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Letter

Epoxidation of styrene by human cyt P450 1A2 by thin film electrolysis and peroxide activation compared to solution reactions

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Abstract

Films of human cytochrome P450 1A2 (cyt P450 1A2) and polystyrene sulfonate were constructed on carbon cloth electrodes using layer-by-layer alternate absorption and evaluated for electrochemical- and H_2O_2-driven enzyme-catalyzed oxidation of styrene to styrene oxide. At −0.6 V vs. saturated calomel reference electrode in an electrochemical cell, epoxidation of styrene was mediated by initial catalytic reduction of dioxygen to H_2O_2 which activates the enzyme for the catalytic oxidation. Slightly larger turnover rates for cyt P450 1A2 were found for the electrolytic and H_2O_2 (10 mM) driven reactions compared to conventional enzymatic reactions using cyt P450s, reductases, and electron donors for cytochromes P450 1A2. Cyt $P450_{cam}$ gave comparable turnover rates in film electrolysis and solution reactions. Results demonstrate that cyt P450 1A2 catalyzes styrene epoxidation faster than cyt $P450_{cam}$, and suggests the usefulness of this thin-film electrolytic method for relative turnover rate studies of cyt P450s.

Keywords: Cytochrome P450; Electrolysis; Epoxidation; Enzyme catalysis; Ultrathin films

1. Introduction

Cytochrome P450 (cyt P450) enzymes in the human liver catalyze the oxidation of lipophilic substrates to relatively hydrophilic metabolites. The metabolites may be therapeutic or genotoxic, and the latter may react with DNA [1–3]. Electrostatic layer-by-layer self-assembly of proteins and oppositely charged polyions is a general method for designing active enzyme films according to predesigned architecture [4,5]. In general, enzymes retain their native structures and activities in these films, and can catalyze conversion of substrates. In 1998, we reported that such films facilitate direct reversible electron exchange between metalloproteins and electrodes [6]. We employed layered films of cyt $P450_{cam}$ and myoglobin (Mb) to catalyze styrene epoxidation driven electrochemi-

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cally or by H_2O_2 [7–9]. These films have been used to investigate catalytic pathways and efficiency for olefin epoxidation [7,10].

Epoxidations with these films are initiated by reversible electrochemical conversion of the enzyme's heme Fe^{III} to Fe^{II}. The Fe^{II} enzyme binds dioxygen, which is reduced to H_2O_2 in an electrochemical catalytic process. H_2O_2 subsequently activates the enzyme, possibly to an oxyferryl state, to catalyze oxidation of the olefin [6,7,10]. The key activating role of H_2O_2 was confirmed by the absence of products in the presence of catalase, which destroyed all the H_2O_2. In addition to olefin epoxidations, cyt P450 3A4 in thin films converted the drugs verapamil and midazolam to their expected liver metabolites using electrolysis [11].

Rates of metabolic oxidations catalyzed by human liver cyt P450s are important in assessing chemical toxicity and human polymorphism [12–14]. We expected that ultrathin enzyme films could be used reliably for relative turnover rate measurements directed toward such ends. Advantages are that only sub-nanomolar amounts of enzyme are needed per film, and that the natural reductase enzymes and electron donors are not needed. Cyt P450 1A2 (CYP1A2) in human liver microsomes is relatively active in metabolizing styrene, but not as active as CYP2E1 or CYP2B6 [15,16]. In this letter, we show that for human liver cyt P450 1A2 and cyt $P450_{cam}$, reasonably similar turnover rates for the enzyme in films and in solution with the reductase are obtained for epoxidation of styrene.

2. Materials and methods

2.1. Chemicals and enzymes

NADPH, glucose-6-phosphate dehydrogenase, glucose-6-phosphate, and sodium poly(styrenesulfonate) (PSS, average MW 70 000) were from Sigma. c-DNA-expressed cyt P450 1A2 (MW 52 000) was isolated and purified from *Escherichia coli* following a published method [17]. NADPH-P450 reductase from rat livers was purified by a published method [18].

2.2. Film construction

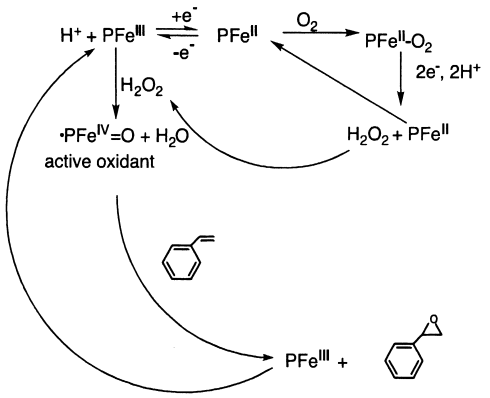
Films were grown on carbon cloth (Zoltek Corp.) by repeated adsorption [3,4] from aqueous solutions of 3 mg/ml PSS and 0.74 mg/ml cyt P450, alternately. Carbon cloth was immersed first for 20 min in PSS solution, then for 20 min in protein solution, with intermediate rinsing in water. The process was repeated to obtain films denoted $PSS/(cyt\ P450/PSS)_2$. In films for voltammetry, PSS solutions contained 0.5 M NaCl to induce coiling [5] to obtain thicker films and more well defined voltammograms.

2.3. Voltammetry, electrolysis and product analysis

A CHI 430 electrochemical analyzer was used for cyclic voltammetry (CV) and a BAS-100B/W electrochemical analyzer was used for electrolysis. The three-electrode CV cell contained a saturated calomel reference electrode (SCE), a Pt wire counter electrode, and a 0.7×1 cm^2 carbon cloth working electrode. Ohmic compensation was $\geqslant 98\%$. Solutions were purged with nitrogen before CV, unless noted otherwise.

Electrolyses were done at an applied potential of -0.6 V vs. SCE at 4 °C in a divided H-cell with a carbon rod counter electrode separated by an agar–KCl bridge, and an SCE reference. Low temperature was used to avoid excessive enzyme denaturation by H_2O_2 formed during reaction. The working electrode was 0.7×6.0 cm^2 enzyme-coated carbon cloth. Ten milliliter of pH 7 phosphate buffer + 10 mM styrene were oxygenated for the first 20 min, then blanketed with oxygen. After 1 h, the reaction mixture was extracted with 3×20 ml hexane, and the combined extract concentrated to 0.5 ml by evaporation. Gas chromatography (GC) was done using an HP-5 capillary column at 50 °C initially for 0.5 min, then 7.5 °C/min to 100 °C, then 50 °C/min to the final T of 250 °C. Average retention times were styrene 4.3 min and styrene oxide 6.6 min.

Assays with dissolved enzyme were done at 4 °C, using 0.6 μM of cyt P450 1A2, 0.6 μM NADPH-P450 reductase, 80 μM dilauroylphosphatidylcholine (DLPC), 0.5 mM NADPH, 5 mM



Scheme 1. Pathway for electrochemical and peroxide-driven catalysis of styrene epoxidation by cyt P450s.

glucose-6-phosphate, 1 U/ml glucose-6-phosphate dehydrogenase in 3 ml 50 mM phosphate buffer pH 7.3+10 mM magnesium chloride. Similar conditions were used for cyt $P450_{cam}$, but putidaredoxin was used as the reductase. Styrene added to final concentration 10 mM started the reaction. Aliquots were extracted with hexane and analyzed by GC as above.

3. Results

Fig. 1 shows a cyclic voltammogram of a PSS/(cyt P450 1A2/PSS)$_2$ film, showing a reduction–oxidation peak pair corresponding to the key reversible PFe^{III}/PFe^{II} conversion under anaerobic conditions. The average midpoint potential at 0.1 V/s in pH 7.0 buffer was −0.31 V vs. SCE (−0.07 V vs. NHE). Plots of peak current vs. scan rate were linear up to 1 V/s with a correlation coefficient close to one, consistent with theory for voltammetry of the enzyme confined in the thin film [9]. Estimates from quartz crystal microbalance weighing of similar films built on gold surfaces [9,10] suggested a film thickness of approximately 30–40 nm.

When oxygen was added to solutions bathing PSS/(cyt P450 1A2/PSS)$_2$ electrodes, the PFe^{III} reduction peak increased greatly, while the oxidation peak disappeared (Fig. 2). This is the classic voltammetric signature for electrochemical catalysis. Here, it reflects reaction of PFe^{II} with dioxygen, followed by reduction of the $PFe^{II}–O_2$ complex formed to give H_2O_2, regenerating PFe^{II} for a further reaction cycle. This behavior has been observed for myoglobin and cyt $P450_{cam}$ in thin polyion films [6–10]. The addition of styrene to this solution caused a further increase in the catalytic reduction current (Fig. 2c), probably because oxygen is utilized faster in the presence

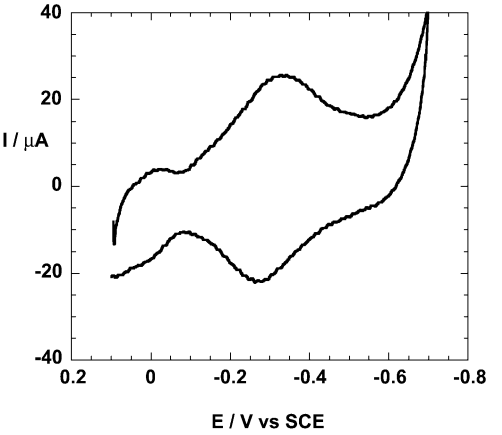


Fig. 1. Background subtracted cyclic voltammogram of PSS/(cyt P450 1A2/PSS)$_2$ film on carbon cloth electrode at 0.1 V/s in 0.1 M KCl pH 7.0 buffer.

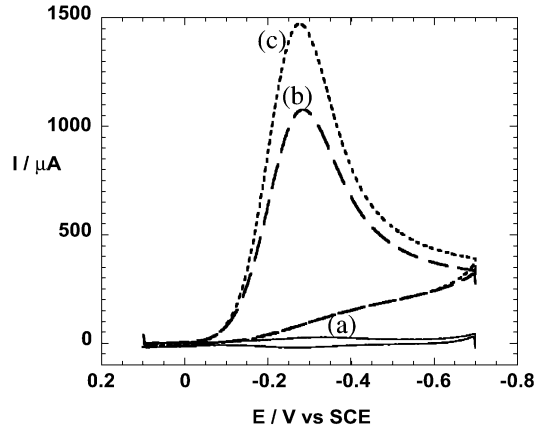


Fig. 2. Background subtracted cyclic voltammograms of PSS/(cyt P450 1A2/PSS)$_2$ film at 0.1 V/s in 0.1 M KCl pH 7.0: (a) under N_2 (as in Fig. 1); (b) after injecting 40 ml of O_2; and (c) after adding a saturating amount of styrene to the oxygenated solution.

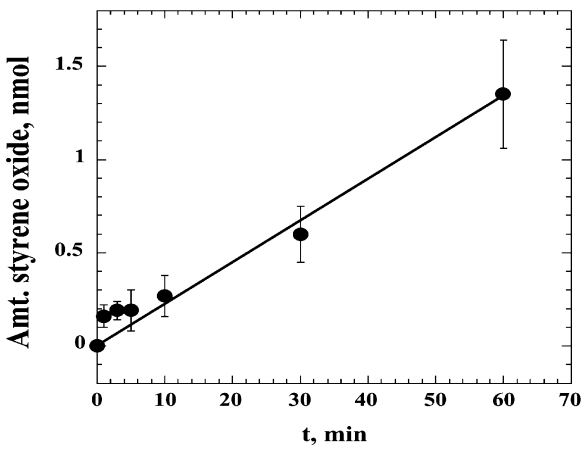


Fig. 3. Production of styrene oxide during solution assays with dissolved cyt P450 1A2.

of styrene via its epoxidation by the oxygenated enzyme in a higher oxidation state. The reduction of oxygen by iron heme enzymes gives H_2O_2, which can contribute to both the catalytic oxidation (Scheme 1) and the catalytic reduction current in Fig. 2c [19].

To measure the turnover rate of the enzyme in a conventional solution assay, cyt P450 1A2 was incubated with styrene, NADPH, and NADPH-P450 reductase at 4 °C. Styrene oxide found increased linearly over 1 h (Fig. 3), with turnover rate 17/h (Table 1). Benzaldehyde is formed by a non-enzymic reaction of styrene with H_2O_2 [7]. Control assays without cyt P450 1A2 showed no detectable styrene oxide. Similar experiments utilizing cyt $P450_{cam}$ and putidaredoxin gave a smaller turnover rate for styrene oxide formation (Table 1).

For enzyme-catalyzed electrolytic generation of styrene oxide, PSS/(cyt P450 1A2/PSS)$_2$-coated cathodes were immersed into aqueous oxygenated buffers containing styrene, and a voltage negative enough to initiate reaction by converting the PFe^{III} enzyme to PFe^{II} was applied under aerobic conditions. H_2O_2 formed electrochemically from reduction of oxygen was detected at approximately 4 mM after 1 h. The cyt P450 1A2 turnover rate for the electrochemical formation of styrene oxide was 39/h (Table 1). Control experiments including catalase to destroy H_2O_2 showed no styrene oxide formation. Similar experiments with films containing cyt $P450_{cam}$ gave styrene oxide with a smaller turnover number.

Use of 10 mM H_2O_2 to initiate epoxidation by PSS/(cyt P450 1A2/PSS)$_2$ films produced styrene oxide at a turnover rate of 53/h, 38% larger than the value obtained by electrolysis. This higher level of peroxide than formed in the electrolysis was able to activate the enzyme in the film for styrene epoxidation, as previously found for cyt $P450_{cam}$ films [10]. Under these conditions, approximately 20% of the enzyme is degraded in the 1 h reaction.

4. Discussion

Figs. 1 and 2 provide electrochemical evidence for the occurrence of initial reaction steps that lead

Table 1
Yields and turnover rates (per nmol styrene oxide/nmol enzyme) for styrene oxidation at 4 °C

Experiment	Enzyme (nmol)	Styrene oxide found (nmol)	Benzaldehyde found (nmol)	H_2O_2 found (mM)	Turnover rate (h^{-1})
P450 1A2 electrolysis[a]	0.134	8.8±2.3	22±4	4	39±10
Electrolysis control[b]	0	3.6	20	4	
P450 1A2+10 mM H_2O_2	0.134	11.6	39	10	54
10 mM H_2O_2 control	0	4.3	33	10	
P450 1A2 assay[c]	0.08	1.35±0.29	0.6±0.1	0.15	17±4
Assay control[c]	0	0	0.56	0.15	
$P450_{cam}$ electrolysis[a]	0.62	12±1	12±1	4	10±1
$P450_{cam}$ assay[c]	0.51	5.2	5.2		10

[a] Electrolysis using an enzyme-coated electrode.
[b] Electrolysis with no enzyme.
[c] Conventional solution reactions with electron donors and reductase (see experimental).

to production of H_2O_2, which activates the enzyme for oxidative catalysis. The reversible cyclic voltammogram (Fig. 1) suggests that electrolysis at −0.6 V will convert the PFe^{III} form of cyt P450 1A2 to the PFe^{II} form. Fig. 2 suggests that oxygen is catalytically reduced via binding to the PFe^{II} form and subsequent reduction of the $PFe^{II}–O_2$ complex to H_2O_2, which was measured at the end of the electrolyses. Catalase shut down the production of styrene oxide during electrolysis using the enzyme films, confirming the importance of peroxide for enzyme activation. Along with the conversion of styrene to styrene oxide in the electrolysis (Table 1), all the data are consistent with our previously suggested pathway of olefin oxidation with iron heme enzymes, including cyt $P450_{cam}$, in thin films on electrodes (Scheme 1) [7,10]. However, minor competing pathways cannot be ruled out at this time.

Electrolytic turnover rates for styrene epoxidation with cyt P450 films were reasonably similar to those found by conventional solution assays with dissolved enzyme (Table 1). These rates are a bit larger with the thin films containing cyt P450 1A2 compared to the solution assay, but just about the same in film and solution with cyt $P450_{cam}$. Turnover rates for enzymes in thin films are not absolute, and depend somewhat on enzyme concentration in the film, applied voltage, and film thickness [10]. Thus, the very good agreement for the cyt $P450_{cam}$ results may be fortuitous. However, if experimental characteristics are kept relatively constant from film to film and enzyme to enzyme, and reaction conditions are identical, reliable comparisons of relative turnover rates should be possible. Comparable turnover rates for cyt P450 1A2 in solution and films suggest that the enzyme has roughly similar activity in both environments.

Acknowledgments

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