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Electrochemistry of mammalian cytochrome P450 2B4 indicates tunable thermodynamic parameters in surfactant films

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Abstract

Electrochemical methods continue to present an attractive means for achieving *in vitro* biocatalysis with cytochromes P450; however fully understanding the nature of electrode-bound P450 remains elusive. Herein we report thermodynamic parameters using electrochemical analysis of full-length mammalian microsomal cytochrome P450 2B4 (CYP 2B4) in didodecyldimethylammonium bromide (DDAB) surfactant films. Electronic absorption spectra of CYP 2B4-DDAB films on silica slides reveal an absorption maximum at 418 nm, characteristic of low-spin, six-coordinate, water-ligated Fe^{III} heme in P450. The Fe^{III/II} and Fe^{II/I} redox couples ($E_{1/2}$) of substrate-free CYP 2B4 measured by cyclic voltammetry are -0.23 V and -1.02 V (vs. SCE, or 14 mV and -776 mV vs. NHE) at 21°C. The standard heterogeneous rate constant for electron transfer from the electrode to the heme for the Fe^{III/II} couple was estimated at 170 s⁻¹. Experiments indicate that the system is capable of catalytic reduction of dioxygen, however substrate oxidation was not observed. From the variation of $E_{1/2}$ with temperature (18 – 40 °C), we have measured entropy and enthalpy changes that accompany heme reduction, -151 J mol⁻¹ K⁻¹ and -46 kJ mol⁻¹, respectfully. The corresponding entropy and enthalpy values are less for the six-coordinate low-spin, imidazole-ligated enzyme (-59 J mol⁻¹ K⁻¹ and -18 kJ mol⁻¹), consistent with limited conformational changes upon reduction. These thermodynamic parameters are comparable to those measured for bacterial P450 from *Bacillus megaterium* (CYP BM3), confirming our prior reports that the surfactant environment exerts a strong influence on the redox properties of the heme.

Keywords

cytochrome P450 2B4; thin film electrochemistry; biocatalysis

1. Introduction

The cytochromes P450 are heme-thiolate monooxygenases that perform highly regio- and stereospecific reactions under physiological conditions. First discovered in 1960, to date more than 500 different P450s have been identified, cloned, and sequenced, resulting in a

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plethora of information on P450 structure, function, and biochemistry [1-3]. P450s play critical roles in mammalian cellular pathways as they are responsible for the biosynthesis of a variety of signaling molecules [4-6]. Owing to their importance in medicine and pharmacology [4], as well as their potential to catalyze challenging oxidations for commercial chemical synthesis [7, 8], there continues to be great potential value in capturing P450 activity *in vitro*. Complicating this endeavor is the intricate electron transfer (ET) machinery required for P450 catalysis, which has posed a significant obstacle for practical applications.

Electrochemical methods are perhaps the simplest way of providing P450s with reducing equivalents for catalysis, resulting in many studies [9-18] and comprehensive reviews [19]. In particular, surfactant films on carbon surfaces are excellent at achieving electronic coupling between heme proteins and electrodes for direct electrochemistry [20-22]. The surfactant is deposited onto the electrode, resulting in the formation of bilayers and micelles into which the protein is incorporated [23, 24]. Flavocytochromes [25], engineered mutants [26], bacterial [27] and mammalian P450s [28], and the related nitric oxide synthases [29] have all been investigated, often yielding mechanistic insights into catalysis. This methodology has evolved into a routine method for interrogating the redox chemistry of heme proteins [30, 31]. Notably, a striking aspect of heme protein electrochemistry in surfactant films is the dramatic shift of the $\text{Fe}^{\text{III/II}}$ couple to positive potentials (up to +300 mV cf. solution). Indeed, such up-shifts are not uncommon for heme protein-surfactant film assemblies on electrodes and appear to vary according to the protein, electrode, surfactant, and solution conditions; possible reasons for this have been the subject of debate [32-34].

Our own investigations using bacterial P450 from *Bacillus megaterium* (CYP BM3) in didodecyldimethylammonium bromide (DDAB) films [35] suggested that there was enhanced hydrogen bonding to the heme axial cysteine ligand within the hydrophobic film, resulting in a weaker iron-thiolate bond. Thiol ligation to the heme iron is the critical factor that separates P450-type reactivity from other heme proteins by modulating the iron redox properties to activate dioxygen for substrate oxidation [36]. Thus, weakening of the iron-thiolate bond results in a shift of the heme redox potential to higher values [37] (diminished “push” effect [38]), thereby affecting the catalytic activity of the enzyme [39]. The results were consistent with studies by Panicco [40] and Todorovic [41], which suggest that immobilization of P450 on modified metal surfaces perturbs the heme potential by dehydration to produce catalytically-inactive cytochrome P420. Thus, formation of P420 on electrode surfaces is a possible explanation for the poor catalytic activity often displayed by surfactant-bound P450 electrode systems [19, 42-44].¹

To complement these prior studies, we used DDAB films on basal plane graphite electrodes to investigate the electrochemistry of the full-length, membrane-bound form of wild-type rabbit cytochrome P450 2B4 (CYP 2B4), the main hepatic P450 induced by barbiturates [45, 46]. As a microsomal form, we were curious to see if the surfactant environment would provide more native-like conditions – thereby leading to catalytic activity – compared to the soluble bacterial forms studied previously. Indeed, prior work by Shumyantseva using CYP 2B4 films with montmorillonite and the non-ionic detergent Tween 80 [47] showed differential catalytic currents with aminopyrine and benzphetamine that paralleled enzymatic specificity for these substrates. Slow ET was observed in the absence of Tween 80, which was attributed to protein aggregation that blocked charge transfer. Herein, we report spectroscopic data and electrochemically-derived thermodynamic parameters for evaluation of and comparison to all of the previous work.

¹Notably, electrochemically-promoted substrate turnover with some mammalian P450s has been reported (see references 19, 42-44). Although not yet practical for large-scale turnover, these systems show promise for biosensor applications.

2. Results and Discussion

DDAB films were made on basal plane graphite electrodes by depositing 5 μL of 10 mM DDAB in water onto the electrode surface, followed by slow drying in air overnight. CYP 2B4 was incorporated into the film by soaking the coated electrode in a 10 μM protein solution (50 mM KPi buffer, pH 7) at 8°C for 30 minutes. Figure 1 displays a voltammogram of CYP 2B4 in DDAB recorded at 50 mV/s. At slow scan rates, well-defined and chemically reversible redox couples are observed at -230 mV^2 and -1020 mV (vs. saturated calomel electrode, SCE; 14 mV and -776 mV vs. the normal hydrogen electrode, NHE) [29]. We have assigned these redox couples to heme $\text{Fe}^{\text{III/II}}$ and $\text{Fe}^{\text{II/I}}$, respectively. Notably, similar potentials for the $\text{Fe}^{\text{II/I}}$ couple have been previously observed for nitric oxide synthase (-1004 mV vs. SCE) [29] and P450 from *Sulfolobus solfataricus* (-995 mV vs SCE) [48]. The latter has been characterized extensively by Farmer, demonstrating that the Fe^{I} species is capable of catalytic dechlorination of carbon tetrachloride at elevated temperatures ($> 50^\circ\text{C}$), producing methane [48].

The $\text{Fe}^{\text{III/II}}$ couple is of primary interest given that it is the physiologically relevant redox couple, and comparative data from solution studies are available. Standard characterization reveals that CYP 2B4 displays properties that are consistent with other reports of heme-thiolate proteins in surfactant films [25, 49, 50]. In particular, the CYP 2B4 $\text{Fe}^{\text{III/II}}$ redox potential in DDAB is shifted to positive values relative to that determined in solution [51] (-532 mV vs. SCE for substrate-free CYP 2B4 in solution,³ see Figure S1 in the Supplementary Data). The protein displays typical thin film electrochemistry, appearing surface confined at slow scan rates (peak current linear with scan rate), with a transition to diffusive behavior at scan rates above 16 V/s (peak current linear with the square root of the scan rate) (Figure S2) [52]. Working within the regime where the enzyme appears surface confined, Laviron's treatment [53] was applied to gain an estimate of the ET rate: the standard heterogeneous rate constant (k° , $G^\circ = 0$) for ET from the electrode to the heme was determined to be 170 s^{-1} [29].⁴ Again, this value is consistent with the aforementioned reports of P450s in surfactant films. However this rapid rate contrasts with the significantly slower rate of reduction of ferric CYP 2B4 by its native redox partner cytochrome P450 reductase, where the rate constant measured in solution is on the order of 4 s^{-1} at 30°C [54].

To evaluate the heme environment, we prepared CYP 2B4-DDAB films on fused silica slides and recorded absorption spectra. The slides were cleaned with "piranha" solution and then coated with isobutyltrimethoxysilane to enhance film adhesion [55]. Reproducible spectra were obtained on these slides. Reported results are difference spectra, with DDAB-only data subtracted from CYP 2B4-DDAB spectra. In a typical spectrum (Figure 2a) a distinct peak is observed at 418 nm, which is analogous to the position of the Soret band in the spectrum of low-spin Fe^{III} CYP 2B4 in solution (Figure 2b) [56], and is unlike that of free heme [35]. This result mirrors our prior report where CYP BM3 was extensively characterized by electronic and infrared absorption spectroscopy [35], and is further complemented by exhaustive studies demonstrating that the heme in myoglobin remains protein-bound [33]. Thus, it appears that the thiol-ligated heme in CYP 2B4 remains intact in the film.

²At faster scan rates a complex voltammogram with two cathodic peaks is observed for the $\text{Fe}^{\text{III/II}}$ couple. This behavior is analogous to that previously found for nitric oxide synthase in DDAB, indicative of water-free and water-bound hemes. See reference 29 for a detailed discussion.

³Redox titrations of the wild-type enzyme yielded E° of -532 mV vs. SCE, similar to the value reported in the literature. Titrations in the presence of surfactant yielded a higher potential, -452 mV , consistent with the potential shift observed to occur on the electrode surface. Notably, no spin state change from low to high spin was observed upon the addition of surfactant, indicating that the heme axial water ligand is not displaced by the surfactant. See Figure S1.

⁴This value represents ET to the five-coordinate heme. See reference 29 for a detailed discussion.

Voltammetry conducted in the presence of dioxygen revealed large catalytic currents at the onset of Fe^{III} reduction (Figure S3). Thus, the system appears competent towards dioxygen reduction; supported by the absorption spectrum, these data suggest that substrate oxidation may be possible. Electrolysis experiments were conducted with a series of substrates (e.g., benzphetamine, styrene), however none of the attempts resulted in substrate oxidation.

Similar to the aforementioned studies of heme proteins in surfactant film assemblies on electrode surfaces, the CYP 2B4 heme also displays a large positive potential shift despite appearing native-like by absorption spectroscopy. Hence, while likely structurally intact (thiol-ligated Fe(III)), there nonetheless appears to be a chemical perturbation that gives rise to the shifted redox potential. To further probe the heme microenvironment, we evaluated the entropy change that accompanies ET at the reaction center (S_{rc}°) [57]. This parameter is accessible from the temperature dependence of the redox potential according to equation (1), which in turn is conveniently measured using a non-isothermal electrochemical cell configuration [58].

$$\Delta S_{rc}^{\circ} = S_{Fe(II)}^{\circ} - S_{Fe(III)}^{\circ} = F \left(\frac{dE^{\circ}}{dT} \right) \quad (1)$$

Non-isothermal voltammetry was conducted in a thermostated cell, with a thermally insulated (room temperature) SCE reference electrode connected to the cell through a salt bridge [35, 58]. Voltammograms were recorded at 25 mV/s between 18.5 – 40 °C. As DDAB gel to liquid crystal film transitions have been shown to occur between 9 and 17 °C [21], we performed our experiments above this range in order to eliminate phase-transition effects. The data reported represent the results of at least five independent experiments for each set of conditions.

A plot of $E_{1/2}$ vs. temperature revealed that the Fe^{III/II} redox potential shifts approximately -0.89 ± 0.03 mV/°C (Figure 3). The change in entropy accompanying heme reduction is equal to the slope of this line multiplied by the Faraday constant, which yields $S_{rc}^{\circ} = -86 \pm 3$ J mol⁻¹ K⁻¹. Taking $S^{\circ}(H_2) = 130.4$ J mol⁻¹ K⁻¹, and assigning $S^{\circ}(H^+) = 0$ [57], we calculate an overall S° for the complete cell reaction (adjusted to the NHE) of -151 J mol⁻¹ K⁻¹. This value enables us to calculate H° from the measured $E_{1/2}$ at 25°C, which is -46 kJ mol⁻¹. Table 1 lists the calculated thermodynamic parameters for CYP 2B4 and values determined for other heme proteins.

Reduction of six-coordinate water-ligated Fe^{III} yields five-coordinate Fe^{II} and expulsion of the axial water ligand [59]. In the reduced form, P450 can more readily convert to P420, presumably because of the weaker bond between the thiolate and ferrous heme. Within the DDAB film, ET to ferric CYP 2B4 is accompanied by a substantial decrease in enthalpy, indicating that the protein is stabilized in the ferrous state. A similarly large decrease in entropy accompanies ET. For comparison, electrochemical measurements of myoglobin at different temperatures yielded $H^{\circ} = -38$ kJ mol⁻¹ [60]; interpretations have focused on tightening of the protein structure, resulting in a more rigid conformation that leads to collapse of the protein around the active site into a more compact state [57, 60-62]. A similar phenomenon occurring for the CYP 2B4-DDAB system would likely be enhanced within the hydrophobic and constrained film environment, resulting in larger enthalpic and entropic changes. Indeed, as these results parallel our prior report of bacterial CYP BM3 in DDAB [35], hydrophobic collapse and the predominant effect of the surfactant environment may be the primary and general result of capturing P450s on electrode surfaces within detergent-like films. Notably, this interpretation appears extendable to related systems: our results are fully consistent with a recent study wherein decreases in enthalpy and entropy are

observed for CYP 2B4 films on carbon generated from colloidal Au-DDAB suspensions (reported $E_{1/2} = -38$ mV vs NHE, or -282 mV vs. SCE)⁵ [63].

To further probe the effect of dehydration, we performed similar electrochemical experiments with CYP 2B4-DDAB films and 500 mM imidazole in solution (Table 1). Imidazole replaces water as the heme axial ligand and remains bound to the heme in both Fe^{III} and Fe^{II} oxidation states [64], unlike water which dissociates upon reduction. Under these conditions, the $\text{Fe}^{\text{III/II}}$ $E_{1/2}$ is essentially temperature independent (0.07 ± 0.1 mV/°C),⁶ with values for S° and H° calculated to be -59 J mol⁻¹ K⁻¹ and -18 kJ mol⁻¹, respectively. Significantly, there is a much smaller change in entropy upon reduction of the CYP 2B4 heme with imidazole bound to the iron, in contrast to the water-ligated species. This dramatic difference is consistent with the likely structural rearrangements that accompany these two redox reactions: whereas reduction of a six-coordinate axially aquated heme triggers water dissociation, ET to an imidazole-bound heme produces no change in coordination, so there is minimal nuclear reorganization. The smaller negative change in enthalpy can be attributed to the preference of imidazole, a better electron donor than water, to bind and stabilize iron in the +3 oxidation state.

3. Summary

CYP 2B4-DDAB assemblies on graphite surfaces display typical thin film electrochemistry, and electrochemical parameters characteristic of other P450-surfactant film systems on electrode surfaces – fast ET, significantly up-shifted $\text{Fe}^{\text{III/II}}$ potentials, and catalytic dioxygen reduction with minimal (in this case no) substrate oxidation. Variable temperature voltammetry revealed substantial changes in entropy and enthalpy upon heme reduction to Fe^{II} , which could be muted through dehydration by replacing the axial heme ligand with imidazole. Despite being a mammalian enzyme that is found natively associated with membranes, the data for CYP 2B4 are consistent with prior studies that suggest a compact protein structure within the film on the electrode surface with a perturbed heme microenvironment, the altered electronics of which would not be capable of properly activating dioxygen for substrate oxidation.

Key differences between CYP 2B4 and CYP BM3 in solution include rate of catalysis (10³ s/min cf. 100³ s/min), redox potential (~70 mV difference for substrate-free species), and substrate specificity (steroids vs. fatty acids). These dissimilarities stand in stark contrast to the comparable thermodynamic parameters we report for the enzymes within DDAB on graphite, resulting in similar half-wave potentials, enhanced ET, and apparent long-term stability. Given that the heme appears protein-bound and thiol-ligated within the films, we suspect that the latter difference, substrate-specificity, is largely intact. The critical catalytic activity governed by the heme redox potential is thus readily influenced by the surfactant environment. Therefore, assuming one can manipulate the film environment to generate catalytically active species, either through the native pathway or via direct heme oxidation [65], it should be possible to tune reactivity by manipulating any combination of the film, electrolyte, and electrode.

⁵A more direct comparison of the thermodynamic parameters is not possible as the values reported by Shumyantseva (2011) have not been corrected for standard conditions. Coupled with the difference in films between the two studies, specific quantitative conclusions drawn from direct comparisons are likely difficult to justify. Nonetheless, decreases in entropy and enthalpy following heme reduction as a consequence of entrapment within films on surfaces may be a general phenomenon.

⁶For comparison, CYP BM3 showed a similar response to imidazole, $E_{1/2} = -247$ mV vs. SCE, -0.08 mV/°C. See Udit and Hagen (2006).

4. Experimental

4.1. Protein Expression and Purification

CYP 2B4 was expressed and purified essentially as previously reported [66]. Modifications to the original protocol are described briefly. Native holo CYP 2B4 (without a hexa-His tag) was expressed in *E. coli* C41 (DE3) using plasmid pLW01-P450, which has a T7 promoter. Thirty minutes after induction with isopropyl- β -D galactopyranoside, α -aminolevulinic acid (0.5 mM final concentration) and ethanol (3% w/v final concentration) were added to the culture. The culture was incubated at 22 °C with shaking at 110 rpm and harvested at 96 hours. The protein was purified by chromatography on DE52, CM52 (this was used instead of Reactive Red Agarose), Bio Beads, and Octyl Sepharose. Detergents were removed on a hydroxyapatite column. The specific content of the preparation used in the experiments was 17.9 nmol P450/mg protein. No cytochrome P420, the inactive form of P450, was present. The protein exhibited a single band on SDS polyacrylamide gels.

4.2. Film Preparation and Voltammetry

Electrodes for voltammetry (0.07 cm²) were made using the basal plane of pyrolytic graphite. The surfaces were prepared by sanding lightly with 600-grit sandpaper, followed by polishing with 0.3 and 0.05 μ m alumina slurries. The electrodes were then sonicated and dried in air. DDAB films were formed by placing 5 μ L of 10 mM aqueous DDAB on the surface of the electrodes, followed by slow drying in air overnight. CYP 2B4 was incorporated into the film by soaking the DDAB-filmed electrode in a solution of enzyme (~10 μ M in 50 mM KP_i pH 7 buffer) for 30 minutes at 8 °C, followed by gentle rinsing with double-distilled H₂O.

A CH Instruments Electrochemical Workstation system was used for voltammetry measurements. Experiments were performed in a 2-compartment cell, using a platinum wire auxiliary and a SCE as the reference. All experiments were performed under argon in thoroughly degassed buffer (50 mM KP_i, 50 mM KCl, pH 7) unless otherwise stated. Experiments at variable temperature were conducted by placing the electrochemical cell in a thermostated water bath (18.5 – 40 °C, $\pm$ 0.1 °C). The reference electrode was held at constant (room) temperature, connected to the thermostated cell with a salt bridge.

CYP 2B4-DDAB films for absorption spectroscopy were prepared on 3.5 $\times$ 40 mm fused silica slides. The slides were made hydrophobic by coating with isobutyltrimethoxysilane using the following protocol. The silica slides were cleaned by boiling in piranha solution (4:1 H₂SO₄:50% H₂O₂) for 20 minutes followed by rinsing with double-distilled H₂O. The following procedure [55] was then performed three times: the slides were placed in a 40:1:1 refluxing solution of 2-propanol:double-distilled H₂O:isobutyltrimethoxysilane for 10 minutes, washed with 2-propanol and then baked in a 100 – 107 °C oven for 10 minutes. CYP 2B4-DDAB films were cast onto the slides by placing 5 μ L of 350 μ M CYP 2B4 and 70 μ L of 10 mM DDAB in double-distilled H₂O onto the glass slide, followed by drying in air overnight. Slides for negative controls were filmed with DDAB only. Absorption spectra were recorded with a Hewlett Packard spectrophotometer by placing the silica slides in a cuvette filled with 50 mM KP_i pH 7 buffer. The resulting absorption spectrum reported is a difference spectrum of slides with and without CYP 2B4.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ET	electron transfer
DDAB	didodecyldimethylammonium bromide
CYP 2B4	rabbit microsomal cytochrome P450 2B4
CYP BM3	P450 from <i>Bacillus megaterium</i>
P450	cytochrome P450
SCE	saturated calomel electrode
NHE	normal hydrogen electrode
KP_i	potassium phosphate

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Highlights

- Direct, reversible electrochemistry within electrode films of P450 2B4, the key barbiturate metabolizer in mammals
- Decreases in entropy and enthalpy following electrochemical reduction, triggering dehydration and yielding a conformationally constrained and stabilized enzyme
- Tunable redox properties dictated by the film environment

Figure 1. Cyclic voltammogram of P450 2B4 in DDAB on basal plane graphite at 50 mV/s and 21°C in 50 mM KP_i / 50 mM KCl / pH 7 buffer.

Figure 2.

a) Absorption spectrum of P450 2B4 on silica within a DDAB film, the spectrum is corrected for absorption of DDAB on silica without enzyme. b) Absorption spectrum of P450 2B4 in solution. All spectra were recorded in 50 mM KP_i pH 7 buffer at room temperature.

Figure 3. Temperature dependence of the heme Fe^{III/II} redox couple for P450 2B4-DDAB films on the surface of basal plane graphite. The slope of this graph is equal to S_{rc}°/nF .

Table 1

Thermodynamic parameters for reduction of heme proteins

	$S^\circ/\text{J mol}^{-1} \text{K}^{-1}{}^a$	$H^\circ/\text{kJ mol}^{-1}{}^b$
CYP 2B4 ^c	−151	−46
CYP 2B4 + imidazole ^c	−59	−18
CYP BM3 ^{c,f}	−163	−47
CYP BM3 + imidazole ^{c,f}	−73	−21
Myoglobin ^d	−148	−38
Horse heart cytochrome c ^e	−127	−64

^aChange in entropy accompanying heme reduction^bChange in enthalpy accompanying heme reduction^cIn DDAB film on graphite electrodes.^dSee Liu and Huang et al 2005 Langmuir 21:375.^eSee Taniguchi 1980 Pure Appl Chem 52:2275.^fSee Udit and Hagen 2006 JACS.