Challenges in Capturing Oxygenase Activity *in Vitro*

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ABSTRACT: Biocatalysis using oxygenase or desaturase enzymes has the potential to add value to native fats and oils by adding oxygen, hydroxyl groups, or double bonds to create regio- and/or stereospecific products. These enzymes are a subset of the large class of oxidoreductase enzymes (from EC subgroups 1.13 and 1.14) involved with biological oxidation and reduction. *In vitro* biocatalytic processing using these enzymes is hampered by the high cost of the stoichiometric cofactors. This article reviews recent progress in developing *in vitro* redox enzyme biocatalysis for commercial-scale syntheses. Coenzyme recycling and electrochemical redox cycling as methods for cofactor regeneration are described and commercial applications indicated. Direct charge transfer without use of mediators is described as the cleanest way of introducing the reducing power into the catalytic cycle. Our electrochemically driven cytochrome $P450_{cam}$ bioreactor is discussed as an example of direct charge transfer to a redox protein. Site-directed mutagenesis in the active site of the $P450_{cam}$ monooxygenase greatly improved performance for the conversion of the nonnative substrate, styrene to styrene oxide. This epoxidation reaction was also shown to give a single product (styrene oxide) in the bioelectrochemical reactor when the diatomic oxygen co-substrate was managed properly.

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A large subset of oxidoreductase enzymes found within the Enzyme Commission (EC) classes 1.13 and 1.14 incorporate oxygen atoms from diatomic oxygen into their substrates. In cases where their catalytic activity is associated with natural substrate(s) and electron-transfer coupled pathways, these enzymes usually produce highly regio- and stereospecific products. Typical stereospecific transformations catalyzed by monooxygenase (single oxygen atom insertion) and dioxygenase (two-oxygen atom insertion) enzymes are shown in Table 1. Desaturase enzymes (EC 1.14.99) are mechanistically related and catalyze desaturation of fatty acids by using diatomic oxygen in the abstraction of adjacent hydrogen atoms, producing a regiospecific double bond in the acyl chain and a molecule of water. These features, coupled with their ability to functionalize nonnative unreactive substrates such as alkanes and aromatics, make these enzymes ideal candidates as catalysts for adding value to native fats and oils.

Common features among oxygenase reactions, such as those shown in Table 1, bring to light the challenges that are faced in converting oxidoreductase biotransformations to cost-effective *in vitro* processes. The first challenge is to avoid the high cost of the nicotinamide-adenine dinucleotide cofactors, NADH or NADPH. The second is to simplify the complicated electron transfer processes of these multiprotein enzyme systems without compromising substrate-to-product stoichiometry. Figure 1 shows that the molecular architecture of these and related oxygenase enzyme systems is a terminal oxygenase, and two additional soluble redox proteins that bring the reducing power from the NAD(P)H cofactors. In many cases, the fidelity of substrate:product stoichiometry, as well as rate and efficiency of electron transfer, is dependent on the right mix of redox partner proteins and oxygenase enzyme. A third challenge is bringing the co-substrate oxygen into the catalytic cycle at the right time and place. Because oxygen is such a strong electrophile, improperly managed oxygen delivery can easily short-circuit the catalytic cycle by draining away reducing power before it reaches the enzyme active site. Improving enzyme stability, product turnover rates, enzyme retention in the bioreactor, and bioavailability of partially miscible substrates may be additional challenges that come into play for particular target products and commensurate process economics.

In this article, we address recent advances pertaining to the first three challenges which are generic to all redox enzymatic conversions for which NAD(P)H and oxygen co-substrate are requirements. The several demonstrations of progress cited in this paper primarily refer to work with oxygenases, although attention to desaturase enzymes has been increasing (1). The similarities between proposed mechanisms for the hydroxylation of methane by soluble methane monooxygenase (2), and the desaturation of C_{18} saturated fatty acid by acyl carrier protein desaturase (1), show that similar technical challenges are involved in developing *in vitro* biocatalytic processes. We review advances in the area of NAD(P)H regeneration, a tech-

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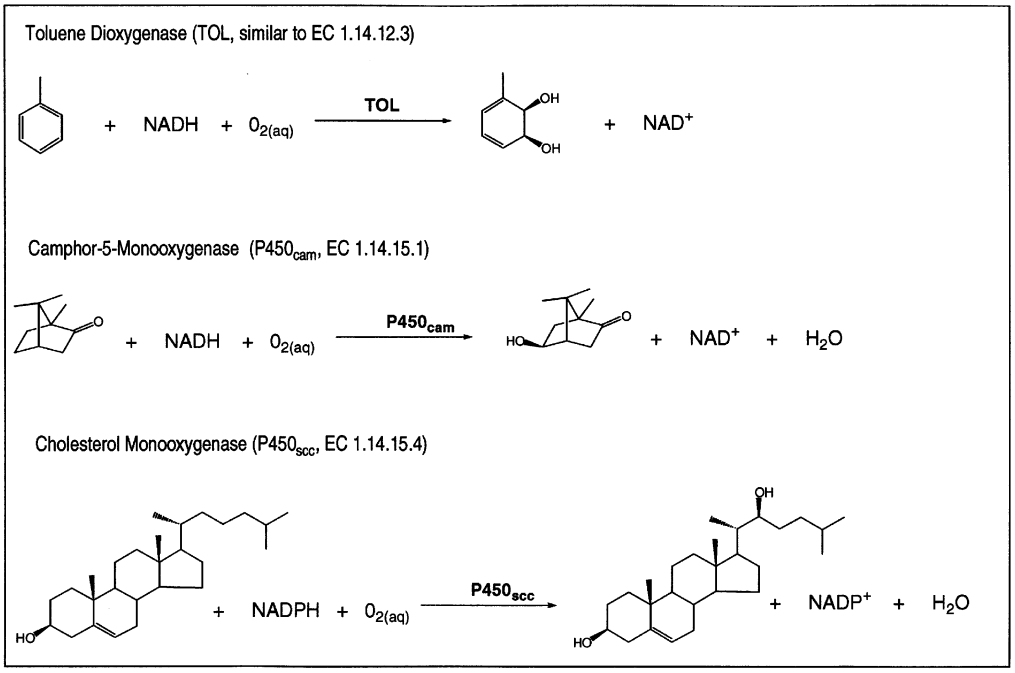
[1]Certain commercial equipment, instruments, and materials are identified in this paper to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that the material or equipment is necessarily the best available for the purpose.

TABLE 1
Examples of Oxygenase-Catalyzed Reactions



nique that has been applied to commercial processes for amino acid synthesis. In another approach, we have developed a bioelectrochemical method for bringing reducing power directly into the P450 camphor-5-monooxygenase (EC 1.14.15.1) cycle that has been simplified by elimination of the ferredoxin reductase redox partner. Finally, site-directed mutagenesis performed on the terminal P450 enzyme will be shown to tailor the enzyme to act on the nonnative substrate styrene, with exclusive conversion to product styrene epoxide.

NAD(P)H REGENERATION

In fermentation, a microorganism is supplied with a nutrient, frequently in the form of sugar, from which the necessary cofactors and metabolic energy requirements needed for oxygenase enzyme activity are produced *in vivo*. For *in vitro* biocatalysis, a fresh cofactor has to be supplied in stoichiometric amounts together with a target substrate. The cost of NADH, which is more than $8,500/mole when purchased in kilogram quantities, serves as an obvious economic limitation for larger-scale preparative processing.

In situ cofactor regeneration by coenzyme recycling (second reagent) or electrochemical oxidation/reduction (reagent-free) are alternatives to stoichiometric cofactor addition in *in vitro* redox biocatalysis. Coenzyme recycling refers to the use of a second enzyme, along with a second substrate, to recycle the cofactor's oxidation state. Homogeneous-phase, second enzyme methods for regenerating pyridine nucleotide cofac-

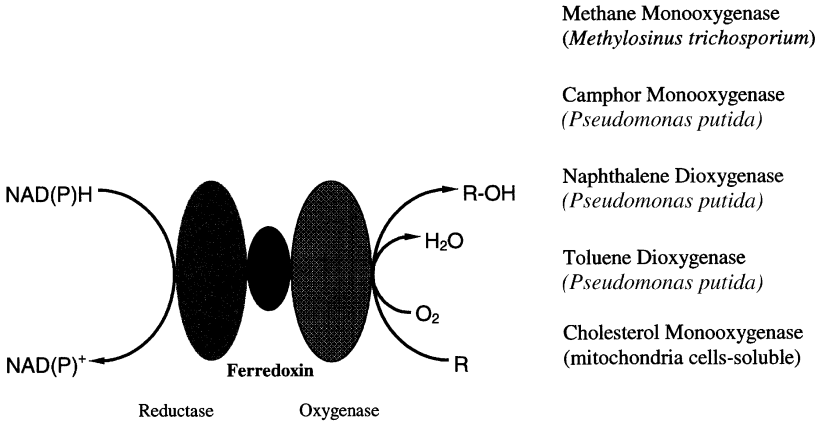


FIG 1. Multiprotein architecture of oxygenase enzyme systems. Oxidoreductase enzyme systems that perform biotransformations of known or potential commercial interest are also indicated. They share a three-protein molecular architecture in which a small {2Fe-2S} ferredoxin provides electron transfer to the terminal oxygenase enzyme.

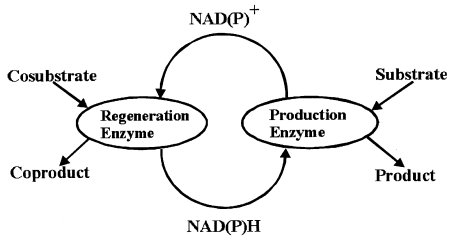


FIG 2. Use of a second enzyme to regenerate the NAD(P)/H cofactor that transfers reducing equivalents to the production enzyme.

tors were pioneered by Whitesides and coworkers (3,4). The generalized scheme for this method is shown in Figure 2. The performance of these systems is quantified by turnover number (TN), which is the number of cycles performed by NAD(P)/H during a unit of reaction time, and by the total turnover number (TTN), which is the total number of cycles completed during a specified reaction duration. Many factors can affect both of these parameters, and these are best dealt with in the context of particular methods for regenerating the cofactors. In all applications however, inherent decomposition of both reduced and oxidized forms of the pyridine cofactors will affect the total number of turnovers and the turnover rate. A recent study measured NADH stability as a function of temperature, pH, and buffer type (5). After 40 min, 0.1 mM NADH decomposition ranged from 0% in 1,4-piperazine-bis[ethane-sulfonic acid) (PIPES) buffer, pH 6.8 and 25°C to 13% in phosphate buffer, pH 7.8 and 30°C. Other studies summarized in Reference 6 report that NADPH is less stable than NADH; that NAD(P)H is stable in base but labile in acid; and that NAD(P) is stable in acid but labile in base.

Although some of the enzymatic cofactor recycling systems are highly developed (4,7), they tend to be quite complicated owing to the addition of extra reagents and the generation of coproduct(s). Also, additional problems associated with different kinetics of several parallel reactions (8), enzyme inhibition by components of the regeneration reaction (3), and varying degrees of stability among all components, are often cited. Many of these problems have been circumvented in the case of the formate/formate dehydrogenase (FDH) coenzyme regeneration system that has been put into commercial application by Degussa AG of Hanau, Germany (9). This is an attractive regeneration system because the additional formate reactant (cosubstrate) and FDH coenzyme are inexpensive, and the carbon dioxide coproduct is easily separated from the main product. Values of TTN range from 600 to 80,000, while the TN values are only of order 1 min^{-1} (10). The Degussa application, synthesis of L-amino acids from their respective α-keto acids, uses NADH-dependent dehydrogenases in membrane reactors.

Not all applications are compatible with the formate/FDH chemistry—pH changes and formate ion inhibition were found to be detrimental to the performance of xylose reductase in a process aimed at producing the sweetener xylitol by reduction of the five-carbon sugar xylose (11). In cases such as these, other coenzyme NAD(P)H regenerating systems have been tried, such as glucose/glucose dehydrogenase (gluconic acid is coproduct) (11), isopropanol/alcohol dehydrogenase (acetone is coproduct), and glucose-6-phosphate/G6P-dehydrogenase [see (7) for more examples].

The electrochemical regeneration of NAD(P)H has to be performed by indirect electrochemical methods, as direct reduction requires high overpotentials and results in production of inactive dinucleotide-dimers (12,13). One approach to overcome dimer formation used a silver electrode at –480 mV vs. NHE covered by an anion-exchange membrane (14). Only three total turnovers for NAD to NADH conversion were reported. More suitably, carefully chosen mediators can prevent dimerization. According to Steckhan (15), such mediators must be able to transfer two electrons in one step, have redox potentials less negative than –0.6 V vs. NHE, and be inactive toward substrate. NAD radical formation is prevented when two electrons are transferred to NAD simultaneously according to the reaction:

$$NAD^+ + H^+ + 2e^- = NADH \quad [1]$$

Several biocatalytic systems that use mediated NADH regeneration have been reported (16,17). Steckhan and coworkers have used tris(2,2′′-bipyridyl) rhodium complexes for very fast hydride ion transfer to NAD (16,18). The regenerated NADH provided the reducing equivalents to D-lactate dehydrogenase for catalytic reduction of pyruvate to D-lactate. Using a carbon foil electrode, they were able to sustain the reaction for 3 h at TN = 0.08 min^{-1} with respect to the mediator (16).

Electrochemical regeneration has also been implemented by using redox proteins as electron shuttles between electrodes and NAD(P)/H cofactors. Redox proteins that have been shown at the laboratory level (15) to conduct two-electron transfers from electrodes to NAD(P) include ferredoxin-NADP-reductase, lipoamide dehydrogenase (diaphorase), and enoate reductases. In some cases, redox protein performance improved if the protein was used in combination with methyl viologen, which serves to transfer one electron at a time to the redox protein before it carries out the two-electron transfer reaction with NAD(P). An electrochemical NADH regeneration system using hydrogenase in both a thin-layer cell reactor (TN = 8 min^{-1}) and in an anaerobic 5-mL preparative-scale batch reactor (TN = 3 min^{-1}) has been described (10). Rapid denaturation of the hydrogenase limited electrolysis duration to about 5 h.

ELECTROCHEMICAL PROTEIN REGENERATION

Given the complexities of NAD(P)/H electrochemical regeneration, much effort has gone into exploring ways to exchange reducing power directly into the enzymatic cycle by way of mediated or direct electrochemical communication with the enzyme. By reducing the number of reaction components, the enzymatic system is simplified while retaining the

regio- and enantioselectivity inherent for the natural cycle. In this section, we review some of the basic lessons about electron transfer to redox centers that are imbedded in apoprotein matrices before describing efforts aimed at achieving direct electron transfer to redox enzymes.

Most redox enzymes are rather bulky molecules with masses ranging from 40 to 850 kDa. Since the rate of electron transfer decays exponentially with distance between redox sites (19), and most redox centers are located far from the outermost surface, the rates of heterogeneous electron transfer are usually unacceptably slow. Research on biosensor applications has established that the maximal electrode-redox center distance should not exceed about 20 Å in order to obtain a detectable current response (20). In addition to this fundamental restriction, the protein should not be perturbed, owing to the adsorptive interaction with the electrode surface, so that an irreversible loss of activity occurs or the electrode becomes fouled with denaturation fragments. The nature of the intervening medium also is important for successful electron tunneling, and the electrochemical response may be critically dependent upon how the protein interacts with the electric field at the electrode–solution interface (21). Despite these restrictions, reversible faradic responses can be obtained for electron-transfer proteins just as they are for small inorganic molecules (22). In this context, the most studied protein is cytochrome c (cyt c), which contains an iron heme active center that is almost entirely buried within the protein except for one exposed edge. Heterogeneous electron transfer from electrodes to both freely diffusing (23) and immobilized cyt c (24) has been measured by a variety of classic electrochemical and spectroscopic techniques. Optimal protein orientation relative to the electrode can be achieved using the electrostatic interaction between excess positively charged lysine groups around the exposed heme edge and the negatively charged electrode (22). Irreversible protein adsorption has been shown to depend strongly on surface hydrophilicity (25,26). Generally, surface properties of the electrode, such as charge density and distribution, hydrophilicity, and strength of interaction between the protein of interest and the electrode surface, are important for direct protein electrochemistry (25). However, at the present time it is not possible to define the universal electrode surface for redox proteins, as the degree of importance of each parameter has to be defined individually for a particular protein–electrode pair. Usually metal electrodes are "tuned" for charge exchange with proteins by adsorbing redox "promoters" (27). In certain instances, a reversible electrochemical response from cyt c on bare gold or glassy carbon has been found just by careful choice of electrolyte solution (28). Rational electrode surface design for protein electrochemistry is increasingly being based on physical studies of the electrochemical interfaces (29–32). Strategies with larger enzymes include use of electroactive groups in solution, or groups that are covalently attached to the electrode (22) or to the protein (19) surface. This creates an electron transfer path to the active site pocket, thus reducing the electron tunneling distance.

DIRECT ELECTROENZYMOLOGY: A P450 MONOOXYGENASE CYCLE

Cytochromes P450 are excellent candidates as catalysts for the synthesis of high-value specialty chemicals that are difficult to synthesize by conventional chemical oxidation or reduction techniques. They can catalyze the oxidation of inactive carbon–hydrogen bonds to give alcohol functionality, the transfer of an oxygen atom to heteroatoms, the epoxidation of olefins and aromatic hydrocarbons (33–35), ω-hydroxylation of long-chain fatty acids (36), and the reduction of halocarbons (37). The native reaction cycle requires reducing equivalents from NADH *via* redox partner proteins, as well as molecular oxygen when operating as a monooxygenase. Estabrook and coworkers (38) have shown mediated electrode-driven biocatalysis for ω-hydroxylation of lauric acid by cytochrome $P450_{BM\text{-}3}$. This is a unique P450 monooxygenase in that it contains all the electron transfer functions on a single polypeptide, so the catalytic cycle needs no external reductase or ferredoxin-type proteins for turnover. For proteins as large as $P450_{BM\text{-}3}$, the active site is sufficiently distant from the surface of the protein (39) that use of a mediator to promote the rate of electron transfer from an electrode becomes essential. Despite the several disadvantages brought on by mediators (e.g., high concentration relative to protein, need to tune selectivity, toxicity), the rates of lauric acid hydroxylation obtained when cobalt(II) sepulchrate was used as a $P450_{BM\text{-}3}$ electrochemical mediator approached those rates in effect when NADPH was used as the source of electrons ($\approx$100 min^{-1}). The reaction was run for 2 h with no significant destruction of the P450 catalyst. Special precautions were required in order to achieve enzyme stability since P450 enzymes operating on nonnative substrates invariably bring electrolytic reduction of molecular oxygen along with the generation of hydrogen peroxide. The formation of reactive oxygen species, such as superoxide and hydrogen peroxide, is detrimental to the P450 catalyst. Therefore, small amounts of catalase enzyme were included in the reaction mixture to catalyze decomposition of these reactive oxygen species. Also, the reduced cobalt(II) sepulchrate mediator is oxidized by molecular oxygen and cannot facilitate further electron transfer. Therefore, the oxygen concentration had to be maintained at about 10 μM level in order to reduce the second-order reaction with reduced mediator. The nature of α-hydroxyl products from lauric acid conversion was not described; however, Schneider *et al.* (36) showed multiple regio-products were formed from the NADPH-driven hydroxylation of pentadecanoic acid by $P450_{BM\text{-}3}$.

In our laboratory we have been pursuing an understanding of the detailed pathway by which cytochrome $P450_{cam}$ (CYP101) transfers electrons through a three-diffusible-protein-subunit system and how this system might be engineered to eliminate nonessential subunits and the costly NADH cofactor (40,41). This soluble cytochrome P450 system is found naturally in the bacterium *Pseudomonas putida* PpG786 when it is cultured on camphor. This enzyme catalyzes stereospe-

cific camphor hydroxylation at the 5-*exo* position and requires stoichiometric amounts of NADH. In addition, the hydroxylase has been shown to accommodate other substrates (35,42), and therefore is thought to have potential for wider synthesis purposes.

The overall biocatalytic cycle is composed of several individual reactions, including two-electron transfer steps as shown in the upper part of Figure 3. The two electrons necessary for the reaction are supplied by reduced putidaredoxin, Pdx, a 2Fe-2S protein (11.6 kDa). In the natural cycle, Pdx mediates the transfer of these electrons from NADH and the FAD-containing putidaredoxin reductase to the heme active center of the cytochrome $P450_{cam}$ (45 kDa). In addition to the electron mediation function, putidaredoxin is an "effector" for product release in the final step of the cycle. This could explain why efforts to do catalysis *via* direct electrochemical reduction of the $P450_{cam}$ hydroxylase using glassy carbon (33) or lipid-modified pyrolytic graphite electrodes (43) results in small to negligible product turnover.

Our strategy has been to use an electrode to reduce Pdx rather than to attempt the direct reduction of the terminal hydroxylase. We eliminated putidaredoxin reductase because of its sole function as electron mediator between NADH and Pdx with no apparent effect on thermodynamic equilibria. Pdx was a viable candidate for direct heterogeneous reduction due to its relatively small size and a redox-active 2Fe-2S center that is close to the surface. Our initial results showed reversible oxidation/reduction cycling of Pdx on modified gold and silver electrodes (21). The electrodes were modified by immobilizing ionizable organic molecules to overcome the repulsive interaction between the electrode and the negatively charged Pdx at the reducing potentials ($E^{0'}_{Pdx} = -220$ mV vs. NHE). However, the response was short-lived as organic electrode modifiers deteriorated while the electrode was held at potentials less than −500 mV. Using *in situ* spectroscopic ellipsometry, we found that Pdx was adsorbing irreversibly on the gold electrodes (29), thus blocking further electron transfer to solution species.

We switched to antimony-doped tin oxide as the working electrode for the continuous reduction of solution Pdx, in part due to a rather negative flat band potential ($E_{fb} = -1.2$ V vs. NHE) that ensures an excess positive charge in the potential range of interest (−300 to −700 mV). For this reason, the electrode was expected to have a favorable coulombic interaction with Pdx and at the same time have a minimal effect on other reaction components. The current response was stable under enzymatic cycle conditions, and there was no irreversible accumulation of protein on the electrode surface as demonstrated by *in situ* spectroscopic ellipsometry (29).

We also measured the electron transfer rate constants from

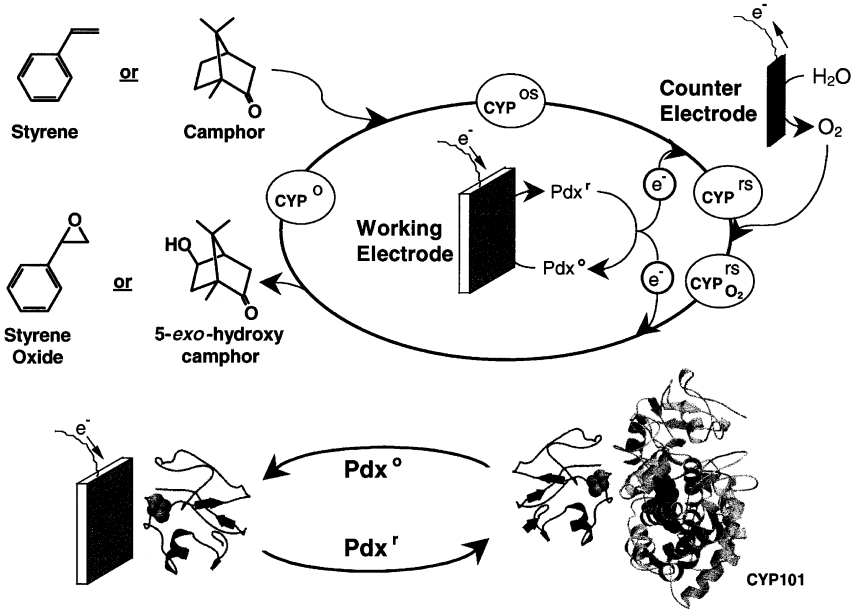


FIG 3. Direct-electrode-driven biohydroxylation begins with substrate **S** (camphor or styrene) binding to oxidized hemoprotein CYP101 (cytochrome $P450_{cam}$, the oxygenase enzyme), forming the protein complex **CYP**^{os}. Valuable 5-*exo*-hydroxycamphor and styrene oxide products are released at the end of the cycle. An anaerobic reactor prevents unwanted oxidation reactions. **CYP** binds electrochemical oxygen and receives two electrons from the reduced iron-sulfur protein putidaredoxin **Pdx**^{r}. The main technical innovation in the process is the development of a semiconductor working electrode, which supplies reducing power to oxidized putidaredoxin **Pdx**^{o}, thereby replacing expensive NADH, and putidaredoxin reductase PdR as reducing components. The lower part of the figure shows the diffusional reorientation of **Pdx**^{r} so that its iron-sulfur cluster is aligned to carry out reduction of the heme cofactor in the active site of **CYP**.

the tin oxide electrode to Pdx, and from Pdx to $P450_{cam}$ (41). These kinetic measurements revealed that the presence of dissolved oxygen, which is utilized by the enzymatic cycle, can significantly retard the reaction. Consequently, the molecular oxygen coreagent must be introduced in a way that minimizes the rapid reoxidation of putidaredoxin (before Pdx can deliver its reducing power to P450), as well as excessive cathodic reduction to hydrogen peroxide, which can diminish protein stability and stereochemical purity of the product. Therefore, we operated the reaction under anaerobic conditions and generated the required stoichiometric oxygen at the platinum (Pt) counterelectrode through the water oxidation reaction. In addition, the working electrode was screened by Pt mesh in order to catalyze decomposition of trace hydrogen peroxide, which could originate during oxygen reduction at this electrode. The optimal potential for the particular electroenzymatic system was –500 mV, as increased overvoltage diminished reaction lifetime.

Control reactions performed in the absence of either Pdx or $P450_{cam}$ in the reaction mixture resulted in undectable product formation and confirmed the crucial Pdx role in the enzymatic cycle. In trials with both Pdx and $P450_{cam}$ present, camphor conversion to the 5-*exo*-hydroxycamphor product exceeded 95%. This result is close to the same product fidelity that is achieved in the natural cycle. The highest total number of turnovers for $P450_{cam}$ observed in our experiments was close to 4000 with an average turnover rate of about 30 min^{-1}. The catalytic cycle was sustained for 16 h at 4°C. The rates achieved were comparable with turnover rates of mediated P450-catalyzed conversion (38).

Styrene is not an easy substrate for $P450_{cam}$, although low levels of styrene oxide and benzaldehyde were produced in our NADH-driven incubations with the full P450 enzyme system. Benzaldehyde is thought to be a nonenzymatic product of styrene and the hydrogen peroxide formed when P450 acts on a substrate that is nonnative (42). In order to improve the activity of $P450_{cam}$ for styrene oxygenation, Nickerson *et al.* (44) replaced the tyrosine residue in the active site with a phenylalanine residue creating a mutant oxygenase P450 (Y96F) with enhanced activity for hydrophobic substrates. We made the same mutation and showed almost a 10-fold improvement in styrene oxide production rate relative to the wild-type enzyme and the complete absence of benzaldehyde co-product formation. In the bioelectrochemical reactor, styrene oxide was the only product produced when the reaction was run under conditions similar to those used for the camphor hydroxylation studies—namely, high Pdx/CYP101 molar ratio, absence of NADH and putidaredoxin reductase, an argon purge, and electrochemical oxygen generation. A final concentration of 30 μM styrene oxide, the exclusive product, was achieved after 3 h and a total of about 75 total enzyme turnovers.

FUTURE PROSPECTS

Widening interest from scientific and industrial communities in cleaner and more stereospecific synthesis implies that significant research and development can be expected for electroenzymatic processes. The above examples illustrate the potential of oxygenase enzymes as *in vitro* catalysts when the electrochemical step can be integrated into the reaction cycle. To a large extent, the technologies are at an initial development stage, but there are few fundamental barriers left to deter rapid growth for fine chemical and pharmaceutical production. A number of engineering solutions will have to be found in order to make the technology competitive on the industrial scale: solid state engineering of electrodes to achieve higher current densities, interfacial engineering to achieve more stable and reactive solid/liquid/protein interfaces, protein engineering to achieve more active, versatile and stable enzymes, just to name a few. Also, organic solvents may be needed to expand the scope of target substrates. Finally, opportunities may exist with extremophile enzymes and nanostructural engineering that will lead to improved enzyme stability.

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