Ferredoxin-Mediated Electrocatalytic Dehalogenation of Haloalkanes by Cytochrome $P450_{cam}$

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Abstract: The potential role of cytochrome $P450_{cam}$ in bioremediation has been extensively studied because of its ability to carry out the nonphysiological reductive dehalogenation of haloalkanes. The reductive dehalogenation catalytic cycle requires the input of two reducing equivalents, typically delivered to the enzyme from NADH via putidaredoxin reductase and putidaredoxin. In this report we present evidence demonstrating that in the presence of polylysine, spinach ferredoxin acts as an efficient electron shuttle between an indium-doped tin oxide (ITO) electrode and substrate-bound cytochrome $P450_{cam}$. The ferredoxin-mediated electrochemical reduction of substrate-bound cytochrome $P450_{cam}$ conduces to the reductive dehalogenation of haloalkanes. Consequently, this strategy permits the replacement of NADH and cytochrome P450 reductase, both expensive and fragile species, with an electrode to catalyze dehalogenation reactions. To accomplish this goal it was necessary to implement a system in which the electrode exchanged electrons with spinach ferredoxin, even in the presence of an excess of cytochrome $P450_{cam}$, which possesses a reduction potential more positive than that of spinach ferredoxin. This molecular discrimination at the electrode surface was achieved by capitalizing on surface electrostatic potentials typically exhibited by electron-transfer proteins. In this particular case, the positive electrostatic potential imparted by polylysine to the electrode surface steers spinach ferredoxin toward the electrode with an orientation optimal for heterogeneous electron exchange, thus acting as a good promoter for its electrochemistry. In contrast, cytochrome $P450_{cam}$ does not exchange electrons with the ITO electrode but instead is readily reduced in solution by accepting an electron from spinach ferredoxin. Digital simulation of the voltammetric experiments aimed at demonstrating the reductive dehalogenation electrocatalytic activity produced important insights into the mechanism of reductive dehalogenations carried out by cytochrome $P450_{cam}$.

Introduction

The cytochrome P450 family of enzymes is involved in a variety of biological oxidation reactions, including hydroxylations, epoxidations, and heteroatom oxidations. Cytochrome $P450_{cam}$ originally isolated from the bacterium *Pseudomonas putida* catalyzes the regio- and stereospecific hydroxylation of *d*-camphor to produce 5-*exo*-hydroxycamphor.[1,2] The monooxygenation reaction requires, in addition to *d*-camphor and molecular oxygen, two reducing equivalents which originate from NADH and are sequentially transferred via the flavin group of NADH-putidaredoxin reductase to the 2Fe-2S-Cys_4 cluster of putidaredoxin and in turn to the heme iron in cytochrome $P450_{cam}$. Consequently, reduced putidaredoxin acts as a direct electron donor to oxidized cytochrome $P450_{cam}$. Although cytochrome $P450_{cam}$ was initially considered to be substrate specific, it was later shown to oxidize molecules closely related to its natural substrate (e.g., adamantanone, adamantane, norcamphor),[3−5] and molecules not related to camphor such as cys-β-methylstyrene,[6] and thioanisole.[7] It has also been shown that cytochrome $P450_{cam}$ can perform nonphysiological reductive dehalogenation reactions[8] and accordingly, it has been extensively studied for potential applications in the bioremediation of halogenated hydrocarbons.[9−11] These findings have led to the postulation that cytochrome $P450_{cam}$ may find useful applications in the regio- and stereoselective synthesis of commodity chemicals as well as in bioremediation schemes. One possible way of achieving these goals may be to electrochemically address cytochrome $P450_{cam}$ at an electrode surface to supply the enzyme with the reducing equivalents needed to carry out its catalytic cycle, hence avoiding the use of NADH, NADH-putidaredoxin reductase, and putidaredoxin.

Significant progress has been achieved toward the electrochemical activation of cytochrome $P450_{cam}$. For example, the cyclic voltammetry of cytochrome $P450_{cam}$ has been observed at low temperatures with an edge-plane pyrolytic graphite electrode by utilizing highly pure and freshly purified enzyme.[12]

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The reversible cyclic voltammetry of cytochrome $P450_{cam}$ incorporated in lipid films has also been reported.[13] It was shown that cytochrome $P450_{cam}$ trapped in these films is capable of catalyzing the electrochemically driven reduction of trichloroacetic acid under anaerobic conditions. It is interesting that the formal potential corresponding to the one-electron reduction of substrate-free cytochrome $P450_{cam}$ trapped in the lipid films is ~180 mV more positive than the formal potential measured for the substrate-free enzyme in solution (−303 mV vs NHE).[13] Although the origin of this large shift in the reduction potential is not clear, the lipid environment and double-layer effects at the electrode surface have been implicated.[13] The direct electrochemistry of cytochrome $P450_{cam}$ has also been obtained with alternate layer-by-layer films deposited on electrode surfaces;[14] these films were also used to electrochemically drive the epoxidation of styrene.

In a different approach to harvest the catalytic power of cytochrome $P450_{cam}$, the mediator cobalt sephulcrate was used to electrolyze the fusion protein containing the heme domain of cytochrome P450 4A1 linked to the flavin domain of NADPH cytochrome P450 reductase.[15] This system was used to perform the electrocatalytically driven ω-hydroxylation of lauric acid. In a different study, a solution containing putidaredoxin and cytochrome $P450_{cam}$ was electrolyzed at an antimony-doped tin oxide electrode in the presence of oxygen and *d*-camphor.[16] It was reported that putidaredoxin is reduced at the semiconductor electrode surface and subsequently transfers the reducing equivalents to cytochrome $P450_{cam}$. It is interesting to note, however, that the hydroxylation of camphor to produce 5-*exo*-hydroxycamphor required the concentration of putidaredoxin in the electrolytic cell to be at least 500-fold larger than that of cytochrome $P450_{cam}$. Although the need for such a large excess of putidaredoxin is not clear, it was postulated to originate from a slow rate of heterogeneous electron transfer (k_s) between the electrode and putidaredoxin. In agreement with this idea, the value of k_s measured for putidaredoxin at the antimony doped tin oxide electrode was reported to be 1.0×10^{-4} cm/s.[16]

An alternative to the use of putidaredoxin as an electron donor to cytochrome $P450_{cam}$ is the utilization of spinach ferredoxin. The active site of this electron-transfer protein also consists of a 2Fe-2S-Cys_4 cluster[17] that exhibits a reduction potential (−430 mV vs NHE)[18,19] sufficiently negative to reduce cytochrome $P450_{cam}$, even in the absence of substrate. It is also known that spinach ferredoxin cannot substitute the effector role that putidaredoxin plays in the monooxygenation reactions performed by cytochrome $P450_{cam}$.[20] However, it has been reported that spinach ferredoxin can transfer the two electrons needed by cytochrome $P450_{cam}$ to carry out the reductive dehalogenation of halogenated hydrocarbons.[21] Accomplishing the electrocatalytic reductive dehalogenation of haloalkanes is highly desirable since recent work has demonstrated the capabilities of naturally occurring cytochromes P450 to reductively degrade compounds such as haloalkanes[9,11,22] and other recalcitrant xenobiotics such as atrazine and thiocarbamate herbicides.[23] Consequently, we explored the possibility of utilizing spinach ferredoxin to mediate the electrochemical activation of cytochrome $P450_{cam}$. The results of this investigation clearly demonstrate that spinach ferredoxin is a suitable electron-transfer protein for shuttling electrons between an electrode surface and cytochrome $P450_{cam}$ to activate cytochrome $P450_{cam}$ toward reductive dehalogenation reactions. The usefulness of this approach resides in abolishing the need for fragile chemicals and enzymes such as NADH and cytochrome P450 reductase. Furthermore, the voltammetric experiments aimed at demonstrating the catalytic activity have also produced important insights into the factors controlling the turnover of reductive dehalogenation reactions.

Experimental Section

Cytochrome $P450_{cam}$ was expressed in *E. coli* as described previously by Unger et al.[24] The recombinant plasmid harboring the cytochrome $P450_{cam}$ gene was a generous gift from Professor Stephen Sligar.[24] Recombinant cytochrome $P450_{cam}$ expressed in *E. coli* was purified to homogeneity by the method reported previously by Gunsalus and Wagner.[2] Protein purity was determined by monitoring the ratio of absorbances A_{418}/A_{280} by electronic spectroscopy; only those chromatographic fractions exhibiting a ratio $A_{418}/A_{280} \geq 1.50$ were used to obtain homogeneous protein. Purified cytochrome $P450_{cam}$ was exchanged into 100.0 mM MOPS, pH 7.0 by dialysis, concentrated to ~0.80 mM by ultrafiltration (Y30 Diaflo ultrafiltration membranes, Amicon), aliquoted, and then frozen at −20 °C. Spinach ferredoxin was purchased from Sigma and subsequently exchanged into 100 mM MOPS buffer, pH 7.0 utilizing a 10DG disposable chromatography column (Bio-Rad Laboratories), concentrated to 1.0 mM, aliquoted, and frozen at −20 °C. Polylysine with average molecular weight of 3400 was purchased from Sigma and used as received.

Cyclic Voltammetry. Cyclic voltammetry was carried out with a BAS-CV50W potentiostat (Bioanalytical Systems, West Lafayette, IN). Glass slides (2.50 cm × 2.50 cm) coated on one side with indium-doped tin oxide (ITO) semiconductor films exhibiting a typical resistance of less than 10 S were purchased from Delta Technologies (Stillwater, MN) and used as working electrodes. A platinum wire auxiliary electrode and a Ag/AgCl reference electrode containing a fiber junction were purchased from Cypress Systems (Lawrence, KS). The electrochemical cell (cross-section shown schematically in Figure 1) was constructed out of plexiglass. To assemble the electrochemical cell, the ITO electrode (a) is fitted into the indentation of the cell base assembly (b), electrical contact with the semiconductor surface is achieved with the aid of an aluminum foil (c). The main body of the electrochemical cell (d), 3.80 cm in diameter, is placed on top of the base assembly so that the O-ring (e) makes contact with the semiconductor surface, hence defining an electrode diameter of 9.3 mm. Finally, the assembled cell is tightened with four screws (f). The solution to be analyzed, typically 300 μL, is placed on the exposed semiconductor surface and the electrochemical cell is capped with cell cap (g). The latter is outfitted with four holes (h) utilized to insert the reference and auxiliary electrodes, and nitrogen inlet and outlet lines. The solution in the electrochemical cell was deaerated by bubbling high-purity nitrogen for 30 min and subsequently blanketed with nitrogen to maintain anaerobicity. Solutions used in the electrochemical studies were typically prepared in 100 mM MOPS, pH 7.0, and contained 100 μM spinach ferredoxin, 300 μM polylysine, and 100−200 μM cytochrome $P450_{cam}$. The ITO working electrode was conditioned by sonicating for 30 min in each of the following solutions: 1% alconox

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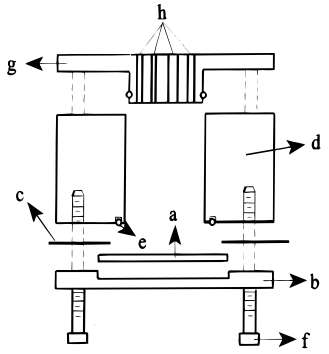

Figure 1. Schematic cross-sectional representation of the electrochemical cell utilized in this investigation: (a) ITO electrode, (b) base assembly, (c) aluminum foil electrical contact, (d) plexiglass cell body (e) O-ring, (f) four screws for cell assembly, (g) cell cap, and (h) ports for reference and auxiliary electrodes and for nitrogen inlet and outlet.

in deionized water, ethanol, and deionized water. The clean electrodes were subsequently dried with a stream of nitrogen and used immediately.

Gas Chromatography−Mass Spectrometry. The identity of the products generated by the catalytic activity of cytochrome $P450_{cam}$ in the electrochemical cell was determined by gas chromatography−mass spectrometry (GC-MS). To this end, the solution in the electrochemical cell was sampled with a 100-μm poly(dimethylsiloxane) solid-phase microextraction (SPME) fiber. The fiber was subsequently placed into the injection port of a Hewlett-Packard (HPG 1800A) GC-MS in order to desorb the extracted material into the GC column, as reported previously.[25]

Results and Discussion

Reversible Electrochemistry of Spinach Ferredoxin. Spinach ferredoxin is an electron-transfer protein whose active site is a 2Fe-2S-Cys_4 cluster. Its molecular surface possesses an asymmetric distribution of acidic residues that impart a highly localized negative charge to the area on the protein surface surrounding the active site. It is well established that this highly localized negative electrostatic field on the surface of spinach ferredoxin is used for molecular recognition and electrostatic binding with physiological protein partners such as nitrite oxidoreductase, sulfite oxidoreductase and thioredoxin.[17] This type of electrostatic recognition among electron-transfer proteins has been successfully utilized to promote their direct electrochemistry at electrodes bearing a charge complementary to that surrounding the active site of the protein.[26−33] At pH values close to 7.0 and above, the surface of an indium oxide electrode is thought to possess negative charge.[34] The negative charge on the surface of indium oxide electrodes was initially exploited by Yeh and Kuwana[35] to obtain the unmediated electrochemistry of cytochrome *c*, a positively charged protein. In subsequent studies, the voltammetry of negatively charged proteins has been

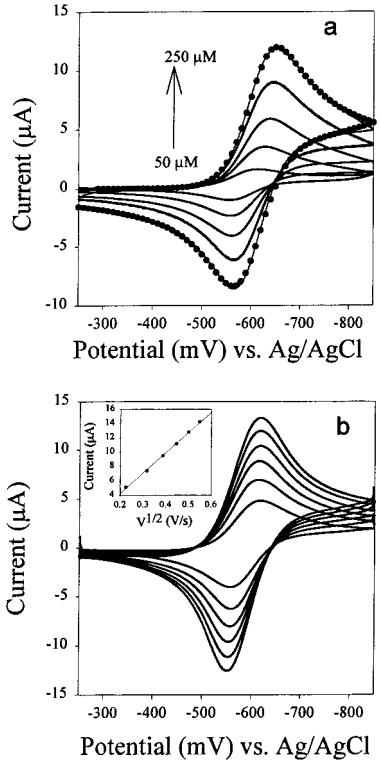

Figure 2. (a) (—) Background-subtracted cyclic voltammograms obtained at an ITO electrode from solutions containing different concentrations of spinach ferredoxin and polylysine (300 μM) in 100 mM MOPS, pH 7.0. Omission of spinach ferredoxin or polylysine results in the absence of a Faradaic response. (●) Digital simulation of the experimental response with the following parameters: $k_s = 0.0025$ cm/s, $D_oFd^{ox} = D_oFd^{red} = 1.6 \times 10^{-6}$ cm^2/s, $\nu = 20$ mV/s, $\alpha = 0.50$. (b) Background subtracted cyclic voltammograms obtained from a solution containing spinach ferredoxin (100 μM) and polylysine (300 μM) at scan rates from 50 to 300 mV/s in 50 mV/s increments. The inset displays the linear correlation between the square root of scan rate and cathodic peak current.

obtained at indium oxide electrodes by addition of a polycation to overcome the electrostatic repulsion between the negatively charged electrode and protein surfaces.[26,36]

Typical cyclic voltammograms obtained at an indium-doped tin oxide electrode from solutions containing spinach ferredoxin and polylysine are shown in Figure 2a. By comparison, omission of polylysine or ferredoxin results in the absence of a Faradaic response, observations that are in agreement with previous reports, demonstrating that the direct electrochemistry of plant ferredoxins at indium oxide electrodes is promoted by polylysine.[36,37] Consequently, the role of polylysine as a promoter is consistent with the negative charge of the ITO electrode surface and the highly localized negative electrostatic field surrounding the active site of spinach ferredoxin.[17] The ratio of the cathodic to anodic peak currents (i_{pc}/i_{pa}) is unity and the peak to peak separation (ΔE_p) is 60 mV. The heterogeneous electron-transfer rate constant (k_s) and the diffusion coefficient (D_o) for spinach ferredoxin were obtained by simulating the cyclic voltammograms with the aid of the program Digisim (Bioanalytical Sytems), a simulator for cyclic voltammetric responses.[38] These values are 2.5×10^{-3} cm/s and 1.6×10^{-6}

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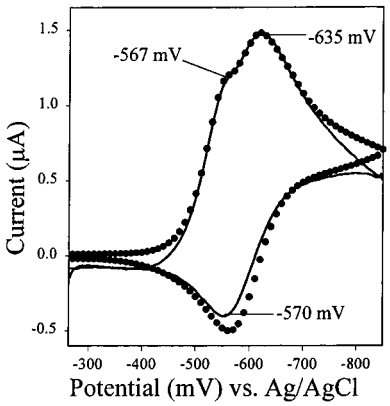

Figure 3. (—) Cyclic voltammogram obtained from a solution containing 100 μM spinach ferredoxin, 100 μM cytochrome P450$_{cam}$, 300 μM polylysine, and saturated with camphor. Scan rate = 3 mV/s. (●) Simulated cyclic voltammogram invoking the mechanism shown by eqs 1 and 2. Parameters for simulation are shown in Table 1.

cm^2/s, respectively, and are in good agreement with previously reported values.[36,39,40] The linear correlation between the square root of the scan rate and the cathodic peak current (Figure 2b) demonstrates that the electrochemical process is diffusion-controlled. In the region of scan rate explored, 10−300 mV/s, the cathodic to anodic peak separation, ΔE_p, is 60 mV and the cathodic to anodic peak current ratio (i_{pc}/i_{pa}) is unity.

Ferredoxin-Mediated Electrochemical Reduction of Camphor-Bound Cytochrome P450$_{cam}$. The cyclic voltammogram of a solution containing an equimolar mixture of spinach ferredoxin and cytochrome P450$_{cam}$ bound to camphor is shown in Figure 3. Two reduction peaks (−567 and −635 mV) are observed in the cathodic scan, and only one oxidation peak (−570 mV) is observed during the corresponding anodic scan. The peaks at −635 and −570 mV correspond to a reversible wave ($E_{1/2}$ = −603 mV) arising from the reduction and subsequent reoxidation of spinach ferredoxin at the electrode surface. The first reduction peak (−567 mV), which is not accompanied by its oxidation counterpart, originates from coupling the electrochemical reduction of spinach ferredoxin to a homogeneous reaction in which an electron is transferred from reduced spinach ferredoxin to camphor-bound ferric cytochrome P450$_{cam}$.[41] Consequently, the first reduction peak, termed a prepeak hereafter, originates from the depletion of substrate-bound ferric cytochrome P450$_{cam}$ in the diffusion layer. This sequence of events, summarized by eqs 1 and 2, and shown schematically in Figure 4, is typical of the EC mechanism:[42]

$$Fd^{ox} + e^- \overset{k_s}{\rightleftharpoons} Fd^{red} \quad (1)$$

$$Fd^{red} + P450^{ox}\text{-}S \underset{k_b}{\overset{k_f}{\rightleftharpoons}} Fd^{ox} + P450^{red}\text{-}S \quad (2)$$

In eq 1, Fd^{ox} and Fd^{red} represent oxidized and one-electron-reduced spinach ferredoxin, respectively, and k_s represents the corresponding heterogeneous electron-transfer rate constant. In eq 2, $P450^{ox}$-S and $P450^{red}$-S represent ferric and ferrous substrate-bound cytochrome P450$_{cam}$, respectively; k_f and k_b are the corresponding forward and backward second-order electron-transfer rate constants. The mechanism summarized by eqs 1 and 2 was digitally simulated using the program Digisim 2.1; the simulated and experimental cyclic voltammograms are depicted in Figure 3 and the parameters obtained from the simulation are listed in Table 1. The agreement between the experimental and simulated responses indicates that the prepeak in the voltammogram shown in Figure 3 originates from the depletion of oxidized camphor-bound cytochrome P450$_{cam}$ in the diffusion layer. The depletion of the latter is a consequence of a homogeneous electron-transfer reaction between electroreduced spinach ferredoxin and oxidized camphor-bound cytochrome P450$_{cam}$, as indicated by eq 2. The values for the

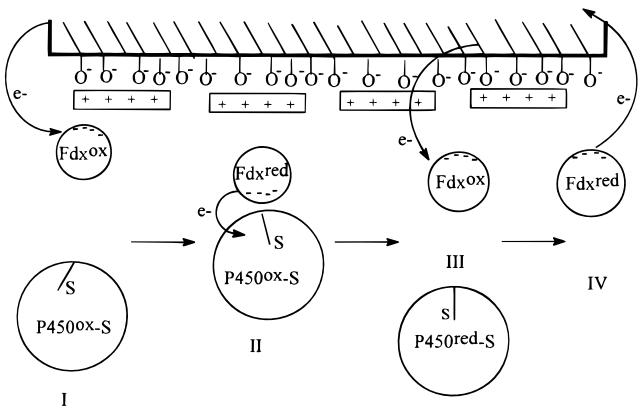

Figure 4. Schematic representation of the sequence of electron-transfer reactions that give rise to the electrochemical response shown in Figure 3: (I) selective electrochemical reduction of spinach ferredoxin in the presence of cytochrome P450$_{cam}$; (II) oxidation of spinach ferredoxin by substrate-bound cytochrome P450$_{cam}$; this reaction gives rise to the depletion of camphor-bound ferric cytochrome P450$_{cam}$ in the diffusion layer and consequently to the pre-peak shown in Figure 3; (III) electrochemical depletion of oxidized spinach ferredoxin that gives rise to the main cathodic peak seen in the voltammogram shown in Figure 3; and (IV) electrochemical reoxidation of spinach ferredoxin that results in the anodic peak in Figure 3.

Table 1. Parameters for Digital Simulation of the Catalytic Response Obtained from a Mixture Containing Spinach Ferredoxin, Cytochrome P450$_{cam}$, and Substrate

	substrate	
	camphor	hexachloroethane
$E^{\circ\prime}$ (Ag/AgCl)	−0.605	−0.605
transfer coefficient, α	0.50	0.50
k_s (cm/s)	$2.5 \times 10^{-3} \pm 2 \times 10^{-5}$	2.5×10^{-3} [a]
k_f	$1.5 \times 10^5 \pm 2 \times 10^4$	
k_b	4.5	
k_{f1} (M^{-1} s^{-1})		2.1×10^7
k_{b1} (M^{-1} s^{-1})		1.5
k_{f2} (M^{-1} s^{-1})		$1.0 \times 10^5 \pm 3 \times 10^4$
k_{b2} (M^{-1} s^{-1})		13.3
k_{f3} (M^{-1} s^{-1})		$1.2 \times 10^4 \pm 2 \times 10^3$
k_{b3} (M^{-1} s^{-1})		5.5
k_{f4} (s^{-1})		$1.5 \times 10^{-2} \pm 3 \times 10^{-3}$
k_{b4} (s^{-1})		2.0
anal. conc Fd^{ox} (μM)	100.0	130.0
D_o Fd^{ox} (cm^2 s^{-1})	$1.6 \times 10^{-6} \pm 4 \times 10^{-7}$	2.0×10^{-6} [a]
D_o Fd^{red} (cm^2 s^{-1})	$1.6 \times 10^{-6} \pm 4 \times 10^{-7}$	2.0×10^{-6} [a]
anal. conc $P450^{ox}$ (μM)	100.0	230.0
D_o $P450^{ox}$ (cm^2 s^{-1})	$5.6 \times 10^{-7} \pm 8 \times 10^{-8}$	6.3×10^{-7} [a]
D_o $P450^{red}$ (cm^2 s^{-1})	$5.6 \times 10^{-7} \pm 8 \times 10^{-8}$	6.3×10^{-7} [a]

[a] During the simulation process these values were allowed to vary within the experimental error shown in the corresponding value listed for camphor. Entries accompanied by standard deviations correspond to the average of four measurements.

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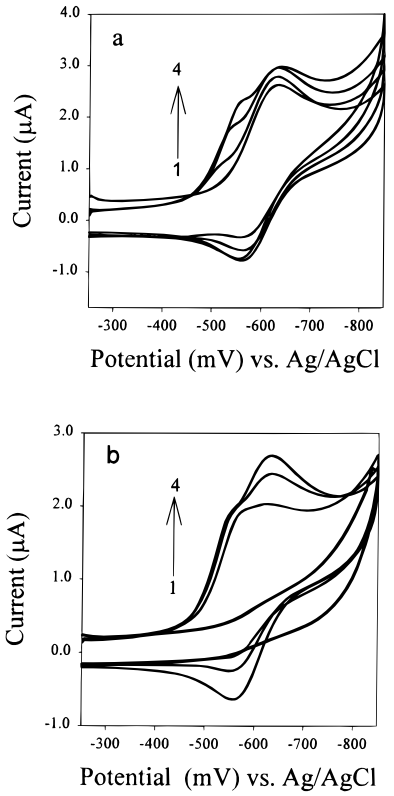

Figure 5. (a) Titration of a solution containing spinach ferredoxin (100 μM) and polylysine (300 μM) in MOPS (100 mM) saturated with camphor (pH = 7.0) with cytochrome $P450_{cam}$: (1) voltammogram obtained in the absence of cytochrome $P450_{cam}$; and (2−4) voltammograms after the addition of cytochrome $P450_{cam}$ to a final concentration of 30, 60, and 100 μM, respectively. (b) Titration of a solution containing cytochrome $P450_{cam}$ (100 μM) and polylysine (300 μM) in 100 mM MOPS saturated with camphor (pH = 7.0) with spinach ferredoxin: (1) voltammogram obtained in the absence of spinach ferredoxin; and (2−4) voltammograms obtained after the addition of spinach ferredoxin to a final concentration of 50, 75, and 100 μM. Scan rates for all voltammograms = 3 mV/s.

heterogeneous electron-transfer rate constant and the diffusion coefficient of spinach ferredoxin utilized in this simulation (Table 1) were obtained experimentally from cyclic voltammograms of solutions containing only spinach ferredoxin and polylysine (see Figure 2). This strategy permits the evaluation of the second-order rate constant (k_f) for the transfer of one electron from reduced spinach ferredoxin to camphor-bound ferric cytochrome $P450_{cam}$ with a higher degree of confidence.

Additional evidence corroborating that the prepeak in the voltammogram shown in Figure 3 originates from a homogeneous second-order electron-transfer reaction in which reduced spinach ferredoxin (generated at the electrode surface) transfers an electron to camphor-bound cytochrome $P450_{cam}$, was obtained as follows: A solution saturated with camphor and containing spinach ferredoxin and polylysine was titrated with cytochrome $P450_{cam}$. The results of this titration, summarized in Figure 5a, show that in the absence of cytochrome $P450_{cam}$ the voltammogram of spinach ferredoxin is devoid of a prepeak. Addition of cytochrome $P450_{cam}$ to the original solution, however, results in the presence of a prepeak whose intensity increases with increasing concentrations of cytochrome $P450_{cam}$. This observation is consistent with the idea that the prepeak originates from the depletion of oxidized cytochrome $P450_{cam}$ in the diffusion layer, as described by eq 2. In a different experiment, a solution saturated with camphor and containing polylysine and cytochrome $P450_{cam}$ was titrated with spinach ferredoxin. The results of this experiment, summarized in Figure 5b, clearly indicate that in the absence of spinach ferredoxin, the voltammetric response originating from the mixture does not produce a Faradaic current. Addition of spinach ferredoxin to the original mixture results in the characteristic voltammogram consisting of a prepeak and a reversible wave. Moreover, the magnitude of the current for the cathodic and anodic peaks corresponding to the reversible wave increases as the concentration of ferredoxin is increased, consistent with the idea that the reversible wave originates from the reduction and oxidation of ferredoxin at the electrode surface. By comparison, the magnitude of the current corresponding to the prepeak does not change, which is consistent with the fixed concentration of cytochrome $P450_{cam}$ during the titration experiment.

To satisfy the requirements for the EC mechanism,[42−44] the reduction of $P450^{ox}$-S at the electrode surface has to be much slower than the forward homogeneous second-order reaction in eq 2, therefore resulting in the negligible reduction of $P450^{ox}$-S at the electrode surface. The discrimination of biomolecules at electrode surfaces leading to the EC mechanism has been reported for a mixture of cytochrome b_5 and cytochrome *c*.[45] In the case of a system containing these two electron-transfer proteins, a gold electrode modified with β-mercaptopropionate was shown to be selective for the positively charged cytochrome *c*. On the other hand, the addition of polylysine to the system resulted in the selective reduction of the negatively charged cytochrome b_5 ($E^{\circ\prime} = -100$ mV), in the presence of cytochrome *c* ($E^{\circ\prime} = 250$ mV). This electrostatic discrimination of biomolecules at the electrode surface resulted in a voltammogram exhibiting a prepeak and a reversible wave.[45] Accordingly, the presence of a prepeak in the cyclic voltammogram shown in Figure 3 demonstrates that although camphor-bound $P450^{ox}$ is thermodynamically more easily reducible ($E^{\circ\prime} = -176$ mV vs NHE) than spinach ferredoxin ($E^{\circ\prime} = -420$ mV vs NHE), the former appears not to interact with the electrode in an orientation that facilitates heterogeneous electron exchange (see above). It is therefore interesting to explore if the discrimination of cytochrome $P450_{cam}$ at the electrode surface may also originate from unfavorable electrostatic interactions with the electrode. To this end, it is useful to visualize the Poisson−Boltzmann (PB) calculations initially reported by Roitberg et al.[46] for cytochrome $P450_{cam}$ (Figure 6A,B) and similar calculations performed on spinach ferredoxin for the purposes of this study (Figure 6C,D).

The results of the PB calculation are readily interpreted by representing the negative electrostatic potential in red, and the positive electrostatic potential in blue. The surface on the "front side" (nearest the active site) of spinach ferredoxin (Figure 6C) reveals the presence of a unique contiguous patch of negative electrostatic potential (red contour at −9 kcal/mol e^-). By comparison, the protein's "back side" (Figure 6D), which is obtained by rotating the view shown in Figure 6C by 180° about the vertical axis of the page, shows a surface with a positive to neutral electrostatic potential. It is therefore possible to infer that the negative electrostatic potential on the "front side" of spinach ferredoxin and the positive electrostatic potential imparted by polylysine to the electrode surface, steer the protein

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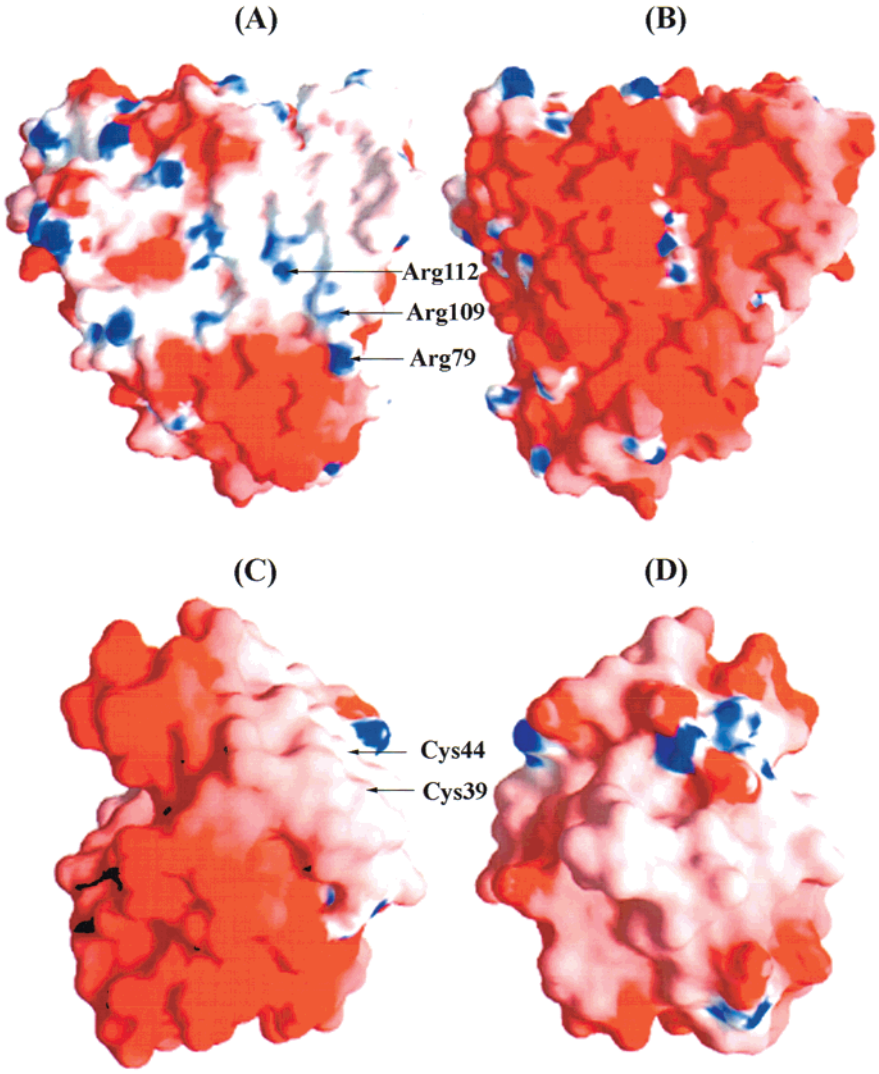

Figure 6. Results of the Poisson Boltzmann electrostatic calculations represented with red contour plots = −9 kcal/mol e^- and blue contour plots = +9 kcal/mol e^-. (A) This view of cytochrome $P450_{cam}$ (PDB ID: 1NOO)[51] shows discrete patches of positive electrostatic potential provided by arginine residues 79, 109, and 112. These residues have been implicated in forming electrostatic interactions between cytochrome $P450_{cam}$ and putidaredoxin before electron transfer. (B) View obtained by rotating (A) 180° about the vertical axis of the page. This view demonstrates that the "backside" surface (furthest away from the heme) in cytochrome $P450_{cam}$ has a large contiguous negative electrostatic potential. (C) This view of spinach ferredoxin (PDB ID: 1A70)[52] shows a large negative electrostatic potential near its $Fe_2S_2Cys_4$ cluster. The position of the outermost cysteine ligands to the Fe_2S_2 cluster (Cys 44 and Cys 39) has been highlighted to show the relative position of the active site. Interactions between this negative electrostatic potential and polylysine at the electrode surface steer ferredoxin with an orientation appropriate for facile heterogeneous electron transfer. (D) This view, obtained by rotating C 180° degrees about the vertical axis of the page, shows a neutral to positive surface electrostatic potential.

toward the electrode with an orientation close to optimum for heterogeneous electron exchange. By comparison, previous work performed with cytochrome $P450_{cam}$ resulted in the identification of a cluster of positively charged residues (Arg-79, Arg-109, Arg-112) that were postulated to encompass the site on the surface of cytochrome $P450_{cam}$ that is utilized for binding with putidaredoxin.[47] Results obtained from Poisson−Boltzmann calculations have shown that this cluster of amino acids produce discrete patches of positive potential (blue in Figure 6A) on the "front side" of cytochrome $P450_{cam}$, near its heme active site.[46] Visualization of the surface on the "back side" of cytochrome $P450_{cam}$ (Figure 6B) demonstrates the presence of a large contiguous patch of negative electrostatic potential.

The PB calculations performed for cytochrome $P450_{cam}$ (Figure 6A,B) suggest that in the absence of polylysine the positive potential on the "front side" of cytochrome $P450_{cam}$ may steer the enzyme toward the negatively charged ITO electrode with an orientation appropriate for fast heterogeneous electron exchange. On the other hand, the presence of polylysine in the electrochemical cell may steer the enzyme toward the electrode surface with an orientation that is nonproductive for heterogeneous electron exchange. Alternatively, cytochrome $P450_{cam}$ may not interact productively with the electrode even in the absence of polylysine. To discern among these alternatives, cyclic voltammetric experiments of solutions containing cytochrome $P450_{cam}$ were carried out in the presence and in the absence of polylysine. Non-Faradaic responses were obtained in both cases. Consequently, it is possible to conclude that in the system consisting of polylysine, spinach ferredoxin, and cytochrome $P450_{cam}$, the presence of polylysine in the electrochemical cell guarantees that the protein with the more negative reduction potential (spinach ferredoxin) is readily reduced at

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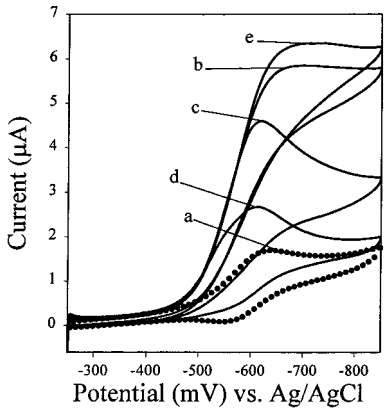

Figure 7. Cyclic voltammograms obtained with a scan rate of 1 mV/s at an ITO electrode: (a) 100 μM spinach ferredoxin, 200 μM cytochrome $P450_{cam}$, and 300 μM polylysine, (b) first scan after the addition of hexachloroethane to the solution in a, (c) second scan after the addition of hexachloroethane, (d) third scan after the addition of hexachloroethane, and (e) first scan after the second addition of hexachloroethane to the solution in d.

the electrode surface. Reduced spinach ferredoxin, in turn, is oxidized by the enzyme possessing a more positive reduction potential (camphor-bound ferric cytochrome $P450_{cam}$) in a homogeneous reaction. The usefulness of this approach to harness the catalytic activity of cytochrome $P450_{cam}$ is described in the next section.

The catalytic cycle of cytochrome $P450_{cam}$ requires the stepwise uptake of two electrons by the enzyme.[1] It is also known that carbon monoxide readily binds to the heme iron of reduced camphor-bound cytochrome $P450_{cam}$, therefore inhibiting the uptake of a second electron by the enzyme.[48] This phenomenon was utilized to corroborate that the electrochemical response shown in Figure 3 corresponds exclusively to the transfer of one electron from spinach ferredoxin to ferric camphor-bound cytochrome $P450_{cam}$. To this end, the voltammetric experiments were conducted under an atmosphere of carbon monoxide (CO) instead of an atmosphere of N_2. The voltammogram obtained from an equimolar mixture of spinach ferredoxin and camphor-bound cytochrome $P450_{cam}$ under an atmosphere of CO is identical to the one obtained under an atmosphere of N_2 (Figure 3). This observation supports the idea that the electrochemical response shown in Figure 3 corresponds to the transfer of one electron from reduced spinach ferredoxin to oxidized camphor-bound cytochrome $P450_{cam}$, as indicated by eq 2.

Ferredoxin-Mediated Electrocatalytic Reductive Dehalogenation Reactions Performed by Cytochrome $P450_{cam}$. The cyclic voltammogram obtained from a solution containing a mixture of spinach ferredoxin and cytochrome $P450_{cam}$ in the absence of substrate is shown in Figure 7a. This voltammogram is identical to that obtained from a solution containing only spinach ferredoxin at the same concentration, hence indicating that reduced spinach ferredoxin does not readily reduce substrate-free ferric cytochrome $P450_{cam}$. This observation is interesting in light of the favorable driving force imparted by the relative reduction potentials of spinach ferredoxin (−420 mV vs NHE)[18,19] and substrate-free cytochrome $P450_{cam}$ (−300 mV).[1] In contrast, when the cyclic voltammetric experiments are carried out with a solution containing a mixture of spinach ferredoxin, cytochrome $P450_{cam}$, and the substrate hexachloroethane (1:2:2), under anaerobic conditions, the catalytic response shown in Figure 7b is obtained. This electrochemical response originates from the ferredoxin-mediated electrocatalytic reductive dehalogenation of hexachloroethane to tetrachloroethylene carried out by cytochrome $P450_{cam}$ (see below). This conclusion is in agreement with previous studies carried out with *Pseudomonas putida* G786, which presented convincing evidence that cytochrome $P450_{cam}$ in *P. putida* catalyzes reductive dehalogenation reactions for a variety of halogenated hydrocarbons.[8,10] Subsequent studies performed in vitro with isolated cytochrome $P450_{cam}$ reconstituted with NADH, putidaredoxin reductase, and putidaredoxin, convincingly demonstrated that cytochrome $P450_{cam}$ is capable of catalyzing the reductive dehalogenation of polychlorinated methanes and ethanes.[11,21]

To obtain additional information concerning the nature of the catalytic reaction giving rise to the electrochemical response shown in Figure 7b, the following sequence of experiments was performed: A cyclic voltammogram was initially obtained with a mixture containing only spinach ferredoxin and cytochrome $P450_{cam}$ (Figure 7a). Addition of hexachloroethane to the electrochemical cell followed by scanning the potential produce the expected catalytic response (Figure 7b). Subsequent scans give rise to the cyclic voltammograms shown in Figure 7c,d. The progressive decrease in the limiting current with each subsequent potential scan originates from the consumption of substrate (hexachloroethane) by the catalytic action of cytochrome $P450_{cam}$. Addition of more hexachloroethane to the solution contained in the electrochemical cell, followed by scanning the potential gives rise to a voltammogram (Figure 7e) almost identical to the one obtained after the first addition of substrate (Figure 7b). Subsequent scanning of the potential after the second addition of substrate result in electrochemical responses similar to those in Figure 7c,d. In fact, the process of adding hexachloroethane to perform its electrocatalytic conversion to tetrachloroethylene can be repeated at least four times (~7 h) without deterioration of the electrochemical response, if the temperature of the cell is maintained at 13 °C. If the temperature of the cell is kept above 20 °C the catalytic current gradually shifts cathodically with each subsequent addition of hexachloroethane and is no longer discernible after the third addition of substrate (~3.5 h). It is likely that the decreased stability of the electrochemical response at temperatures above 20 °C is related with the long-term stability of the proteins.

A GC-MS analysis of the solution contained in the electrochemical cell corroborated that the product of the catalytic activity of cytochrome $P450_{cam}$ is tetrachloroethylene. Furthermore, GC-MS analysis of the electrolyzed solution as a function of time demonstrates that as the concentration of hexachloroethane decreases the concentration of tetrachloroethylene increases (Figure 8a). Additional evidence demonstrating the connection between the electrochemical response and the catalytic current is shown in Figure 8b, which shows that the catalytic current is proportional to the concentration of substrate (hexachloroethane) in the electrochemical cell. In this plot, the current measured in the absence of hexachloroethane is due to the reduction of spinach ferredoxin, as discussed above (Figure 7a). It should also be pointed out that similar results are obtained with pentachloroethane as substrate, except that the product of reductive dehalogenation is trichloroethylene (data not shown).

The following control experiments were carried out to provide additional evidence that the catalytic reductive dehalogenation of hexachloroethane in the electrochemical cell is carried out by cytochrome $P450_{cam}$ with electrons provided by the ITO electrode via spinach ferredoxin. Omission of ferredoxin from

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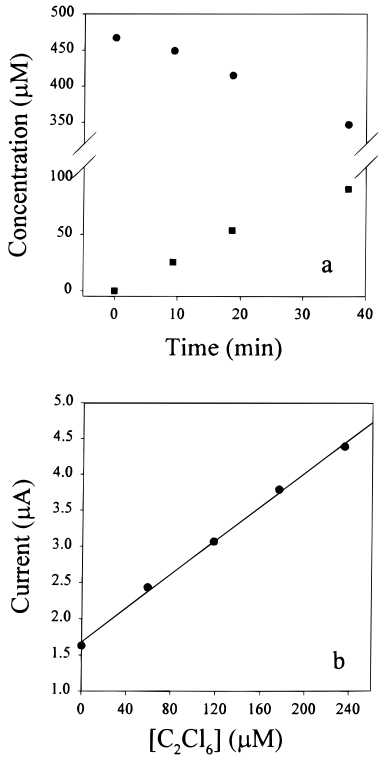

Figure 8. (a) GC-MS analysis of the solution (100 μM spinach ferredoxin, 200 μM cytochrome P450$_{cam}$, 300 μM polylysine, and 470 μM hexachloroethane) contained in the electrochemical cell as a function of electrolysis time: (●) hexachloroethane, and (■) tetrachloroethylene. (b) Limiting current as a function of the concentration of substrate (hexachloroethane).

the electrochemical cell resulted in the absence of a Faradaic response, whereas the omission of cytochrome P450$_{cam}$ resulted in a Faradaic response identical to that seen with experiments containing only spinach ferredoxin (see Figure 2). Furthermore, GC-MS analysis performed on the solutions utilized for these control experiments after the potential had been cycled three times demonstrated a nondetectable concentration of tetrachloroethylene. Experiments with solutions containing only polylysine and hexachloroethane resulted in the absence of Faradaic response. GC-MS analysis of these solutions after three potential cycles showed the presence of only hexachloroethane. Identical results were obtained when polylysine, spinach ferredoxin, and cytochrome P450$_{cam}$ were omitted.

Digital Simulation of the Electrocatalytic Response. The reductive dehalogenation of hexachloroethane to produce tetrachloroethylene and two chloride ions requires two electrons. In the electrochemical cell, these electrons are shuttled from the ITO electrode to hexachloroethane-bound cytochrome P450$_{cam}$ by spinach ferredoxin in two distinct electron-transfer reactions; ferrous substrate-bound cytochrome P450$_{cam}$, in turn, transfers the electrons to hexachloroethane. Assuming that the transfer of an electron from ferrous cytochrome P450$_{cam}$ to its bound substrate is faster than the reduction of substrate-bound ferric cytochrome P450$_{cam}$ by spinach ferredoxin, the minimum mechanism summarized by eqs 3−7 was utilized to simulate the catalytic response in Figure 9A. In this mechanism, Fdx^{ox} and Fdx^{red} represent oxidized and reduced spinach ferredoxin, respectively, P450^{ox}-S represents ferric cytochrome P450$_{cam}$ bound to substrate, cytochrome P450^{red}-S represents ferrous cytochrome P450$_{cam}$ bound to substrate, and cytochrome P450^{ox}-P represents ferric cytochrome P450$_{cam}$ bound to product.

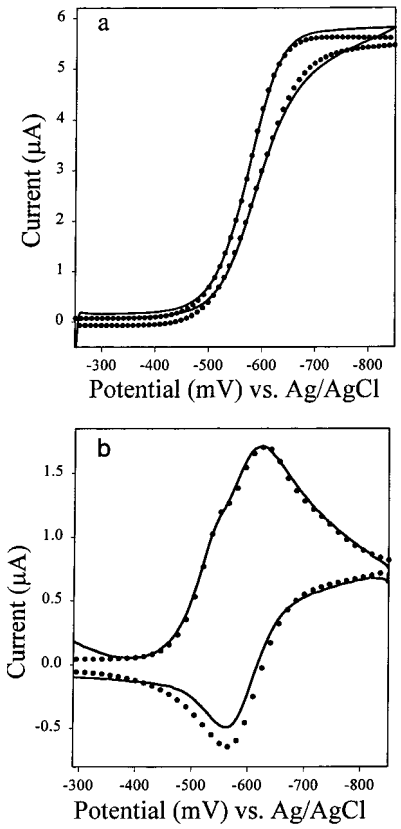

Figure 9. A: (−) Background-subtracted cyclic voltammogram obtained at an ITO electrode with a solution 100 μM in spinach ferredoxin, 200 μM in cytochrome P450$_{cam,}$ 300 μM in polylysine and saturated with hexachloroethane. (●) Simulated cyclic voltammogram obtained by invoking the catalytic cycle shown by eqs 3−7. Parameters for simulation are shown in Table 1. B: Background-subtracted cyclic voltammogram obtained with conditions identical to A under an atmosphere of CO. (●) Simulated cyclic voltammogram obtained by invoking the mechanism described by eqs 1 and 2.

$$\mathrm{Fdx^{ox}} + e^- \overset{k_s}{\rightleftharpoons} \mathrm{Fdx^{red}} \tag{3}$$

$$\mathrm{P450^{ox}} + \mathrm{S} \underset{k_{b1}}{\overset{k_{f1}}{\rightleftharpoons}} \mathrm{P450^{ox}\text{-}S} \tag{4}$$

$$\mathrm{Fdx^{red}} + \mathrm{P450^{ox}\text{-}S} \underset{k_{b2}}{\overset{k_{f2}}{\rightleftharpoons}} \mathrm{Fdx^{ox}} + \mathrm{P450^{red}\text{-}S} \tag{5}$$

$$\mathrm{Fdx^{red}} + \mathrm{P450^{red}\text{-}S} \underset{k_{b3}}{\overset{k_{f3}}{\rightleftharpoons}} \mathrm{Fdx^{ox}} + \mathrm{P450^{ox}\text{-}P} \tag{6}$$

$$\mathrm{P450^{ox}\text{-}P} \underset{k_{b4}}{\overset{k_{f4}}{\rightleftharpoons}} \mathrm{P450^{ox}} + \mathrm{P} \tag{7}$$

The parameters used to obtain the simulated catalytic response shown in Figure 9A are summarized in Table 1. Several of these parameters were obtained independently by performing the following experiments: (a) The values corresponding to the heterogeneous electron-transfer rate constant (k_s) and to the diffusion coefficient of spinach ferredoxin were obtained from cyclic voltammetric experiments carried out with solutions containing only this protein (see Figure 2). (b) The values corresponding to the rate constant for the first electron transfer from reduced spinach ferredoxin to hexachloroethane-bound ferric cytochrome P450$_{cam}$ (k_{f2}) and to the diffusion coefficient of cytochrome P450$_{cam}$ were obtained from experiments carried out with solutions containing spinach ferredoxin, cytochrome P450$_{cam}$, and hexachloroethane under an atmosphere of CO. Under these conditions, the electron-transfer step from reduced

ferredoxin to $P450^{ox}$-S is manifested in the appearance of a prepeak in the corresponding cyclic voltammogram (Figure 9B). By comparison, electron transfer between reduced spinach ferredoxin and $P450^{ox}$ in the absence of substrate does not take place at a discernible rate (see above). Furthermore, the presence of an oxidation peak in the anodic scan shown in Figure 9B indicates that reduced spinach ferredoxin is oxidized at the electrode surface, thus providing additional evidence for the inhibition of catalytic activity produced by CO. Consequently, the digital simulation of the voltammogram shown in Figure 9B provides an independent measurement of the second-order electron-transfer rate constant for the oxidation of spinach ferredoxin by hexachloroethane-bound cytochrome $P450_{cam}$.

Utilizing the independently measured constants, k_s, k_{f2}, and the diffusion coefficient values obtained previously, the digital simulation of the catalytic response (Figure 9A) permits to evaluate the constants, k_{f3} and k_{f4}, with a relatively high degree of confidence. The results of this simulation, summarized in Table 1, indicate that the rate constant for the transfer of the second electron from reduced spinach ferredoxin to hexachloroethane-bound cytochrome $P450_{cam}$ (k_{f3}) is ~8 times smaller than the rate constant for the transfer of the first electron (k_{f2}). It is also interesting to note that the analysis described above indicates that the rate-limiting step in the catalytic conversion of hexachloroethane to tetrachloroethylene is the release of the latter from the catalytic site in cytochrome $P450_{cam}$, as indicated by the value of k_{f4}. In support of this idea, it is known that hexachloroethane binds to the catalytic site in cytochrome $P450_{cam}$ with a binding affinity slightly larger than that measured for camphor.[21] Furthermore, the binding of hexachloroethane also results in the concomitant trigger of the well documented[1] spin shift from $S = {}^1/_2$ to $S = {}^3/_2$ that accompanies the dehydration of the distal heme site in cytochrome $P450_{cam}$.[21] The product of reductive dehalogenation (tetrachloroethylene), therefore, is formed inside the catalytic site of cytochrome $P450_{cam}$ and must egress from it before a new molecule of hexachloroethane is admitted in order to initiate a new catalytic cycle. The insights gained from digital simulation of the catalytic response indicate that tetrachloroethylene egress from the active site appears to proceed relatively more slowly than any of the previous steps in the mechanism, therefore suggesting that this step regulates the overall rate of reductive dehalogenation. Rate-limiting product release in the catalytic activity of cytochrome $P450_{cam}$ has been previously proposed for the oxidation of toluene to benzyl alcohol on the basis of kinetic deuterium isotope effect studies.[49] Finally, it is also interesting to pointout that the value of the rate constant for the formation of the hexachloroethane complex of cytochrome $P450_{cam}$ is verysimilar to that measured for the binding of camphor to cytochrome $P450_{cam}$ (2.5×10^7 M^{-1} s^{-1}).[50]

Tetrachloroethylene as Substrate. As mentioned previously, an important early step in the cytochrome $P450_{cam}$ hydroxylation reaction cycle is the binding of substrate, which triggers the consequent conversion of the heme iron from a low-spin to a high-spin state.[1] The change in spin state is thought to result from expelling water, including the water molecule coordinated to the heme iron, from the distal heme cavity in cytochrome $P450_{cam}$. This occurs with an accompanying 150 mV anodic shift in the reduction potential of the enzyme (from $-$ 320 to $-$ 170 mV vs NHE).[1] Substrates such as camphor and adamantanone bind in the catalytic site of cytochrome $P450_{cam}$ and expel the coordinated water,[4] with a concomitant >95% conversion of the spin state and a 130 mV shift in the reduction potential of the enzyme.[3] On the other hand, substrates such as camphane and norcamphor, which allow the coordinated water to remain in the catalytic site, result in lower (~50%) spin-state conversion and in considerably smaller shifts in the reduction potential of the enzyme.

In this context, it is interesting to compare the binding affinities and percentage of spin-state conversion that result from the binding of hexachloroethane and tetrachloroethylene to ferric cytochrome $P450_{cam}$. Hexachloroethane binds with $K_d = 0.7$ μM and results in a >95% conversion to the high spin state, whereas tetrachloroethylene binds with a $K_d = 150$ μM resulting in only 40% spin-state conversion.[11] By comparison, camphor binds with $K_d = 0.84$ μM and results in >95% conversion to the high-spin state.[3] It is also important to consider that the more highly chlorinated compounds (e.g., hexachloroethane vs tetrachloroethylene) exhibit more positive reduction potentials, thus making them more susceptible to reduction than their less chlorinated counterparts.[22] It is therefore possible to explain the relatively efficient dehalogenation of hexachloroethane by the large anodic shift in the redox potential of the enzyme−substrate complex (i.e., increasing the driving force for the oxidation of reduced spinach ferredoxin) and by the positive reduction potential of hexachloroethane. In contrast, although the reasons behind the lack of reactivity toward tetrachloroethylene are not as obvious, it might originate from (a) a modest anodic shift in the reduction potential of the tetrachloroethylene-bound cytochrome $P450_{cam}$ complex, which is expected to result from a 40% spin state conversion or (b) from the inability of the one-electron reduced P450 complex ($P450^{red}$-S) to catalyze the reductive dehalogenation of tetrachloroethylene due to the less positive reduction potential of this substrate.

In an attempt to elucidate the reasons underlying the lack of reactivity exhibited by tetrachloroethylene, cyclic voltammetric experiments were carried out with solutions containing ferredoxin, cytochrome $P450_{cam}$, and tetrachloroethylene. The voltammograms obtained from these experiments (Figure 10) exhibit a prepeak in addition to the cathodic and anodic waves originating from the electrochemistry of spinach ferredoxin. The presence of a prepeak in the voltammogram originates fromthe depletion of ferric tetrachloroethylene-bound cytochrome $P450_{cam}$ that occurs via the selective reduction of spinach ferredoxin at the electrode surface. Furthermore, the separation between the prepeak and the main peak in the cathodic scan is proportional to the electron-transfer rate constant between the electrogenerated species and the species not interacting with the electrode.[42,45] The presence of a prepeak almost not separated from the main peak, indicates that reduced spinach ferredoxin is capable of reducing tetrachloroethylene-bound cytochrome $P450_{cam}$, albeit more slowly than when the substrate is hexachloroethane. Simulation of the electrochemical response shown in Figure 10 with a mechanism identical to that shown in eqs 1 and 2 demonstrates that the electron-transfer rate constant for this process is 2.0×10^4 M^{-1} s^{-1}. This value is 1 order of magnitude slower than that measured with hexachloroethane as a substrate. It is therefore possible to conclude that cyto-

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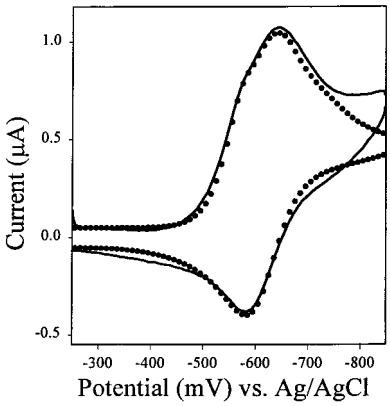

Figure 10. (−) Background-subtracted cyclic voltammogram (scan rate = 3 mV/s) obtained at an ITO electrode with a solution 100 μM in spinach ferredoxin, 200 μM in cytochrome $P450_{cam,}$ 300 μM in polylysine, and saturated with tetrachloroethylene. (●) Simulated cyclic voltammogram invoking the mechanism shown by eqs 1 and 2. Parameters for simulation are identical to those listed in Table 1 under camphor, except that the value of the second-order rate constant for the reduction of tetrachloroethylene-bound cytochrome $P450_{cam}$ by spinach ferredoxin, k_f, is 2.0×10^4 M^{-1} s^{-1}.

chrome $P450_{cam}$ bound to tetrachloroethylene can indeed be reduced by spinach ferredoxin, but that the resultant ferrous heme in the ($P450^{red}$-S) complex cannot transfer an electron to tetrachloroethylene.

Conclusion

The fortuitous ability of cytochrome $P450_{cam}$ to catalyze reductive dehalogenation reactions attests to its well-known catalytic versatility. This lack of specificity for just one substrate has led to an intense effort to harness the catalytic ability of cytochrome $P450_{cam}$ in novel biosynthetic and bioremediation schemes. The catalytic activity of cytochrome $P450_{cam}$, whether involved in oxidative or reductive chemistry, necessitates the input of two reducing equivalents that in vivo are typically provided by NADH via the two accessory proteins, putidaredoxin reductase, and putidaredoxin. Consequently, most of the in vitro studies have been carried out with a reconstituted system consisting of NADH, the two accessory proteins and cytochrome $P450_{cam}$. In this report conclusive evidence has been presented demonstrating that spinach ferredoxin can act as an effective electron shuttle between an electrode surface and cytochrome $P450_{cam}$, hence eliminating the need for expensive and fragile species such as NADH and putidaredoxin reductase, to sustain reductive catalysis.

Two important considerations are necessary to make this strategy successful: First, it is important to choose the appropriate electron-transfer protein for shuttling electrons between an electrode and cytochrome $P450_{cam}$. Spinach ferredoxin was chosen to play this role because in addition to being readily accessible, it possesses the appropriate reduction potential and surface electrostatic potential that permits it to transfer electrons to cytochrome $P450_{cam}$. Second, it is necessary to accomplish the selective reduction of the electron-transfer protein in the presence of an excess of substrate-bound cytochrome $P450_{cam}$, which by necessity possesses a more positive reduction potential. Cytochrome $P450_{cam}$ does not exchange electrons with the ITO electrode in the presence or in the absence of polylysine but it is readily reduced by spinach ferredoxin in a homogeneous second-order electron-transfer reaction.

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