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Electrochemistry of Cytochrome P450 BM3 in Sodium Dodecyl Sulfate Films

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Abstract

Direct electrochemistry of the cytochrome P450 BM3 heme domain (BM3) was achieved by confining the protein within sodium dodecyl sulfate (SDS) films on the surface of basal-plane graphite (BPG) electrodes. Cyclic voltammetry revealed the heme $Fe^{III/II}$ redox couple at −330 mV (*vs*. Ag/AgCl, pH 7.4). Up to 10 V/s, the peak current was linear with scan rate, allowing us to treat the system as surface-confined within this regime. The standard heterogeneous rate constant determined at 10 V/s was estimated to be 10 s^{-1}. Voltammograms obtained for the BM3-SDS-BPG system in the presence of dioxygen exhibited catalytic waves at the onset of Fe^{III} reduction. The altered heme reduction potential of the BM3-SDS-graphite system indicates that SDS is likely bound in the enzyme active-site region. Compared to other P450-surfactant systems, we find redox potentials and electron transfer rates that differ by ~ 100 mV and > 10-fold, respectively, indicating that the nature of the surfactant environment has a significant effect on the observed heme redox properties.

Introduction

The cytochromes P450 (P450s) are ubiquitous heme-thiolate proteins that catalyze stereo- and regiospecific reactions under physiological conditions.[1] The enormous potential of *in vitro* P450 systems is widely recognized: synthesis of medicinal drugs and fine chemicals,[2] use in biosensors,[3] bioremediation,[4] and production of drug metabolites[5] are all possible applications for P450 biocatalysis. While attractive, *in vitro* commercial exploitation of P450s is hindered by an inability to adequately replicate the complex electron transfer (ET) machinery the enzyme requires for catalysis, which includes relatively unstable or expensive cofactors (*e.g.*, NADPH) and reductase proteins.

Direct electrochemical reduction is a potential method for providing P450s with reducing equivalents, thereby eliminating the native ET machinery. One problem to overcome is the relatively weak polypeptide-mediated electronic coupling between redox cofactors and an electrode surface;[6–8] this is especially true for P450, where the heme is buried deep within the folded protein. It follows that a successful electrochemical method must be one which increases the heme-electrode electronic coupling. Several investigations have described methods for preparing modified electrodes that result in enhanced heme-electrode ET rates, including use of polished edge-plane graphite,[9] mercaptan films on gold,[10] polyion layers on carbon,[11, 12] covalent surface modification,[13] clay on carbon,[14] and rhodium-graphite electrodes.[15] In each instance the voltammetric response was relatively strong, allowing in-depth characterization of the P450 heme ($Fe^{III/II}$ and $Fe^{II/I}$) redox couples.

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Surfactant assemblies on carbon electrodes have been shown to facilitate direct ET with various P450s.[16–19] Protein is incorporated into surfactant layers deposited on the electrode. Typical surfactants for P450 electrochemistry include biological phospholipids,[20] synthetically-derived molecules (*e.g.*, didodecyldimethylammonium bromide) (DDAB),[21] and surfactant-polyion mixtures (*e.g.*, didodecyldimethylammonium polystyrenesulfonate) (DDAPSS).[4] Interestingly, the nature of the surfactant has been shown to have a profound effect on the redox properties of the protein: significant variations in heme redox potential (in the absence and presence of various heme ligands), ET rates, and catalysis have been observed. Herein, we report electrochemical investigations of the cytochrome P450 BM3 heme domain from *Bacillus megaterium* (BM3), a fatty acid hydroxylase, in sodium dodecyl sulfate (SDS) surfactant films on the surface of basal-plane graphite (BPG) electrodes. BM3 is a good P450 candidate to study for commercial applications given its relative stability (*cf.* other P450s), impressive turnover rates (~ 10^3 min^{-1}), and the ability to mutate it to accept a variety of substrates.[5, 22–24]

Results and Discussion

The interaction of SDS with BM3 was examined by electronic absorption spectroscopy (Figure 1). In the absence of SDS, BM3 displays an absorption maximum at 418 nm, characteristic of a six-coordinate low-spin Fe^{III} heme. The absorption spectrum develops a new feature at 390 nm as SDS is titrated into a BM3 solution, indicative of a five-coordinate high-spin Fe^{III} heme. Notably, BM3 appears capable of tolerating a relatively high local concentration of SDS. In fact, turnover experiments using holo BM3 (heme-reductase fusion) and NADPH resulted in sub-terminal hydroxylation of SDS at the ω-1 and ω-2 positions. The observation that SDS is a substrate for BM3 is not surprising given the good catalytic activity BM3 displays in reactions with lauric acid, a structurally similar molecule.[25]

Protein films for voltammetry were formed by applying a mixture of BM3, surfactant, and buffer to the BPG surface, followed by slow evaporation of water under high humidity. Cyclic voltammetry of the filmed electrode revealed a well-defined redox couple with $E_{1/2}$ = −330 mV (*vs.* Ag/AgCl, pH 7.4) (Figure 2). With no enzyme, no film, or BPG alone, there were no apparent redox reactions. We have assigned the observed voltammetric signal to the heme $Fe^{III/II}$ redox couple, which is shifted ~ +200 mV relative to (six-coordinate, low-spin) BM3 in solution.[26] P450 potential shifts on electrode surfaces are commonly observed; for BM3-SDS-BPG, there are two likely reasons for the altered heme potential. First, since a large excess of SDS is present during the filming process, an SDS molecule would bind in the active site, causing the typical substrate-induced low- to high-spin shift in the heme. Indeed, this process is known to increase the heme potential by at least 100 mV (depending on the substrate), and is the key mechanistic feature gating P450 ET and the overall catalytic cycle.[26] Second, local electrostatic effects (*e.g.*, electrolyte, hydration) also play a role, as previously described.[16]

Prior work with P450 confined in DDAB films on graphite revealed that the $Fe^{II/I}$ redox couple also is accessible.[27] However, in experiments scanning BM3-SDS-BPG electrodes to −1.5 V, we found no evidence for Fe^{I} species. In addition, the $Fe^{III/II}$ redox couple was no longer observable after scanning to −1.5 V. Most likely, polarizing the electrode to such a negative potential results in desorption of negatively charged SDS molecules, with subsequent loss of the protein signal. A related observation has been made in experiments involving DDAB films on graphite: polarizing the electrode by applying a positive potential causes a phase transition in DDAB (a cationic surfactant) to a more liquid-like state.[28] Given the high solubility and relatively high critical micelle concentration of SDS,[29] a similar phase transition would likely cause SDS to desorb from the graphite surface, resulting in subsequent loss of the P450 voltammetric response.[30]

The system displayed peak current-scan rate behavior characteristic of thin-film voltammetry: the peak current was linear with scan rate up to 10 V/s, after which it was linear with the square root of the scan rate, indicating finite diffusion of the protein within the film.[31, 32] Our subsequent data analysis reflects the surface-confined regime. The cathodic to anodic peak current ratio ($I_{p,c}/I_{p,a}$) is 0.9 (Figure 2), fully consistent with electrochemical reversibility: the redox active sites that are reduced during the cathodic sweep are reoxidized on the reverse scan.[32] Regarding surface coverage, the number of moles of electroactive protein reduced during the cathodic sweep can be determined by integrating under the peak in Figure 2. A value of 3 μC was found, corresponding to a surface coverage of 2×10^{-10} mol/cm^2, which in turn indicates that approximately 33% of the total protein deposited during filming was electroactive. Loading more protein onto the surface did not increase the electrochemical response. The calculated surface coverage, which is comparable to other systems (*e.g.*, 1×10^{-10} mol/cm^2 for hemoglobin[33] and 7×10^{-10} mol/cm^2 for P450 from *Pseudomonas putida* (CAM)[16]), corresponds to multiple layers on the electrode surface.

Using Laviron's treatment, we estimated the heterogeneous rate constant k^o ($\Delta G^o = 0$; at 10 V/s) to be 10 s^{-1}.[34] For comparison, k^o values of 221 and 250 s^{-1} were obtained for BM3 in DDAB[21] and DDAPSS,[35] respectively (Table 1). Although the heme-electrode ET rate in SDS is not as high as that in DDAB, it still exceeds that found for direct electrochemistry of P450 in solution (~ 0.01 s^{-1}).[14, 36]

The redox properties of BM3 in surfactant-electrode systems are strongly influenced by the nature of the surfactant. For example, films using the DDA^+ cation yield heme $Fe^{III/II}$ redox potentials that are 100 mV greater than that observed in SDS, whereas the heme-electrode ET rate in DDA^+ is much higher (Table 1). Indeed, extensive investigations have been made on heme protein-DDA^+ systems in an attempt to account for the dramatically altered heme redox properties. Several proposals have been put forth, ranging from a surfactant-induced perturbation of the active-site environment[20, 37] to heme dissociation from the protein.[38] With regards to the BM3-SDS-BPG system, it would appear that SDS has a less dramatic effect: the altered heme potential is fully consistent with substrate binding (and minor local electrostatic perturbations), while the heterogeneous ET rate approaches a value that is more consistent with direct protein electrochemistry.

Voltammograms of BM3-SDS-BPG in experiments with increasing concentrations of dioxygen are shown in Figure 3. We find that: 1) the voltammograms become increasingly less reversible as more dioxygen is added, indicating oxidation of the reduced heme by dioxygen; 2) the cathodic peak shifts to more positive potentials with increasing amounts of dioxygen in solution, illustrating the known potential shift that accompanies dioxygen binding to the heme;[39] and 3) the cathodic peak current increases with increasing concentrations of dioxygen, indicating catalytic reduction of dioxygen. In the absence of protein, dioxygen reduction proceeds slowly at more negative potentials.

Our observation of P450-catalyzed dioxygen reduction led us to investigate bioelectrocatalytic oxygenation of substrates. For these experiments, films were prepared on larger pieces of graphite or carbon cloth (see Experimental Methods). Electrolysis reactions were conducted at −0.6 V (*vs.* Ag/AgCl) in a stirred cell in air. Several substrates were examined, including lauric acid and styrene. We also assayed for SDS oxidation. Employing wild-type BM3, we found no evidence for substrate oxygenation in any of the trials.

Previous work with CAM determined that catalysis within films on electrode surfaces occurs through the peroxide shunt, with the required hydrogen peroxide formed by P450-mediated reduction of dioxygen.[12] To determine if BM3 is competent for substrate oxidation within SDS films, catalysis experiments were conducted using BM3 mutant 5H6, which has enhanced

catalytic activity through the peroxide shunt pathway.[40] However, as with wild-type BM3, experiments with 5H6 did not yield any oxygenated substrates. The inability of BM3 to oxidize added substrates can possibly be explained by the substrates preferentially partitioning into the SDS film, or by an inability of the substrates to access the active site due to a bound SDS molecule. Indeed, the upshifted BM3 redox potential in the absence of exogenous substrates and the insensitivity of this potential to added substrates indicates that an SDS molecule is bound in the active site. Given this scenario, one would expect to observe oxidation of the SDS film itself, but this was not the case. Voltammetry after electrolysis suggests a reason for this: scanning in the region of the $Fe^{III/II}$ redox couple resulted in no observable waves, indicating complete desorption of the BM3 film. Thus, it can be inferred that polarizing the electrode at a negative potential results in film desorption before significant substrate oxidation can occur.

Experimental Methods

Protein Expression and Purification

Expression and purification of wild-type and mutant BM3 heme domains followed literature procedures.[25]

Electrode Preparation and Voltammetry with BM3-SDS Films

Electrodes were made using the basal plane of pyrolitic graphite (Union Carbide) (0.2 cm^2). The surfaces were prepared by abrasion with sandpaper, followed by brief sonication in double-distilled H_2O and drying with a heat gun. Protein films were applied by placing 20 μL of a solution containing 6 μM BM3, 15 mM SDS, and 8 mM KP_i pH 7.4 onto the electrode surface. The electrodes were covered under water-saturated air for 6 h, followed by uncovering and drying in air overnight. Prior to voltammetry, the films were thoroughly dried under vacuum for 1 h

A CH Instruments Electrochemical Workstation was used for the reactions. Voltammetry experiments were performed in a three-compartment cell, using a platinum wire auxiliary and a Ag/AgCl reference (BAS). The buffer, 30 mM KP_i/130 mM NaCl/pH 7.4, was thoroughly degassed by evacuating and backfilling with argon multiple times. All experiments were performed under argon unless otherwise stated.

Electrode Preparation for Bulk Electrolysis with BM3-SDS Films

Carbon cloth electrodes (ElectroChem, Inc.) were cleaned by soaking in concentrated nitric acid for 1 h, followed by repeated rinsing with double-distilled H_2O and drying with a heat gun. Films were formed by soaking the electrode in a solution of 10 mM SDS, 5 μM BM3, and 6 mM KP_i pH 7.4 for 5 h, followed by drying in air overnight.

Pyrolytic graphite for electrolysis was obtained from GE Advanced Ceramics. The surface was prepared by abrading with sandpaper, followed by brief sonication in double-distilled H_2O and drying with a heat gun. Protein films were formed by placing 250 μL of a solution containing 6 μM BM3, 15 mM SDS, and 8 mM KP_i pH 7.4 onto the electrode surface. The electrodes were covered under water-saturated air overnight, followed by uncovering and drying in air overnight.

Electrolysis Protocols and Activity Assays

Electrolysis reactions were performed in a two-compartment cell separated by a glass frit, with the working and reference (Ag/AgCl) electrodes in one compartment and the Pt auxiliary in the second compartment. The potential was set to −0.6 V, and the cell was stirred in air. The reaction solution typically consisted of 1–5 mM of substrate (lauric acid, styrene) in 30 mM KP_i/130 mM KCl/pH 7.4 buffer, in a final volume of 10 mL. The reaction was allowed to

proceed for 1 h, after which it was extracted and analyzed by GC/MS. GC/MS protocols for lauric acid and SDS were performed as described.[25]

For styrene reactions, 5 μL of a 5 mM solution of 1-hexanol in methanol (internal standard) were added to the reaction solution after electrolysis, followed by extraction with 400 μL $CHCl_3$. The extract was then analyzed by GC/MS using an Innowax column. Styrene, styrene oxide, and benzaldehyde were quantified using a calibration curve constructed from standards, and relating the area of these standards to that of the internal standard.

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Abbreviations

BM3
heme domain cytochrome P450 BM3

ET
electron transfer

SDS
sodium dodecyl sulfate

BPG
basal plane graphite

DDAB
didodecyldimethylammonium bromide

DDAPSS
didodecyldimethylammonium polystyrenesulfonate

CAM
cytochrome P450 from *Pseudomonas putida*

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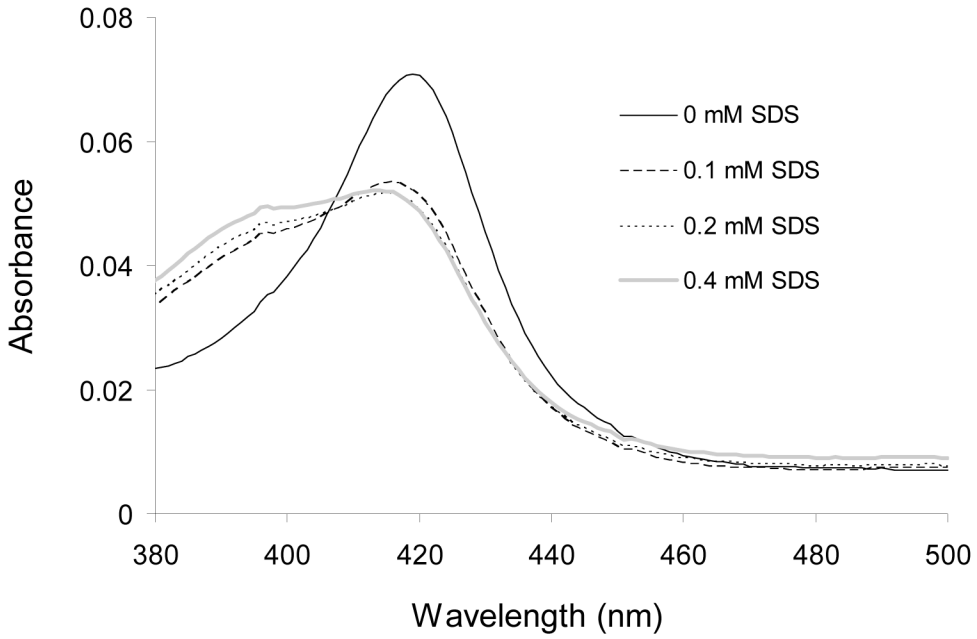

Figure 1.
Absorption spectra of BM3 (0.5 μM) as a function of SDS concentration.

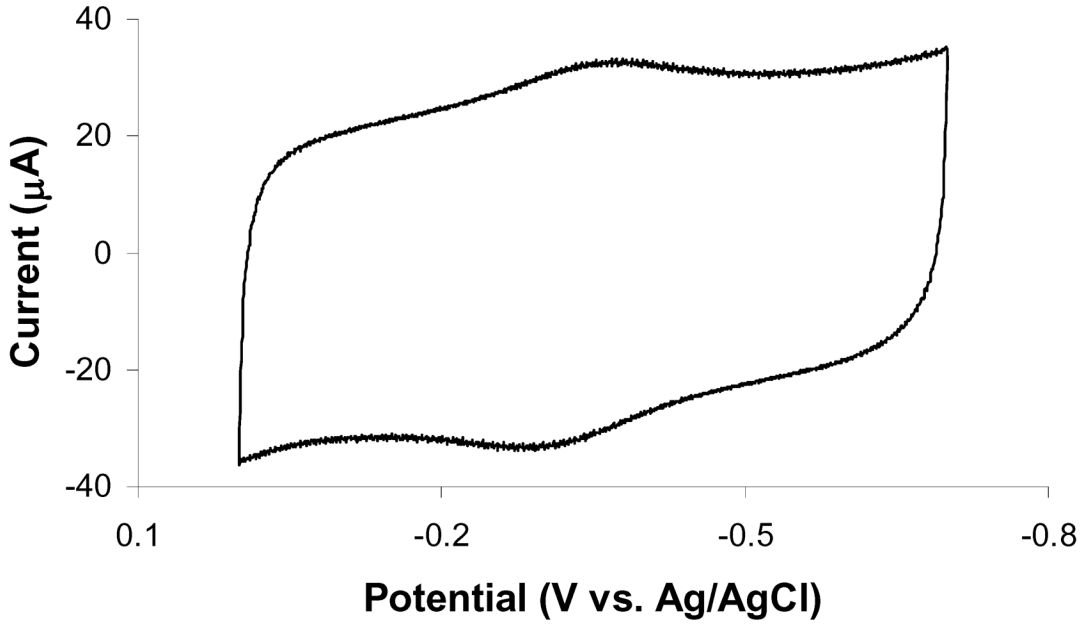

Figure 2.
Cyclic voltammogram of BM3 in SDS on BPG (0.2 cm^2) at 200 mV/s in 30 mM KP_i/130 mM NaCl/pH 7.4.

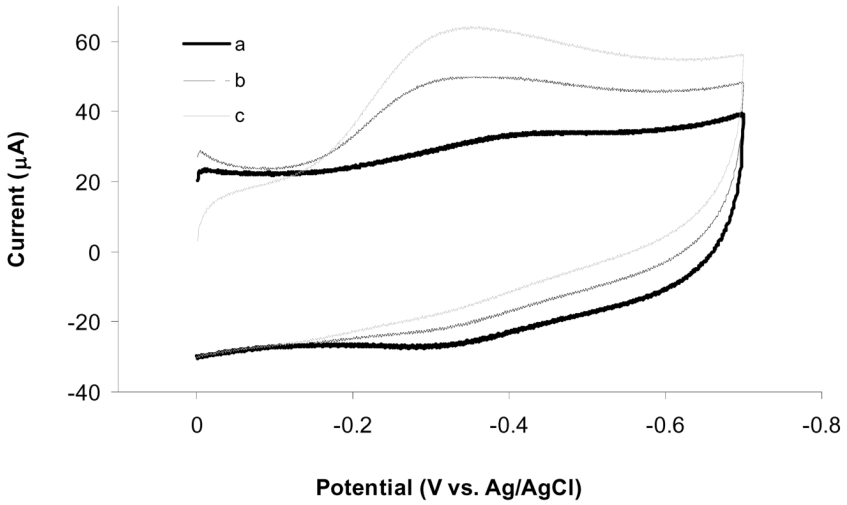

Figure 3.
Cyclic voltammograms of BM3 in SDS on BPG as a function of dioxygen concentration: (a) 0 μM, (b) 42 μM, (c) 83 μM.

Table 1

Redox properties of heme proteins in films on electrode surfaces

Protein	Film	E^o ($Fe^{III/II}$) (mV *vs.* NHE)	k^o(s^{-1})	ref.
BM3	SDS	−133[a]	10	this work
BM3	DDAB	−9[b]	221	21
BM3	DDAPSS	4[c]	250	35
Myoglobin	SDS	−35[d]	0.001[g]	41
Myoglobin	DDAB	0[e]	48	42
Myoglobin	DDAPSS	5[f]	28	19

[a] by CV, pH 7.4

[b] by CV, pH 7.4

[c] by CV, pH 7

[d] by CV, pH 5.5

[e] by SWV, pH 7

[f] by CV, pH 7

[g] reported in cm/s