errors (e.g., analyte absorption onto glass vials). Two hundred microliters of saturated KCl, followed quickly by 110 μL of chloroform and 10 μL of *n*-decane in chloroform (internal standard, Sigma-Aldrich, St. Louis, MO) were added to each microvial gravimetrically by injection through the septum. Vigorous shaking was achieved by immobilizing the vials sideways on a Vortex Genie (Daigger, Inc., Lincolnshire, IL) and agitating at the highest setting for 10 min. Centrifugation for 10 min at 5000 $\times$ *g* efficiently broke the protein emulsion and separated aqueous and organic layers. Aliquots (1.0 μL) of the chloroform phase were injected into a HP 5890 GC chromatograph, fitted with an HP-5 cross-linked 5% phenylmethyl silicone glass capillary column (Hewlett-Packard, Wilmington, DE). The injector temperature was 250 °C, and the FID detector temperature was 270 °C. Carrier gas velocity was 100 cm/s, measured at 60 °C. The initial temperature was 60 °C, held for 1 min postinjection. The temperature was increased 12 °C per min to 130 °C, then 20 °C per min to 225 °C. Retention times were established with authentic samples (Sigma-Aldrich, St. Louis, MO) for styrene (2.2 min), benzaldehyde (3.0 min), *n*-decane (3.5 min), phenylacetaldehyde (4.0 min), styrene oxide (4.3 min), α-hydroxyacetophenone (6.4 min), and styrene glycol (7.1 min). Response factors were calculated for styrene and styrene oxide from calibration solutions. Linearity was established between 5 and 500 μM for styrene and styrene oxide.

Hydroxyacetophenone Identification. The identity of the compound formed by P450 catalysis of styrene oxide was elucidated by GC-MS analysis performed under contract (SAIC, Inc., NCI-FCRDC, Frederick, MD). Electron impact source energy was 70 eV, and GC conditions were identical to the conditions stated above. A reaction to obtain large quantities of the doubly oxidized compound was carried out under the following conditions. A 4 mL solution was composed of racemic styrene oxide (2 mM), NADH (10 mM), catalase (2000 U/mL), and P450 proteins (5 μM Pdx, 1 μM PdR, 5 μM Y96F P450cam). After 2 h of incubation at 22 °C, the reaction mixture was extracted as described above. GC analysis of the extract showed that approximately 85 mol % of the styrene oxide was converted to a doubly oxidized compound, demonstrating that either enantiomer of styrene oxide was capable of being converted. GC-MS analysis of this extract and the TMS derivative were consistent with the doubly oxidized product having the identity of α-hydroxyacetophenone (CAS 582-24-1). Later, commercially available samples of α-hydroxyacetophenone (Sigma-Aldrich, St. Louis, MO), were analyzed by GC-MS and produced equivalent ion fragmentation patterns and molecular ion peaks.

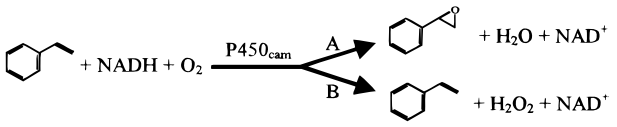
Mass Balance and NADH Coupling. Incubations for the quantification of styrene consumption and/or styrene oxide production were performed in 1.0 mL septum glass conical microvials (Kimble, Vineland, NJ). This prevented evaporation of analytes and improved extraction reproducibility. All solutions were formed by gravimetric addition directly into each microvial with glass/steel microsyringes (Hamilton, Reno, NV). Solutions for NADH coupling determinations were composed of 10 μM P450cam (wild-type or Y96F), 10 μM Pdx, 1 μM PdR, 2000 U/mL bovine liver catalase, and Buffer T saturated with styrene (Sigma Chemical, St. Louis, MO). NADH concentrations ranging from 200 to 500 μM were used to initiate the reaction. The incubation volume was 0.6 mL, allowing for ample headspace to maintain oxygen saturated conditions throughout the experiment. The reaction was quenched by chloroform extraction after the NADH was entirely consumed (0.2−2 min). Corrections for the small amount of Pdx auto-oxidation that occurred during the reaction were made using an auto-oxidation turnover rate of 0.2 min^{-1}, as determined using established protocols (*24*). Calibration solutions for styrene oxide quantification were made by adding known amounts of styrene oxide (Sigma Chemical, St. Louis, MO) to solutions that were identical to the reaction mixtures except that NAD^+ was substituted for NADH. Mass balance measurements were done in solutions containing 3 μM Pdx, 0.3 μM PdR, 0.3 μM Y96F P450cam, and 2000 U/mL catalase. NADH concentration was in excess at 3 mM. The reaction was initiated by adding styrene in dimethyl sulfoxide (DMSO) to give an initial reaction concentration of 180 μM styrene and approximately 100 mM DMSO. The use of DMSO to make up stock styrene solutions was seen to improve the accuracy of the initial styrene concentration measurement, and control experiments showed no effect of its use on reaction rate or product profile. Reactions were quenched by chloroform extraction at time points between 2 and 20 min. Calibration solutions analogous to the NADH coupling calibration solutions were used to quantify both styrene and styrene oxide.

Electrode-Driven P450cam Biocatalysis. Electrolysis in 1 mL of solution was conducted in a 1-cm path length quartz spectrophotometric cell. The cell was equipped with two 1 cm $\times$ 5 cm antimony-doped tin oxide working electrodes, shielded by a Pt mesh, and a Pt wire counter electrode, separated by a glass frit from the main solution volume. Solution was thoroughly purged with argon at open circuit potential, and a gas blanket was maintained in the headspace during the electrolysis. The cell top was sealed using Parafilm. Mixing was provided by a small Teflon coated spinbar driven by a magnetic stirrer. Potential of the working electrode was maintained at −0.7 V vs Ag/AgCl reference electrode, and the potential on the counter electrode was adjusted to +1.0 V by controlling the extent of submergence at fixed working electrode potential. Additional experimental details on the P450cam electroenzymatic reactor are as previously described (*18*). A typical solution contained 0.1 M Tris−HCl, 0.2 M KCl, 20 mM $MgCl_2$, and varying amounts of Pdx and Y96F P450cam. Styrene was introduced in pure form at the solubility limit (approximately 3 mM). Temperature was maintained at 22 °C by a circulating water bath (Neslab, Newington, NH). Samples for analysis (100−300 μL) were taken via a glass/steel microsyringe (Hamilton, Reno, NV) and placed gravimetrically into a septum glass conical microvial. Extraction and quantification were performed as previously described.

Results and Discussion

Characterization of Y96F P450cam. *Styrene Epoxidation.* The active site of wild-type P450cam is composed of several hydrophobic residues and one hydrogen bonding residue, tyrosine 96. X-ray crystallographic structures show that this residue hydrogen bonds with the camphor keto group (*25*). When nonnatural hydrophobic substrates such as ethylbenzene or styrene are introduced to the active site of wild-type enzyme, the lack of optimal substrate orientation coupled with increased water access results in nonspecific oxy-

genation, hydrogen peroxide generation, and decreased efficiency of NADH utilization (*15*). In the case of styrene biocatalysis by P450cam, NADH is turned over to form either styrene oxide or hydrogen peroxide according to the reactions



Replacing tyrosine with phenylalanine makes P450cam more accepting of nonpolar hydrophobic substrates such as styrene. Table 1 compares NADH utilization rate, coupling efficiency, and product formation rate between wild-type P450cam and the Y96F mutant. The NADH efficiency and NADH utilization rate are increased for the Y96F mutant, and styrene oxide formation rate is nearly an order of magnitude higher.

Our measurements and those of Nickerson et al. (*8*) show that the Y96F mutant is an improved biocatalyst for styrene epoxidation. However, our results and theirs do not compare well quantitatively with regard to NADH utilization rate (130 vs 310 min^{-1}) and coupling efficiency (54 vs 32 mol %). The difference in NADH utilization rate is probably due to differences in the protein concentrations, especially with respect to the ratio of Pdx to P450cam (or the Y96F mutant). For our measurements with wild-type, the P450cam concentration was 0.5 μM and the PdR:Pdx:P450cam ratio was 2:20:1; for our measurements with the Y96F mutant, the P450cam Y96F concentration was 0.1 μM and the PdR:Pdx:Y96F ratio was 10:100:1. We chose these conditions in order to keep the Pdx-dependent NADH oxidation rate less than 20% of the total NADH oxidation rate. In Nickerson et al., the concentration used in both wild-type and Y96F mutant measurements was 0.05 μM and the PdR:Pdx:P450cam ratio was 10:320:1, thereby allowing for a greater influence of Pdx auto-oxidation on the measured rate of NADH utilization. We believe the differences in coupling efficiency can be attributed to different strategies used by each group for calibrating analyte GC responses in determining the NADH efficiency measurements. Whereas Nickerson et al. utilized an enzyme + NADH + styrene oxide solution to calibrate styrene oxide, the present work utilized an enzyme + NAD^+ + styrene + styrene oxide calibration solution. Systematic errors (elevated NADH consumption) can result from the calibration method of Nickerson et al. if P450cam reacts with styrene oxide in the presence of NADH, whereas our method eliminates possible reaction by substituting NADH with NAD^+. Evidence will be presented in the next section that shows P450cam does indeed react with styrene oxide.

Absorption Spectra. Figure 1 shows camphor-free and camphor-bound absorption spectra for wild-type and Y96F mutant P450cam. Differences in the spectra for the wild-type and Y96F mutant, along with concern about sensitivity in the Soret region of the spectrum to the buffer composition (especially potassium concentration), temperature, and pH, led us to undertake measurement of the absorptivity coefficients. These measurements appear not to have been done since before the advent of recombinant protein technology (*20, 26*). Spectra were measured in 50 mM Tris−HCl, 0.2 M KCl, 1 mM DTT, with or without 1mM camphor, pH 7.4, at 22 °C. These conditions are known to saturate the enzyme with respect to potassium and camphor (*27*), so that small changes in potassium or camphor concentration do not lead to large fluctuations in spectra. It was determined previously that the addition of 1 mM DTT had no effect on the camphor-bound P450cam spectrum from 350 to 600 nm. Concentration of P450cam was independently determined by perchloric acid/hydrogen peroxide protein digestion followed by spectrophotometric iron determination with phenanthroline (*23*). The measured absorptivity coefficients are shown in Table 2. Camphor-bound Y96F P450cam was not determined as a result of the split Soret peak (see Figure 1). Absorptivities for camphor-free wild-type and mutant enzyme are in close agreement and about midway between the range of previous reports. Our measurements for camphor-bound wild-type enzyme are systematically smaller than the literature reports at all three wavelengths. These discrepancies could be caused by different buffer conditions or the purity or activity of enzyme preparations.

Table 1. Improved Styrene Epoxidation Biocatalysis by Y96F P450cam

reaction parameter	wild-type	Y96F
NADH utilization rate[a], min^{-1}	56 (7)	130 (15)
NADH coupling[b], mol %	14 (1)	54 (1)
product rate[c], min^{-1}	8 (1)	70 (8)

[a] NADH utilization rate measured in Buffer T (50 mM Tris−HCl, 0.2 M KCl, pH 7.4, 22 °C) containing 2 mM styrene, 1 μM PdR, 10 μM Pdx, either 0.5 μM wild-type P450cam or 0.1 μM Y96F mutant, and 2000 U/mL bovine liver catalase. The reaction was initiated by the addition of 250 μM NADH and was followed spectrophotometrically at 340 nm using 6.22 mM^{-1} cm^{-1} absorptivity (extinction) coefficient. Correction for NADH consumption to support unwanted Pdx auto-oxidation was incorporated to give the value reported in the table. Standard deviations are indicated in parentheses. [b] Coupling reactions run under NADH-limiting conditions were {200−500 μM NADH, styrene saturation (≈3 mM at 20 °C), 1 μM PdR, 10 μM Pdx, 10 μM P450cam (wild-type or Y96F mutant), 2000 U/mL catalase in Buffer T}. NADH coupling determined by measuring styrene oxide production after NADH was totally consumed. Results expressed as ratio styrene oxide produced to total NADH consumed. [c] Rate of styrene oxide formation calculated from NADH utilization rate × NADH coupling efficiency.

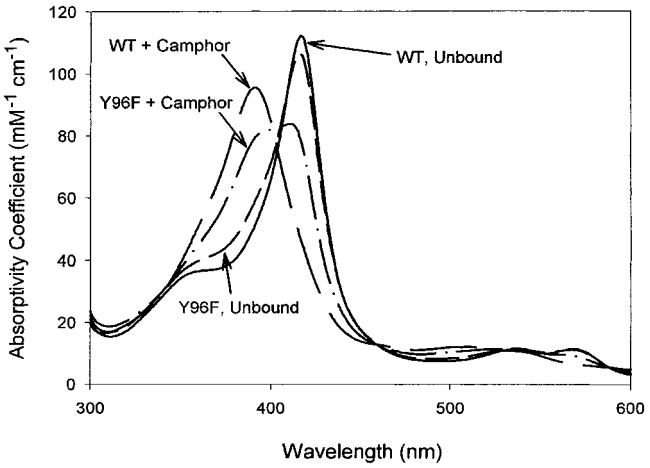


Figure 1. Absorption spectra of wild-type and Y96F mutant cytochrome P450cam in 50 mM Tris−HCl, 0.2 M KCl, 1 mM DTT, pH 7.4, 22 °C, with or without saturated camphor.

NADH-Driven Styrene Epoxidation. The stoichiometry of styrene conversion to styrene oxide by P450cam has not been previously determined. Fruetel et al. (*6*) report several products from styrene incubation with wild-type P450cam. In order of decreasing yield, these products were: benzaldehyde > styrene oxide > phenylacetaldahyde and ring-hydroxylated isomer. Benzalde-

Table 2. Absorptivity Coefficients for Wild-type and Mutant (Y96F) P450cam

wavelength, nm	ϵ (mM^{-1} cm^{-1}), wild-type: this study[a]	wild-type: lit. values	Y96F mutant: this study[a]
	Camphor-Free		
417	112	115[b], 105[c]	107
535	11.2	11.6[b], 10.3[c]	11.3
568	11.4	11.9[b], 10.4[c]	11.2
	Camphor-Bound		
391	96.2	102[b], 104[c]	nd[d]
509	11.6	13.1[b], 13.0[c]	nd[d]
535	10.1	11.2[b], 11.4[c]	nd[d]

[a] Spectra taken from free and camphor-bound wild-type and Y96F P450cam enzymes in 50 mM Tris−HCl, 0.2 mM KCl, 1 mM DTT, pH 7.4. Protein concentration was quantified by spectrophotometric determination of iron using phenanthroline (see Materials and Methods). Uncertainty analysis results in a relative error of 3% for each absorptivity coefficient. [b] Measurements from ref 20 were made in 50 mM potassium phosphate buffer. [c] Measurements from ref 26 were made in 20 mM potassium phosphate buffer, 0.1 M KCl, pH 7.4. [d] Camphor-bound Y96F P450cam was not determined (nd) as a result of the split Soret peak (see Figure 1).

hyde was formed from reaction of styrene with the H_2O_2 generated as a result of epoxidation uncoupling. Nickerson et al. (*8*) eliminated benzaldhyde formation by adding catalase as a peroxide scavenger to the wild-type and Y96F P450cam incubation mixtures, but a full stoichiometric analysis was not performed.

In our work, only Y96F P450cam was used for mass balance determinations, as the much less active wild-type enzyme made quantification of styrene oxide less reliable. There are several challenges to quantifying concentrations of styrene and styrene oxide in aqueous enzyme solutions. First, styrene oxide is labile in water and is efficiently acid- and base-catalyzed to form styrene glycol (about 2 mol % per hour at pH 7, (*28*)). Second, nonspecific absorption of styrene and styrene oxide to denatured protein caused less than 100 mol % extraction efficiency from aqueous protein solutions using various organic solvents. Third, analyte recovery was found to be strongly dependent on protein concentration. The influence of these effects on the measurements were minimized by using (i) short incubation times that reduced the extent of styrene oxide hydrolysis and (ii) the low-volatility solvent, chloroform, as reaction quenchant and extractant for products and unreacted styrene, thereby reducing systematic errors associated with solvent evaporation.

Varying ionic strength, protein concentrations, and styrene concentration resulted in an optimized reaction protocol. Reaction rates were maximized at high ionic strength (200 mM KCl, pH 7.4), excess NADH (3 mM), and a Pdx to P450cam ratio of at least 10. Sufficient headspace was present in the septum glass microvials to allow for saturated oxygen levels throughout the reaction. Catalase was added to all reactions to decompose the hydrogen peroxide formed during the reaction. The effect of cosolvents such as DMSO and dioxane on reaction rate was assessed based on previous work showing that these cosolvents accelerated the P450cam-catalyzed hydroxylation of tetralin to *R*-1-tetralol (*14*, *19*). However, for styrene conversion, DMSO had no effect on reaction rate, and dioxane had a detrimental effect. Because styrene solubility is approximately 3 mM and tetralin is only about 0.3 mM, cosolvent effects are less influential on the enzymatic conversion rate of styrene relative to the tetralin conversion rate.

Table 3. Styrene Epoxidation by Mutant (Y96F) P450cam: Mass Balance at Low Conversion

reaction time[a] min	styrene oxide concn, $[SO]_{final}$ μM	$\frac{[styrene]_{final} + [SO]_{final}}{[styrene]_{initial}}$ %
3	9.2 ± 0.4	100 ± 3
4	12.8 ± 0.5	101 ± 2
5	16.9 ± 0.6	102 ± 2
8	26.6 ± 0.8	95 ± 3
11	47 ± 1	97 ± 2

[a] Reactions performed under styrene-limiting conditions (initial solution composition: 3 mM NADH, 180 μM styrene, 100 mM DMSO, 0.3 μM PdR, 3 μM Pdx, 0.3 μM Y96F P450cam, 2000 U/mL catalase in Tris−HCL/KCl buffer). Styrene was introduced via a concentrated stock solution in DMSO to start the reaction. Chloroform extraction was used to quench the reaction, and styrene (aqueous solubility ≈ 3 mM) and styrene oxide (aqueous solubility ≈ 23 mM) were quantified by GC/FID.

Results of the mass balance experiments are listed in Table 3 for styrene to styrene oxide conversions up to 25 mol %. From these measurements, we concluded that at low conversion, styrene is converted solely to styrene oxide. In addition, no other chromatographic peaks were observed in these reaction extracts. However, when styrene conversion exceeded about 25 mol %, the styrene conversion was no longer accounted for by the molar production of styrene oxide or benzaldehyde, and a new peak appeared in the chromatograms of the reaction extracts.

The second product peak exhibited a retention time between those of styrene oxide and styrene glycol, suggesting that this compound has a polarity intermediate to these two analytes. This fact served to constrain the search for compound identity, which was established by GC-MS to be α-hydroxyacetophenone. Identification was confirmed by demonstrating that GC-MS analysis of commercially available samples of α-hydroxyacetophenone produced equivalent ion fragmentation patterns and molecular ion peaks.

Figure 2 shows an example of substrate and product profiles for a reaction going to high styrene conversion. After approximately 20 mol % styrene conversion, styrene oxide formation levels off with a concomitant appearance and increase of α-hydroxyacetophenone. Follow-up incubations in equivalent enzyme solutions showed greater than 50 mol % conversion of commercially available racemic styrene oxide to α-hydroxyacetophenone. These results suggest an A → B → C reaction mechanism. Since the other works on styrene oxidation by P450cam used saturated styrene conditions in their reactions (*6*, *8*), such an effect would never be seen. A similar sequential reaction mechanism was observed in the substrate/product profile for the P450cam reaction with tetralin to form *R*-1-tetralol at high tetralin conversion (*14*) and in the hydroxylation of fatty acids by P450BM3 (*29*).

Electroenzymatic Styrene Epoxidation. The electroenzymatic method we used earlier for camphor hydroxylation (*18*) was applied to styrene epoxidation with the Y96F mutant. Since hydrogen peroxide elimination was imperative to prevent enzyme damage and peroxide-driven conversion of styrene to benzaldehyde, the working tin oxide electrode was fitted with an uncharged platinum mesh shield that catalytically decomposes peroxide. This eliminated the need to add catalase to the electroenzymatic reactor solution. Initial experiments showed that additional buffering capacity was needed, as the pH of the solution decreased to 2.0 after a few hours. Therefore, conditions were modified slightly from the camphor hydroxylation conditions. Tris−HCl was

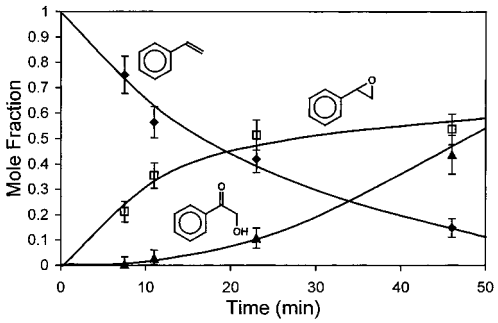


Figure 2. Substrate and product profiles for high styrene conversion by Y96F P450cam. Results are expressed as mole fraction of initial styrene concentration (180 μM). Experimental uncertainties for quantities of styrene (◆), styrene oxide (□), and α-hydroxyacetophenone (▲) were obtained by combining, in quadrature, the response factor uncertainty and the uncertainty due to variation for each analyte. The profiles are consistent with the sequential reaction styrene → styrene oxide → α-hydroxyacetophenone.

used at 100 mM, with 0.2 M KCl and 0.02 M $MgCl_2$, pH 7.4 (22 °C). Initial styrene concentration was added at saturation levels, and styrene conversion levels were kept low in order to maximize the epoxidation reaction rate and to eliminate the formation of α-hydroxyacetophenone.

As in camphor hydroxylation, oxygen control was crucial to the success of electrode-driven P450cam formation of styrene oxide, as shown by the results presented in Table 4. In the second experimental result listed, the reaction was run with dissolved oxygen concentration at atmospheric saturation. The final reaction mixture contained no detectable styrene oxide but did contain a large amount of benzaldehyde. Evidently, high oxygen concentrations elicit large peroxide formation rates, which proceed faster than the peroxide decomposition rate from the platinum mesh shield, resulting in peroxide attack of styrene. In the first experiment labeled as control and in the last two experiments shown in Table 4, there was an initial argon purge of the reaction solution before electrolysis was begun, after which the electroenzymatic reactor was argon blanketed and electrochemical oxygen generation begun at the platinum counter electrode. The potential at the counter electrode was held at +1.0 V by controlling the submergence at the fixed working electrode potential, thereby allowing evolution of oxygen. The first experiment was a control, devoid of P450cam to ensure that the styrene oxide detected in the reactions was enzymatically formed. The last result shown in the table indicates the effect of increasing the Pdx to P450cam ratio. Styrene oxide concentration after 3 h of electrolysis is higher than in the 4-h electrolysis experiment, even though Y96F P450cam concentration is reduced from 30 to 0.4 μM. This is consistent with the hypothesis that the limiting step in this reaction system is due to insufficient reduced Pdx concentration caused by Pdx auto-oxidation, slow heterogeneous electron transfer, and/or slow bulk solution transport rates. We did not attempt galvanostatic electrolysis, as potential of the working electrode had to be maintained within the limits imposed by tin oxide stability from the negative side and cycle thermodynamics from the positive side.

Conclusions

Cytochrome P450 enzymology is remarkable in that a wide number of nonactivated substrates are oxidized regio- and stereospecifically. P450cam has been the most studied cytochrome P450 to date and serves as a model for other P450 enzyme systems. P450cam exhibits wide-ranging substrate specificity, at the expense of slower reaction rates and less efficient cofactor coupling. For P450 technology to be attractive for industrial use, accurate assessments of product profiles for nonnatural substrates must be made, reaction rates for nonnatural substrates must be increased, and the expense of cofactor utilization must be addressed. This work represents the first successful application of direct electrode-driven biocatalysis to the oxidation of nonnatural substrates by an engineered P450 enzyme. Site-directed mutagenesis was used to modify the wild-type enzyme to make it more suited to oxygenate hydrophobic substrates such as styrene. Mass balance experiments accurately quantifying styrene and styrene oxide before and after enzyme reactions illuminated two key characteristics of P450cam styrene oxidation previously not appreciated. At low conversions of styrene, styrene oxide is formed stoichiometrically. At high conversions, a second P450cam attack on styrene oxide forms a doubly oxidized compound (α-hydroxyacetophenone), never before seen. With similar results found in P450cam oxidation of tetralin (*14*), it is likely that this phenomenon is present in most nonnatural transformations of P450cam and possibly other P450s such as the fatty acid hydroxylase P450BM3 (*29*). Direct electrode-driven P450cam oxidation of styrene is too slow to be used in industry directly but provides a very crucial first step in that this is the first successful demonstration of noncofactor mediated P450 oxidation of a nonnatural substrate. Further engineering of the P450cam active site for nonnatural substrates and optimizations of the electroenzymatic reactor may yield an industrially viable biocatalysis system in the future. We are currently investigating other oxide materials for increasing the rate of Pdx reduction, and other reactor designs to prevent the direct interaction of Pdx with oxygen prior to its interaction with CYP101.

Table 4. Electroenzymatic Styrene Epoxidation Using Y96F P450cam

Pdx:P450cam (μM)	oxygen control (electrolysis time[a])	$[SO]_{final}$ (μM)	P450cam turnovers
290:0 (control)	O_2 generation (4 h)	0	0
290:30	no purging (4 h)	benzaldehyde only	0
290:30	O_2 generation (4 h)	19	na
500:0.4	O_2 generation (3 h)	30	75

[a] Electrolysis conducted for 3−4 h in an argon-purged electroenzymatic cell with buffers (0.2 M KCl, 0.10 M Tris−HCl, 0.02 M $MgCl_2$, pH 7.4, 22 °C) developed for camphor hydroxylation (*18*). Saturated styrene was used in order to maximize rate of styrene epoxidation and to preclude formation of α-hydroxyacetophenone. A noncharged platinum mesh shield was used to catalytically decompose hydrogen peroxide formed during oxygen reduction and enzyme decoupling. The two parameters shown to be most important for successful catalysis are oxygen control and Pdx to P450cam ratio.

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