Direct electrochemistry of human, bovine and porcine cytochrome P450c17

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Abstract

The direct electrochemistry of human, bovine and porcine cytochrome P450c17 (CYP17) has been examined on an edge-oriented pyrolytic graphite electrode. The recombinant protein was immobilized on an electrode modified with a surfactant to simulate the environment of a biological membrane, and hence physiological electron-transfer conditions. The P450 enzymes all retained 'electron-transfer' activity while immobilized at the electrode surface as assessed by the presence of catalytic signals under aerobic conditions. The redox potentials for porcine P450c17 were more positive (anodic) than both the human and bovine forms, perhaps reflecting the differences in substrate specificity for these species. In addition, these enzymes were all influenced by pH, consistent with a single proton associated with the single electron-transfer event. Ionic strength of the buffer medium also shifted the redox potentials towards positive, suggesting that electrostatic forces contribute to the protein environment required for the electron-transfer process. The effect of substrate on the redox potential for each P450c17 was measured in the presence of pregnenolone, progesterone, 17 α -hydroxypregnenolone and 17 α -hydroxyprogesterone. However, no influence on the redox parameters was observed.

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Introduction

The ubiquitous cytochrome P450 enzymes (P450s) are a superfamily of enzymes involved in the oxidation of a wide range of endogenous sterols, fat soluble vitamins and fatty acids, as well as a variety of xenobiotics (Estabrook 2003, Johnson *et al.* 2005, Omura 1999). The vast majority of reactions catalyzed by P450s involve the two-electron reduction of molecular oxygen, the energetically taxing insertion of an oxygen atom into a substrate C–H bond, and the production of water (Li 2001). Much has been learned about the biochemistry of these proteins from studies on prokaryotic P450s, but there are fundamental differences between prokaryotic and eukaryotic P450s. Prokaryotic P450s are soluble and often self-supporting, whereas P450s expressed in eukaryotes are membrane-bound, with some in the endoplasmic reticulum (microsomal forms) and others in mitochondria. Most microsomal and all mitochondrial P450s require distinct accessory proteins to transfer electrons from the donor to activate oxygen- and metabolize-bound substrates. Therefore, while undoubtedly sharing inherent similarities, essential differences exist among P450 enzymes that might be reflected in even the most fundamental mechanisms controlling substrate metabolism.

Among eukaryotic enzymes, the so-called steroidal P450s generally metabolize a narrower range of substrates than other members of the superfamily. Cytochrome P450s expressed in the adrenal cortex and gonads catalyse critical steps in the synthesis of adrenal corticoids and the sex steroid hormones. 17 α -hydroxylase/17,20-lyase cytochrome P450 (P450c17), a microsomal enzyme, is particularly noteworthy among them for several reasons. P450c17 is not only essential in the production of both adrenal and gonadal steroids, it occupies a key regulating branch point in the pathway, leading to either corticoid or androgen production (Conley & Bird 1997). Importantly, P450c17 is one of only three P450s involved in steroid synthesis that catalyzes concerted oxygenation reactions culminating in cleavage (lyase activity) of a side-chain group. Moreover, this 17,20-lyase activity is profoundly substrate-specific, and variably so across mammalian species (Fevold *et al.* 1989). For instance, porcine P450c17 readily metabolizes 17 α -hydroxyprogesterone to androstenedione and metabolizes 17 α -hydroxypregnenolone to dehydroepiandrosterone, but the human and bovine enzymes only utilize the latter pathway to any significant degree (Brock & Waterman 1999, Conley & Bird 1997, Fevold *et al.* 1989). These recognized species differences in the competence of P450c17 to catalyze

formation of dehydroepiandrosterone and androstenedione have profound physiological consequences for the control of steroidogenesis in the gonads and adrenals.

To date, studies directly comparing the functional properties of P450c17 from different species have focused almost exclusively on the substrate-specificity of the 17,20-lyase reaction (Fevold *et al.* 1989, Lee-Robichaud *et al.* 1995b, Brock & Waterman 1999, Arlt *et al.* 2002, Gilep *et al.* 2003). The few direct comparisons of turnover rates suggest that human P450c17 hydroxylates pregnenolone at almost twice the rate of the porcine enzyme, but that cleavage of 17 α -hydroxypregnenolone to dehydroepiandrosterone is 3.5 times faster in reactions with porcine than human P450c17 (Lee-Robichaud *et al.* 1995a). Human P450c17 hydroxylates progesterone seven times faster than pregnenolone (Brock & Waterman 1999), even though it can only cleave 17 α -hydroxypregnenolone effectively, as noted above. Hydroxylation of pregnenolone and progesterone by rat P450c17 occurs at roughly equal rates, but at half the rate of 17 α -hydroxyprogesterone synthesis by human P450c17. Subsequent cleavage of hydroxylated products by rat P450c17 is much slower, but again, in marked contrast to the human enzyme, occurs at roughly equal rates for 17 α -hydroxypregnenolone and 17 α -hydroxyprogesterone (Brock & Waterman 1999). Although substrate metabolism reduced peroxide formation, there was no obvious correlation between the rate of peroxide formation and substrate turnover rates by the rat and human enzymes (Brock & Waterman 1999, 2000). The physical basis of these differences in substrate specificity, turnover and peroxide formation remains unknown.

Unlike many other microsomal P450s expressed in the liver, the physical properties of P450c17, for instance, changes in spin states or the coordination number of the iron heme, have received little or no attention. No studies known to us have examined the direct electrochemistry of these enzymes; nor have any compared physical properties of any kind of the same enzyme from different species. P450s are particularly challenging enzymes as their active sites tend to be well buried within a large protein matrix (Fantuzzi *et al.* 2004), and membrane-bound proteins are notoriously difficult to examine by direct electrochemistry (Stevens & Arkin 1999, Hunte *et al.* 2000). In this study, a pyrolytic graphite (PGE) electrode was modified with a surfactant that both binds strongly to the electrode and mimics a hydrophobic membrane environment. The electron-transfer properties of human, bovine and porcine P450c17 were then evaluated in the presence and absence of substrate and these results evaluated in terms of an electron-transfer model for P450c17.

Materials and methods

Recombinant protein over-expression in *Escherichia coli*

The cDNA constructs encoding human, bovine and porcine P450c17 were generously provided by Dr Michael Waterman (Department of Biochemistry, Vanderbilt University, Nashville, TN, USA; Barnes *et al.* 1991, Imai *et al.* 1993), each modified at the 5' end for expression in *E. coli* and His-tagged at the 3' end before sub-cloning into the pCWOri+ vector. Plasmid DNA was used to transform *E. coli* strain DH5 α and a fresh colony was grown overnight in 5 ml LB broth containing 100 $\mu\text{g}\cdot\mu\text{l}^{-1}$ ampicillin. The overnight culture was diluted 1:200 into 200 ml Terrific Broth medium containing 100 $\mu\text{g}\cdot\mu\text{l}^{-1}$ ampicillin and 1 mM thiamine in a 2 l flask and grown at 37 °C until the OD₆₀₀ was 0.3–0.5. The culture was induced with 1 mM isopropyl-1-thio- β -D-galactopyranoside (IPTG) in the presence of 0.5 mM δ -amino-levalulinic acid (ALA) and grown for 30–48 h at 30 °C with gentle shaking. The bacteria were pelleted at 2000 *g*, resuspended in 50 ml cold 100 mM Tris/acetate, pH 7.6, 500 mM sucrose, 1 mM EDTA and 0.5 $\text{mg}\cdot\text{ml}^{-1}$ lysozyme and stirred slowly on ice for 30 min. After centrifugation at 5000 *g* the pellet was resuspended in 10 ml cold 100 mM potassium phosphate, pH 7.4, 6 mM magnesium acetate, 0.1 mM dithiothreitol, 20% glycerol and 0.2 mM phenylmethylsulfonyl fluoride (PMSF), placed in a salt/ice bath and sonicated at 50% power with six 20-s bursts, with intermittent cooling, followed by centrifugation for 1 h at 150 000 *g*. The membrane-containing pellet was solubilized in 12 ml 50 mM phosphate buffer, pH 7.4, 20% glycerol, 1% Triton X-100, 0.1 mM PMSF and 40 μM pregnenolone for 1 h on a rotator at 4 °C. The supernatant was collected following a 1 h 100 000 *g* spin. Cytochrome P450 iron heme content was determined by CO difference spectra.

Protein purification

The solubilized membrane fraction was thawed on ice and combined with 8.3 mg imidazole (10 mM final) and 2 ml Ni²⁺-nitrilotriacetate agarose for 2 h at 4 °C on a rotator. The resin was packed by gravity flow at room temperature into a 5 ml disposable column and washed with four column volumes of 20 mM imidazole and then four column volumes of 40 mM imidazole in solubilisation buffer (comprising 50 mM potassium phosphate, pH 7.4, 20% glycerol, 1% Triton X-100, 0.1 mM PMSF and 40 μM pregnenolone). The His-tagged protein was eluted in a total of 3 ml of 200 mM imidazole in solubilisation buffer and dialysed overnight against 1 l of 100 mM phosphate buffer, pH 7.4, 20% glycerol, 2-mercaptoethanol and 1 mM CHAPS. The iron heme content of the P450s was again measured by CO

difference spectra and total P450 protein content was determined using the BCA protein reagent and the proteins were separated on an 8% SDS/PAGE gel and visualized using GelCode Blue stain reagent. Purification yielded enzyme preparations containing 10–12 nmol P450/mg protein. There was little or no P420 by spectral analysis and turnover rates of up to 50 min^{-1} were observed at substrate saturation.

CO difference spectra

The dialysed eluate was diluted 1:1 in dialysis buffer and divided between the sample and reference cuvettes for spectral analysis on a split-beam Shimadzu 2401 spectrophotometer. A baseline was recorded between 400 and 500 nm. The sample cuvette was saturated with CO, a few crystals of sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) were mixed by inversion into both the reference and sample cuvettes and the spectra were again recorded. The cytochrome P450 concentration was calculated as $(A_{450} - A_{490})/0.091 = \text{nmol} \cdot \text{ml}^{-1}$.

Materials

Didodecyldimethylammonium bromide (DDAB; >99%) was purchased from Sigma and used without further purification. All other chemicals were analytical reagent grade and were used without further purification. Milli-Q water was used both as a solvent and in all washing steps. Solutions were buffered in the pH range 5.8–8.2 with phosphate buffer. The pH was determined using a calibrated Radiometer PHM210 pH meter.

Electrochemical apparatus and procedures

All experiments were undertaken in a nitrogen-filled glove box to alleviate complications associated with oxygen catalysis and subsequent hydrogen peroxide formation. Cyclic voltammograms were recorded using a BAS Model 100B electrochemical analyser, and were obtained at least four times with fresh buffer and different electrodes. Scan rates typically varied between 2 and $1500 \text{ mV} \cdot \text{s}^{-1}$. Voltammetric data were obtained using an edge-oriented PGE electrode; radius, $\sim 2.0 \text{ mm}$. Before each experiment, the electrode surface was polished with an alumina/water slurry (alumina particle size was $1 \mu\text{m}$, followed by $0.3 \mu\text{m}$), washed, and sonicated for 1 min to remove adsorbed alumina. The reference and counter electrodes were Ag/AgCl (3 M KCl) and platinum wire respectively. The reference electrode was regularly calibrated against the $[\text{Fe}(\text{CN})_6]^{3-/-4-}$ couple in 1 M KNO_3 (Johnson *et al.* 2003). Midpoint potentials (E_{mid}) were calculated as the average of the oxidation (E_{p_a}) and reduction (E_{p_c}) peaks, the peak separation (ΔE) was calculated as the difference between E_{p_a} and E_{p_c} , and the peak current

ratio ($i_{\text{p}_c}/i_{\text{p}_a}$) was calculated by dividing the cathodic peak current (i_{p_c}) by the anodic peak current (i_{p_a}). Potentials have been corrected to the normal hydrogen electrode.

A typical experiment proceeded as follows: a solution of DDAB was applied to the clean electrode surface ($1 \mu\text{l}$ of a 0.1 M stock in chloroform) and allowed to dry. The protein ($3 \mu\text{l}$; stock solutions were $3\text{--}5 \mu\text{M}$) was then applied to the electrode surface, and the electrode allowed to stand for 2 min before immersing into the buffered solution at the required pH. Phosphate buffer (0.1 M) was typically used, but the influence of ionic strength was also evaluated by examining voltammetric behavior at several pH values in 1.0 M phosphate buffer.

Substrate binding was evaluated in three different ways at a variety of concentrations between $10 \mu\text{M}$ and 100 mM in 1% DMSO, with comparable results. (1) The electrode was modified with a protein film as above and then placed into a solution of phosphate buffer containing the desired substrate. (2) An electrode was modified with a protein film as described above, and then the electrode was left to stand for 5–15 min prior to $3 \mu\text{l}$ of the substrate solution being added. The film was then allowed to dry for $\sim 10 \text{ min}$ before being placed into a buffered solution. (3) Protein ($10 \mu\text{l}$) was incubated with a solution of the substrate at 4°C for 10 min (incubation periods up to 16 h were also attempted), before being applied to the electrode surface as per a typical experiment. In each case, cycling for several hours revealed no significant changes in midpoint potential.

Results

A thin film of purified cytochrome P450c17 was immobilized at an edge-oriented PGE electrode pretreated with DDAB. DDAB is a cationic surfactant comprising a positively charged dimethyl-ammonium head group capable of electrostatically interacting with negatively charged surface residues at a freshly polished PGE surface, and a hydrophobic didodecyl tail-group moiety capable of mimicking the environment of a biological membrane. Cyclic voltammograms of a PGE electrode modified with a film of DDAB in the absence of P450 revealed no redox response in the potential range considered. However, voltammograms of the PGE/DDAB/P450c17 film were redox active and showed good signal-to-noise responses for scan rates between 2 and $1500 \text{ mV} \cdot \text{s}^{-1}$ (Fig. 1a). The cyclic voltammograms obtained for the human, bovine and porcine forms of P450c17 were all typical of that expected for direct electron exchange between the electrode and the iron heme 'active site' (Rusling 1998, Aguey-Zinsou *et al.* 2003, Fleming *et al.* 2003) with midpoint potentials (E_{mid}) of -48 , -54 and -3 mV

respectively (pH 7.4, 0.1 M phosphate buffer). Cyclic voltammograms differed slightly between the first few cycles as the molecular structure at the surface appeared to reorganize into an *optimal configuration*, after which reproducible voltammograms could be obtained for several hours, indicating a stable, active enzyme film. The surface reorganization did not influence any of the measured electrochemical parameters of interest; E_{mid} , ΔE , $i_p/c/i_{p_a}$ and k_s were consistent throughout. The small changes primarily relate to variation in the capacitive currents and these are typical for electrochemistry of thin films containing biological components (Armstrong 1990, Armstrong *et al.* 1997). Typically, the PGE/DDAB/P450c17 films were used over ~ 3 h of continuous cycling between the ferric and ferrous forms, suggesting that the film integrity, composition and amount of bound protein remains constant throughout this period (Fig. 1a). Figure 1b highlights a linear relationship between scan rate (v) and peak current (i_p), indicative of an immobilized redox-active species with little or no influence of mass transport of redox-active material away from or towards the electrode. The 'reversibility' of electron-transfer process was evaluated by dividing the cathodic current by the anodic current (i_{p_c}/i_{p_a}), revealing a slight dominance of the cathodic wave, typically $\sim 5\%$ greater than the anodic current. The magnitude of the Faradaic current was influenced by the amount of immobilized protein applied and also by the number of alternate layers of DDAB and P450 applied to the electrode. Optimal peak currents were obtained using two distinct layers of the DDAB/P450 repeating unit. Additional layers appeared to have no impact on the observed peak currents.

The surface coverage (Γ) of the electroactive, immobilized protein for a single DDAB/P450 layer was evaluated by integration of the anodic and/or cathodic peak employing the relationship $Q = F\Delta\Gamma$, where Q is the charge, obtained by integration of the oxidative voltammogram, F is Faraday's constant and A is the electrode area. The electroactive surface coverage was calculated to be $9.21 \times 10^{-12} \text{ mol}\cdot\text{cm}^{-2}$. Assuming that individual P450c17 molecules retain their fundamental geometry when they bind to the electrode, a single monolayer of P450 affords a surface coverage of

Figure 1 (a) Typical cyclic voltammograms of porcine P450c17 obtained at a PGE/DDAB electrode in the absence of oxygen. The stability of the film is highlighted, with scans taken at hourly intervals after first putting the electrode into buffer. Scans at 1, 2, 3, 4, 5 and 10 h are overlaid (outer traces). Inner trace is the voltammogram after removal of capacitive (baseline) current, showing only Faradaic current. Buffer was 0.1 M potassium phosphate, pH 7.4. Scan rate, 50 $\text{mV}\cdot\text{s}^{-1}$. (b) Variation of peak currents (i_{p_a} , $\blacklozenge$ and i_{p_c} , $\blacksquare$) with scan rate (v) for bovine P450c17. The linear relationship indicates that the protein is confined as a thin film at the electrode surface. Buffer was 0.1 M potassium phosphate, pH 7.4.

Figure 2 Midpoint potentials of human, bovine and porcine P450c17 adsorbed at a PGE/DDAB electrode as influenced by pH and ionic strength. The porcine midpoint potential differs to the human and bovine forms at each pH value considered and is shifted by ionic strength. The slopes of each plot, in mV per pH unit are: bovine 0.1 M (◆), -52.1; bovine 1 M (◇), -56.1; human 0.1 M (■), -53.8; human 1 M (□), -55.1; porcine 0.1 M (▲), -56.9; porcine 1 M (△), -56.5.

$1.01 \times 10^{-11} \text{ mol}\cdot\text{cm}^{-2}$, based on the crystal structure dimensions of P450 cam (Lei *et al.* 2000). Although only an approximate calculation, this suggests that around 91% of adsorbed protein contributes to the electrochemical signal.

For all species examined here, the P450c17 reduction potentials were influenced by both pH and the ionic strength (*I*) of the phosphate buffer solution (Fig. 2). Variation of pH led to a shift in E_{mid} of between -51 and -57 mV per pH unit, close to the theoretical value of -59 mV per pH unit predicted for the coupling of a single proton transfer to the electron-transfer event from the Nernst equation. This trend was observed for the human, bovine and porcine samples, and was comparable at 4 and 20 °C. Interestingly, this effect was consistent in the presence and absence of substrate. The influence of *I* was less dramatic, with a 10-fold increase in *I* (0.1–1 M phosphate buffer), prompting an anodic

Figure 3 Influence of pH value on peak separation (ΔE) for bovine (▲), human (■) and porcine (●) P450c17 adsorbed at a PGE/DDAB electrode. In each case, the midpoint potential was minimized at a higher pH, perhaps reflecting efficiency of electron transfer. Ionic strength, 0.1 M; scan rate, 50 mV·s⁻¹.

shift of ~15 mV for the porcine P450c17 and smaller changes for the bovine and human forms. The influence of *I* on the porcine sample was approximately linear across the pH range considered; all three species showed the greatest anodic shift in E_{mid} at higher ionic strength (*I*=1.0) and at low pH (pH 5.8), the lowest pH value examined here.

Each of the P450c17 isoforms showed generally faster electron-transfer rates at higher pH (Fig. 3). The electron-transfer rate constant (k_s) was estimated (Laviron 1979) for each of the P450c17 (human, bovine and porcine) enzymes and found to be very similar; 164, 157 and 153 s⁻¹ respectively. It should be noted that these rate constants were measured in the absence of oxygen and thus do not necessarily reflect enzymic activity rates during substrate turnover.

P450c17 has several well-known substrates, including progesterone, pregnenolone, 17 α -hydroxypregnenolone

and 17 α -hydroxyprogesterone. These substrates were used to evaluate the influence of substrate binding on the redox properties of PGE/DDAB/P450c17-modified electrodes. Interestingly, the voltammetric parameters defining the redox process (E_{mid} , ΔE , $i_{\text{p}_c}/i_{\text{p}_a}$, k_s) were unchanged over a wide range of experimental conditions, including the presence of substrate. This may indicate that, in the absence of oxygen, the substrate docks with the enzyme some distance from the active site, or alternatively that substrate binding has a negligible effect on the electronic environment of the heme redox centre at the active site of P450c17.

Discussion

We report for the first time direct (without mediators) electrical communication between an artificial electron donor (an electrode) and cytochrome P450c17. Three important observations were made. Firstly, the redox potential of all P450c17 enzymes investigated was considerably higher than that of the microsomal redox partner protein NADPH-cytochrome P450 oxidoreductase. In itself, this finding suggests that no increase in redox potential is required to facilitate enzyme reduction. Secondly, no shift in redox potential was observed upon the addition of known substrates. Thirdly, the porcine P450c17 exhibited a much higher redox potential than either the human or bovine enzyme. This suggests that there are species differences in charge density surrounding the iron heme moiety, despite the fact that molecular modelling predicts a highly conserved active site (Auchus & Miller 1999, Burke *et al.* 1997). Charged residues near the iron heme may well influence substrate orientation, determine accessibility to activated oxygen or directly influence oxygen insertion by some as yet undetermined manner favouring 17 α -hydroxyprogesterone only for the porcine P450c17.

A point mutation selectively altering 17,20-lyase activity of human P450c17 was recently reported to involve neutralization of a positively charged glutamate residue predicted to lie near the heme in the active site (Sherbet *et al.* 2003). While implicating charged residues in 17,20-lyase activity, this residue is conserved across mammalian species and is unlikely to explain the species differences in substrate specificity. Our electrochemical results are also consistent with the notion that the ferrous form of the porcine enzyme is stabilized to a greater degree than the human or bovine forms. Lewis and Hlavica (2000) described the influence of the local dielectric constant and protein radius relative to that of the heme moiety on the predicted redox potentials. It is possible that the porcine P450c17 is more hydrophobic overall at the active site compared with the human and bovine isoforms and that this effect results in a

stabilization of the ferrous form. How this influences substrate binding and/or affinity is unclear at present, since no shift in redox potential was observed with any of the substrates investigated here. These observations should direct future site-directed mutagenesis to target charged residues predicted to be near the heme in the active site to identify those residues involved in species-specific substrate preferences of mammalian P450c17 enzymes.

The redox characteristics of P450s have been considered for over 20 years, but unmediated electron transfer has only been obtained for a few species and under limited experimental conditions. During the past decade, direct electrochemistry has been obtained at edge-plane PGE without redox mediators (Kazlauskaitė *et al.* 1996), in lipid films (Ivanov *et al.* 2001, Zhang *et al.* 1997), using sol-gels (Iwuoha *et al.* 2000), in colloidal clay (Lei *et al.* 2000) and with surface-confined promoters (Aguey-Zinsou *et al.* 2003, Lvov *et al.* 1998, Munge *et al.* 2003). Numerous other redox studies have examined P450s in solution. Taken together the redox characteristics of P450 enzymes vary widely with experimental conditions, emphasizing the importance of reproducing more physiological conditions during electrochemical measurements. Modification of electrodes with DDAB provides a pseudo-membrane surface and may be more reflective of the environment of membrane-bound P450s. Our results suggest that some alignment or re-orientation ('bedding down') occurs initially at the electrode surface. P450s are predicted to have surface-exposed hydrophobic patches (Graham & Peterson 1999, Peterson & Graham 1998) and thus may be capable of adopting more than one favourable orientation at the electrode surface. Theoretically, a one-electron transfer of an immobilized redox active species in a homogenous orientation gives rise to a peak width at half height ($\omega^{1/2}$) of 90 mV (Laviron 1979). The broadness of the redox waves for all the P450c17 samples ($\omega^{1/2} \sim 130$ mV) suggests that several stable enzyme orientations may contribute to the overall electrochemical response observed here.

The P450c17 E_{mid} was found to vary linearly with pH over the range 5.8–8.2, with the potential shift between –52 and –57 mV per pH unit, depending on species and ionic strength. These values indicate that a single proton transfer is coupled to the electron-transfer event, a phenomenon for which a potential shift of –59 mV per pH unit is predicted by the Nernst equation. Similar pH profiles have been observed for other P450s, with the dependence of –33 mV per pH unit for P450BM3 (Fleming *et al.* 2003), and –52 mV per pH unit for P4502C9 (Johnson *et al.* 2005) at a DDAB-modified electrode. This pH profile has been attributed to protonation of either an amino acid residue in the immediate vicinity of the active site or a functional group coordinated to the iron heme (Aguey-Zinsou *et al.*

2003), and is a fundamental requirement for the completion of the catalytic cycle, providing the proton required for a hydroxylation reaction. However, the nature of the functional group involved in the proton transfer at the active site remains unclear, as its pK_a lies outside of the pH range used in this study. A similar conclusion was reported for P450 cin (Aguey-Zinsou *et al.* 2003), where the pK_a was thought to lie at a value >10 , indicating that reduction from ferric to ferrous liberates a strong base close to the heme active site. The redox potentials of all cytochromes studied to date vary between -400 and $+400$ mV, a magnitude equivalent to changing the pK_a of a functional group by ~ 14 pH units (Mao *et al.* 2003). Thus, the high pK_a for P450c17 enzymes suggested by the current data are not unique.

The pH profiles for the three P450c17 enzymes examined in this study were also very similar in the presence and absence of substrate. This suggests that the coordination number of the iron heme probably remains constant with or without substrate. The absence of any shift in redox potential with substrate suggests that the spin-state conversion for the ferric ion upon substrate binding may occur independently of redox potential. Indeed, the correlation between E_{mid} and spin equilibrium has been predicted to be a relatively small effect (Cant *et al.* 1975), notably less significant than inductive or substituent effects that are often observed (Sligar 1976, Fisher & Sligar 1985). Redox potentials are predominantly influenced by the binding constants to both oxidized and reduced forms of the enzyme. Consequently, it has been proposed that the anodic substrate shift observed for some P450s may simply indicate that the substrate binds with stronger affinity to the reduced form of the enzyme, raising the E_{mid} . Conversely, the absence of a shift may simply indicate that the substrate does not preferentially bind to either the oxidized or the reduced forms of the enzyme, rather with equal affinity to both forms (Rusling 1998). The possible relevance of a substrate-induced change in E_{mid} has been questioned (Fleming *et al.* 2003). Since these experiments require anaerobic conditions, oxygen binding to the heme *in vivo* would surely influence redox potential, with or without substrate present, as observed for binding of CO to the iron heme in the absence of substrate. This is consistent with biochemical studies on several purified rat liver P450s from which it was concluded that no correlation between spin state of the iron heme or kinetics of ferric reduction could explain the differences observed (Guengerich 1983, Backes & Eyer 1989, Eyer & Backes 1992).

Substrate docking has also been proposed to act as a thermodynamic trigger for P450 turnover, inducing an anodic shift in redox potential of the P450 from below to above that of the physiological donor (Sligar 1976, Reipa *et al.* 2002). However, no substrate-induced

anodic shift was observed with any of the P450c17 enzymes in the current study, and this may not be true for all P450s. Direct electrochemical studies with P450 cam, P450 scc, P450 3A4 and P450 cin have not universally confirmed a substrate-induced shift in redox potential (Table 1), despite spin conversion observed in some cases (Yamazaki *et al.* 1996). These apparent discrepancies might be explained if either (i) P450 conformation in the presence of lipids or surfactants is different to that in solution, possibly influencing the interaction of P450c17 with substrate (French *et al.* 1980, Miwa & Lu 1981, Ruckpaul *et al.* 1982, Omata *et al.* 1987, Imaoka *et al.* 1992, Voznesensky & Schenkman 1994, Yun *et al.* 1998) or (ii) the substrate binds at a location remote from the redox 'active site' with minimal change in local electronic structure. Certainly caution must be exercised when comparing results across studies using different methodologies and experimental conditions. The redox potential measured for P450 cam shifts by 130 mV upon camphor binding in solution (Sligar & Gunsalas 1976), just 23 mV at a hydrophobic electrode (Iwuoha *et al.* 2000) and none at all at a clay-modified electrode (Lei *et al.* 2000). Nevertheless, immobilized P450 enzymes turn over under aerobic conditions in many of these cases (Iwuoha *et al.* 2000, Estavillo *et al.* 2003, Joseph *et al.* 2003, Munge *et al.* 2003, Fantuzzi *et al.* 2004, Immoos *et al.* 2004). Crystallographic evidence documents marked changes in P450 structure upon ligand binding; of P4502C9 with warfarin (Williams *et al.* 2003) and P450 2B4 with imidazole (Scott *et al.* 2004). For these xenobiotic P450s, there may be multiple substrate-binding modes (Williams *et al.* 2003, 2004), only some of which might alter redox potential or spin state. However, with the narrow substrate specificity observed for P450c17 this is less likely.

Interestingly, increasing the ionic strength (I) of the phosphate buffer also had a greater effect on the porcine isoform than either the human or bovine isoforms. Increasing I from 0.1 to 1.0 M induced a 15–20 mV shift in E_{mid} (porcine) compared with only 0–7 mV (human or bovine). The effect of I for P450c17 has been investigated previously, in particular its role in the interaction with P450 reductase (Jenkins & Waterman 1998), where an increase in I led to a decrease in P450c17 activity, attributed by the authors to disruption of electrostatic interactions needed for electron delivery to the P450 enzyme. In this study, experiments were undertaken above and below the pI of P450c17 of ~ 6.5 (Kuwada 1999, Kuwada *et al.* 2001). It seems likely that individual charged amino acids near the heme group influence the E_{mid} of P450c17; however, the anodic shift we observed with increasing I is unusual, since most proteins exhibit a cathodic shift with increasing I (Alberty 2001). Data obtained in this work using three species of P450c17 enzymes, together with previous reports on P450 scc (Nicolini *et al.* 2001) and also a

Table 1 The substrate-induced shifts in midpoint potential for several P450 enzymes. There appears to be two clear groups: those species that undergo a substrate shift, and those that do not. Three trends are worth noting: (1) when a substrate shift is observed, it is typically over 100 mV; (2) the studies that have observed a substrate shift have been almost exclusively conducted in solution phase; (3) experiments on P450 enzymes immobilized at membrane-like electrodes rarely show a substrate-shift

Enzyme	Substrate	E _{mid} (mV)	E _{mid} with substrate (mV)	Substrate shift (mV)	Technique	Reference
P450cam	Camphor	-303	-173	130	Pot., solution phase	Silgar and Gunsalas (1976)
	Camphor	-284	-148	136	D.E., pH 7.4 no promoter	Kazlauskaitė et al. (1996)
	Camphor	-306	-170	136	Pot., pH 7.4, solution phase	Reipa et al. (2002)
	Camphor	-356	-333	23	D.E., pH 7.5, DDAB	Iwuoha et al. (2000)
	Camphor	-139	-139	0	D.E., pH 7, clay electrode	Lei et al. (2000)
P450BM3	Pyrene	-356	-343	13	D.E., pH 7.5, DDAB	Iwuoha et al. (2000)
	Styrene	-306	-265	41	Pot., pH 7.4, solution phase	Reipa et al. (2002)
	Palmitate	-368	-239	103	Pot., pH 7.0, solution phase	Daff et al. (1997)
	Arachidonate	-30	-30	0	Pot., pH 7.0, solution phase	Daff et al. (1997)
	Various	-412	-305	107	D.E., pH 7.4, DDAB	Fleming et al. (2003)
P450sc	Cholesterol	-37	-37	0	Pot., pH 7.4	Light and Orme-Johnson (1981)
	22R-OH Cholesterol	-412	-387	25	D.E.	Nicolini et al. (2001)
	20,22-OH Cholesterol	-412	-306	106	Pot., pH 7.4	Light and Orme-Johnson (1981)
	Pregnenolone	-412	-370	42	Pot., pH 7.4	Light and Orme-Johnson (1981)
	Cineole	-182	-182	0	Pot., pH 7.54	Aguey-Zinsou et al. (2003)
P450 3A4	Cineole	-60	-60	0	D.E., pH 7.4, DDAB	Aguey-Zinsou et al. (2003)
	Testosterone	-316	-312	4	Dye, pH 7	Yamazaki et al. (1996)
	Ethylmorphine	-316	-320	-4	Dye, pH 7	Yamazaki et al. (1996)
	Verapamil	98	98	0	D.E., pH 7.4, PDDA	Joseph et al. (2003)
	Prog	-50	-50	0	D.E., pH 7.4, DDAB	This study
P450c17 (human and bovine)	17α-OH Pregnenolone	-50	-50	0	D.E., pH 7.4, DDAB	This study
P450c17 (porcine)	Pregnenolone	-3	-3	0	D.E., pH 7.4, DDAB	This study
	17α-OH Pregnenolone	-3	-3	0	D.E., pH 7.4, DDAB	This study

D.E., direct electrochemistry; PDDA, poly(dimethyldiallyl) ammonium chloride; Pot., potentiometry.

cytochrome b_5 mutant (Reid *et al.* 1984), are consistent with the ferrous (reduced) state being stabilized at higher I , indicating an decrease in negative charge near the heme group. Additionally, all of the P450c17 enzymes examined in this study exhibited rapid but very similar rates of electron transfer: $k_s \sim 150 \pm 30 \text{ s}^{-1}$ at pH 7.4. Rates from electron donors to P450s vary apparently over a large range, from 0.45 s^{-1} for P450 scc (Nicolini *et al.* 2001) to $25\text{--}84 \text{ s}^{-1}$ for P450 cam (Fisher & Sligar 1985, Zhang *et al.* 1997, Mouro *et al.* 1999, Lei *et al.* 2000) and 223 s^{-1} for P450BM3 (Fleming *et al.* 2003). Interestingly, in the current study electron-transfer rates generally increased with pH (data not shown), indicating that P450c17 turnover should be maximized at high pH as observed previously for P450 cam and myoglobin (Munge *et al.* 2003). However, optimal P450c17 17 α -hydroxylase activity is obtained at pH 8.25 for pig testicular microsomes (Swinney & Mak 1994) and at 7.7 for human testicular microsomes (Hosaka *et al.* 1980). These data suggest that electron-transfer rates do not limit substrate turnover, consistent with data suggesting that C–H bond breakage is at least partially limiting in many mammalian P450 reactions (Guengerich *et al.* 2004).

In summary, we have measured the redox potentials for human, bovine and porcine P450c17 at graphite electrodes modified with the surfactant DDAB. A linear dependence of the redox potentials with pH was observed, consistent with a one-electron/proton-coupled transfer process. Increased I of the buffer solution led to an anodic shift in midpoint potential for all species, consistent with a stabilization of the reduction process. Measured values for the midpoint potentials were similar for the human and bovine enzymes, but was significantly higher for porcine P450c17 at all pH values, perhaps identifying differences in charge density, and stabilization of the reduced and oxidized forms of the enzymes. How the noted variations in redox potential influence known differences in substrate utilization requires further investigation.

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