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High-Temperature Electrocatalysis Using Thermophilic P450 CYP119: Dehalogenation of CCl_4 to CH_4

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Chloro-organics, such as chloroform and methylene chloride, are produced on the hundreds of millions of pounds per year scale.[1] These hepatotoxic and carcinogenic solvents[2] have been released into the environment causing stratospheric ozone depletion[3,4] and contamination of agricultural land. The widespread use of chlorinated solvents has resulted in their presence at most of the hazardous waste sites on the EPA's national priority list,[5] and carbon tetrachloride has specifically been banned from industrial use in many nations by the Montreal Protocol, effective January 1, 1996.[6] Carbon tetrachloride is still generated as a major byproduct during chloroform and methylene chloride production. Bioremediation has had only limited success as the dechlorination turnover is inhibited by increasing concentrations of CCl_4.[7]

Dehalogenations utilizing chemical reductants and biocatalysts, such as cobalamin (Co), coenzyme F_{430} (Ni), and $P450_{cam}$ (Fe), have been demonstrated for various chlorinated toxins.[8−11] Likewise, several groups have studied electrocatalytic dehalogenation using heme protein or porphyrin electrocatalysts.[12−16] However, complete dehalogenation of polychlorinated substrates is difficult for the C_1 halocarbon solvents. In this communication, we describe efficient multiple dehalogenations of C_1 halocarbons at elevated temperatures, electrochemically catalyzed by a thermophilic enzyme.

Extremophilic enzymes are of wide interest because of their stability at extremes of temperature, pressure, and pH which may enable their use under harsh industrial applications. Cytochrome P450 CYP119 was discovered in the genome of *Sulfolobus solfataricus*, an extremophilic archaebacteria found in sulfurous volcanic hot springs. CYP119 has an unusually high denaturation point of ca. 90 °C and tolerance for extreme pHs and pressures for extended periods in solution.[17−21] We have previously demonstrated that CYP119 gives excellent electrochemical response when immobilized in a dimethyldidodecylammonium bromide (DDAB) film on a pyrolitic graphite electrode (PG)[20] and have utilized it for the catalytic reduction of NOx species such as nitrite and nitric oxide.[22]

To determine the effect of temperature on the electrochemical activity of CYP119, thermostable immobilization schemes using sol−gels and polymer-based surfactants were investigated. P450cam, immobilized in a methyltriethoxysilane sol−gel film on graphite as an electrochemical sensor for camphor and pyrene, retains prolonged stability in organic solvents.[23] Likewise, alkylammonium poly(*p*-styrene sulfonate) are thermostable and insoluble in aqueous and organic solutions making them suitable for a varied array of electrochemical environments;[24] the combination of dimethyldidodecylammonium poly(*p*-styrene sulfonate), or DDAPSS, to protein voltammetry was recently demonstrated for myoglobin.[13]

As determined electrochemically, the CYP119/sol−gel film remained stable until ca. 60 °C (S1, Supporting Information), but the anodic current drops off at higher temperatures to ca. 45% of that at 30 °C, Figure 1.

At 80 °C, CYP119/DDAPSS films retain 93% of the current at the $Fe^{III/II}$ couple at 30 °C, and only a moderate loss of signal is observed between 80 °C and 90 °C. Variable temperature absorption measurements demonstrate that the heme Soret band does not shift with temperature in either system (S2B, Supporting Information). Over the accessible temperature range, observed $E_{1/2}$ for the $Fe^{III/II}$ couple decreases linearly with increased temperature, from −213 mV at 25 °C to −278 mV at 80 °C, $\Delta E_{1/2} = -1.07 \times 10^{-3}$ V/°C (S4, Supporting Information). This represents a somewhat larger temperature shift than those seen for other thermophilic and nonthermophilic redox proteins[25,26] but may contain contributions from the film dielectric.

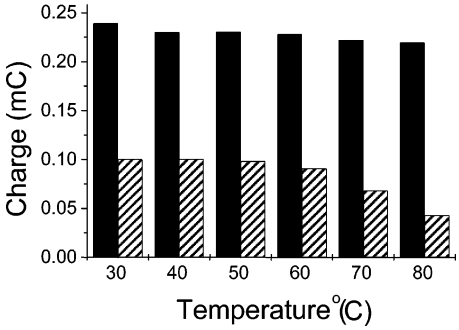

Figure 1. Comparison of the $Fe^{III/II}$ anodic currents at various temperatures for CYP119 modified electrodes for identical loadings: solid bars denote CYP119/DDAPSS in 100 mM pH 6 iP buffer; striped bars denote CYP119/sol−gel/DDAB in 50 mM pH 4 iP buffer. Conditions: 500 mV/s, Pt counter electrode, Ag/AgCl reference electrode.

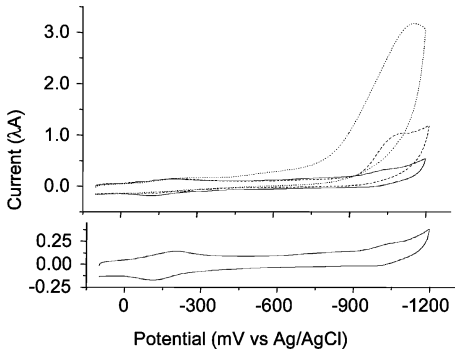

Figure 2. Electrocatalytic reductive dehalogenations of C_1 chlorocarbon substrates at room temperature. (Top) CYP119/DDAPSS in the presence of 10 mM CH_2Cl_2 (solid), $CHCl_3$ (dashed), and CCl_4 (dotted). (Bottom) Voltammogram of catalyst in absence of substrate. Conditions: 100 mM pH 6 iP buffer, 20 mV/s, and Pt counter electrode, Ag/AgCl reference.

At room temperature, the presence of C_1 chlorinated substrates cause large increases in cathodic current in voltammograms of CYP119/DDAPSS, indicative of catalytic reductions of these compounds, Figure 2. The catalytic currents increase linearly with substrate concentrations well beyond the substrates solubility in water, indicative of preconcentration of the hydrophobic compounds within the film (S5, Supporting Information). GC−MS analysis of

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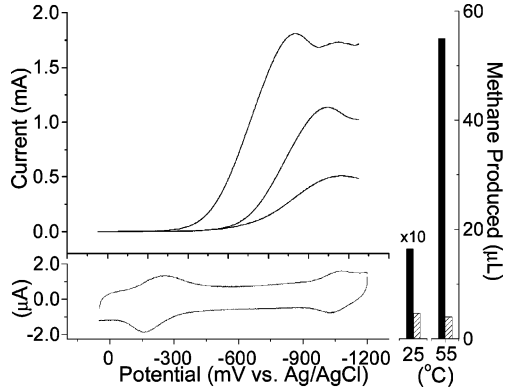

Figure 3. Effect of temperature on catalytic dehalogenation of CCl_4. (Top) Linear sweep voltammograms of CYP119/DDAPSS in the presence of saturated CCl_4 at (a) 25 °C, (b) 55 °C, (c) 75 °C. (Bottom) Voltammogram of CYP119/DDAPSS at 200 mV/s. (Right) Volume of methane produced during electrocatalysis of CCl_4 at 25 °C (× 10) and 55 °C after electrolysis for 20 min at −1150 mV, average of three trials. Solid bars denote CYP119 catalysis, and striped are control experiments in absence of CYP119.

products resulting from bulk electrolysis identified sequentially dechlorinated C_1 products but gave no evidence of C_2 products, e.g., chloroethanes (S6, Supporting Information). The electron turnover per protein at equivalent concentrations of substrate are 52.1 s^{-1} for CCl_4, 27.5 s^{-1} for $CHCl_3$, and 4.5 s^{-1} for CH_2Cl_2, following the differences in electron affinity of the substrates.[27] The catalytic waves occur close to the $Fe^{II/I}$ couple, suggesting that the Fe^{I} state is the active catalyst and that the reactions proceed via an overall two-electron reduction process, eq 1.

$$Fe^{I} + RCl + H^{+} \rightarrow Fe^{III} + RH + Cl^{-} \qquad (1)$$

Catalytic electrolysis of CH_2Cl_2 in D_2O yielded signals attributable to CD_3H and CD_2H_2 by MS analysis, suggesting that complete dechlorination was achievable (S6, Supporting Information). We therefore investigated the effect of increasing temperature on the catalytic reduction of CCl_4 using GC-FID analysis. Varying the temperature from 25 °C to 55 °C increased the maximum catalytic current observed in linear scan voltammograms, from 0.51 to 1.14 mA; likewise, the onset of the catalytic current shifted positively, as seen in Figure 3. The observed changes in catalytic current include kinetic enhancement as well as increased diffusion and substrate solubility at the elevated temperature. But most impressively, a 35-fold increase in methane production is seen in the headgas, from 1.65 μL at 25 °C to 55.00 μL at 55 °C over a 20 min electrolysis at −1.15 V vs Ag/AgCl, Figure 3. In combination with the lack of C_2 products, this dramatic increase in multiple dehalogenations suggests that the substrate is constrained within the redox active heme pocket during electrocatalytic turnover.

We have recently reported similarly dramatic differences in the electrocatalytic reduction of nitrite by myoglobin and CYP119.[22] Within the first 30 min of catalysis, myoglobin produces mainly nitrous oxide with less than 20% conversion to ammonia; within the same period, CYP119 catalysis produces almost quantitative ammonia. The reduction of nitrite to ammonia requires an overall six-electron reduction of each substrate. CYP119 is here demonstrated as a superior catalyst for the reduction of CCl_4 to CH_4, an overall eight-electron reduction. The advantage over other catalysts may lie within the protein structure itself, i.e., that the rigidity and stability of the thermophilic protein traps the substrate within the active pocket, facilitating multiple reductions of individual substrates.

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Supporting Information Available: Experimental details, including characterizations of the protein-modified electrodes, temperature dependence of the $Fe^{III/II}$ couple, methods of analysis of the products of bulk electrolysis, control experiments, and a comparison of nitrite reduction at elevated temperatures; seven pages. This material is available free of charge via the Internet at http://pubs.acs.org.

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