

Cytochrome P450 immobilisation as a route to bioremediation/biocatalysis

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Abstract The diverse substrate specificity of the cytochrome P450 (P450; CYP) enzyme superfamily offers the opportunity to develop enzymatic systems for environmental detoxification and biotransformations of drugs, pesticides and fine chemicals. Here we report on the immobilisation of a fusion protein between plant cytochrome P450-71B1 (CYP71B1) and its electron donor, plant NADPH cytochrome P450 reductase using an oil-in-water macro-emulsion, termed polyaphron, which contains a proportion of internal organic phase (ϕ) greater than 0.74. Efficiency of P450 immobilisation was greater than 85%, and in this state enzymatic activity could be measured for more than 24 h at 15°C. Chlortoluron, a recalcitrant herbicide pollutant in the environment, was shown to be metabolised, with the major metabolite (*N*-monodemethylated chlortoluron) being separated from the substrate due to partitioning into the aqueous phase. The turnovers exhibited superactivity compared with those obtained using free enzyme located in membranes prepared following heterologous expression in *Saccharomyces cerevisiae* and *Escherichia coli*. The potential to exploit the unprecedented catalytic diversity of the P450 superfamily in biocatalysis is discussed.

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Key words: Cytochrome P450; Colloidal liquid aphron (CLA); Immobilization; Fusion protein

1. Introduction

The cytochrome P450 superfamily is widely distributed in various forms of life and participates in a range of oxygenation reactions of diverse substrates including pharmaceuticals, carcinogens, pesticides and pollutants [1,2]. Its unique catalytic chemistry and substrate specificity are well known and exploited in whole-cell biotransformations, such as the hydroxylation of corticosteroids by fungi [3]. However, using biological engineering techniques to exploit P450 systems for the synthesis of highly specific chemicals and detoxifying environmental pollutants remains a challenge in biotechnology.

Enzyme immobilisation is a technique that facilitates biocatalyst separation from the product, localisation within a reactor, improved enzymatic stability/activity retention, continuous operation over extended periods of time, and the possibility of obtaining enzyme superactivity [4,5]. The use of polyaphrons as an immobilisation technique has been previously described for other enzymes with various degrees of success. The immobilisation of lipase from *Candida cylindracea* was successful with over 80% [6] of the enzyme being effectively retained on the support and activity comparable to the free enzyme being achieved. However, for α -chymo-

trypsin, a large proportion of the enzyme could be immobilised, but with virtually no resulting activity [7].

Polyaphrons are similar to oil-in-water High Internal Phase Ratio (often termed 'concentrated') emulsions, containing a greater proportion of internal organic phase than is associated with hexagonal close packing of spheres (ratio of internal/continuous, $\phi = 0.74$) [8]. In this type of polyaphron formulation, they exist as polyhedral cells separated by thin, planar films of continuous aqueous phase [9,10]. They are formed by mixing a relatively non-polar organic solvent containing surfactant into an aqueous surfactant solution, with stable formulations lasting for many months without visible deterioration. The polyaphron phase can be homogeneously dispersed into a bulk aqueous phase with only minimal agitation, and under these conditions form discrete micron sized droplets of organic solvent (typical Sauter mean diameter 18 μm), which can then be referred to as Colloidal Liquid Aphrons (CLAs). The general applicability of this technique in the immobilisation of commercially important biocatalytic enzymes remains unresolved.

In this work, the immobilisation of plant cytochrome P450-71B1 fused to its electron donor, NADPH cytochrome P450 reductase, in an active form, is described. The great diversity of organic structures that can serve as substrates of P450s makes their application in enzyme-based biotechnological processes attractive, including the rapid prediction of the metabolic fate of drugs and pesticides metabolised by human and plant P450s. In addition, P450s combine the advantages associated with biocatalysts for organic syntheses, i.e. chemoselectivity including stereoselectivity and regioselectivity. The investigation of P450 immobilisation is a logical route towards more efficient biocatalysis/bioremediation technologies, and here we report on the successful immobilisation of a purified eukaryotic P450 monooxygenase fused to its electron donor.

2. Materials and methods

2.1. Genetic engineering

Plasmid pSP19g10L, which is derived from pSPORT, was used for P450 expression. This plasmid contains the inducible *tac* promoter [11]. Expression constructs containing a fusion protein comprising both plant CYP71B1 and NADPH-cytochrome P450 reductase have been described previously [12]. For successful bacterial expression, modifications to the N-terminal region of CYP71B1 were carried out to encode the codons representing the initial eight amino acids of CYP17A [13]. All manipulations were checked by diagnostic restriction analysis, and the successful constructs, CYP71B1:CPR, were sequenced to confirm the integrity of the clone.

2.2. Enzyme preparation

E. coli suspensions from a 500-ml culture were resuspended in 25 ml 10 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA and 20% (v/v) glycerol. Lysozyme (0.5 mg/ml) was added and the suspension left on ice for 30 min. Phenyl methyl sulphonyl fluoride (PMSF) was added to a final concentration of 1 mM and the mixture

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was sonicated on ice with a Soniprep cell disruptor using six 20-s pulses with 20-s intervals at 80% of the maximum power. Cell debris was removed by centrifugation at $5000 \times g$ for 10 min and the membranes pelleted by centrifugation at $100\,000 \times g$ for 60 min. *E. coli* membranes were diluted to a concentration of 3.0 mg/ml in 10 mM potassium phosphate buffer (pH 7.5) containing 20% (v/v) glycerol, 0.5 mM EDTA, 0.1 mM dithiothreitol, and 1.0% Emulgen 911 (w/v). All procedures were carried out at 4°C. The solubilised membrane was stirred for 30 min and subjected to centrifugation at $100\,000 \times g$ for 60 min. The clarified supernatant was then dialysed with 10 mM potassium phosphate buffer (pH 6.5) containing 20% glycerol (v/v) and applied to a hydroxyapatite column that had been equilibrated with the dialysis buffer. For removal of detergent and PMSF, the column was washed extensively with the equilibration buffer, and the detergent monitored at 280 nm. P450 (red colour) was eluted from the column with 500 mM potassium phosphate buffer (pH 7.5) containing 20% (v/v) glycerol and 0.1 mM DTT. The P450 fractions were dialysed against 10 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 0.1 mM DTT and 20% (v/v) glycerol to lower the phosphate concentration. The protein was stored at -80°C until further use.

2.3. Polyaphron preparation

Polyaphron phases were prepared by the dropwise addition of the organic phase containing non-ionic surfactant (1% (w/v) Softanol 30) into a foaming aqueous solution containing another non-ionic surfactant (1% (w/v) Synperonic A20 in RO water), and the desired concentration of the enzyme. The initial volume of the aqueous phase was typically 2 ml, which was stirred at approximately 800 rpm, and the organic phase was added at an average flow rate of 0.5 ml/min until the desired phase volume ratio of 4 was reached ($\text{PVR} = V_{\text{org}}/V_{\text{aq}}$). The polyaphrons formed containing the enzyme were used on the day of manufacture.

2.4. Determination of protein loss

Loss of CYP71B1:fusion from the CLAs after dispersion was determined at 417 nm using a Shimadzu UV2101 double beam spectrophotometer with an accuracy of $\pm 8\%$. The polyaphron phase was dispersed into a 50 mM buffer solution of known pH at a volume ratio of 1:1 by gentle shaking. After 1 h, most of the aphrons had risen forming a separate phase, the bottom aqueous phase was then filtered through a syringe filter (Sartorius, $0.2 \mu\text{m}$) to obtain a CLA-free sample of the continuous phase. The enzyme concentration in the aqueous phase was estimated according to Omura and Sato [14], and the percentage loss of the enzyme determined by mass balance.

2.5. Determination of cytochrome P450 activity

Polyaphrons were formulated containing enzyme, and dispersed into buffer (pH 7) containing NADPH (final concentration 1 mM), and finally [^{14}C]chlortoluron was added. The reaction was incubated at 37°C for 3 h, with the reaction vessels being continually stirred throughout the experiment. The CLAs were then allowed to separate from the aqueous phase and the products tested and identified by autoradiography by reference to known products. For quantification by TLC, the metabolite bands were excised and radioactivity assessed by liquid scintillation counting. The reaction for erythromycin was also similar; 5 ml of erythromycin (1 mM solution) was mixed with an equal volume of NADPH (1 mM final concentration). The polyaphron phase (5 ml) was then added, giving a volume ratio of 1:1, and incubated at 37°C for 30 min. A sample was then taken, filtered and tested for formaldehyde using the NASH reagent. Control experiments involved incubation with CLAs without the enzyme and CLAs immobilised with enzyme prepared from *E. coli* membranes expressing the null vector.

Table 1
Rates of plant CYP71B1:reductase fusion-catalysed erythromycin N-demethylation and chlortoluron metabolism

Compound	Activity (nmol product(s) formed/min/nmol CYP71B1)		
	Control	Free	Immobilized
Erythromycin	–	1.2 ± 0.8	11.0 ± 1.2
Chlortoluron	–	0.9 ± 0.7	10.2 ± 1.5

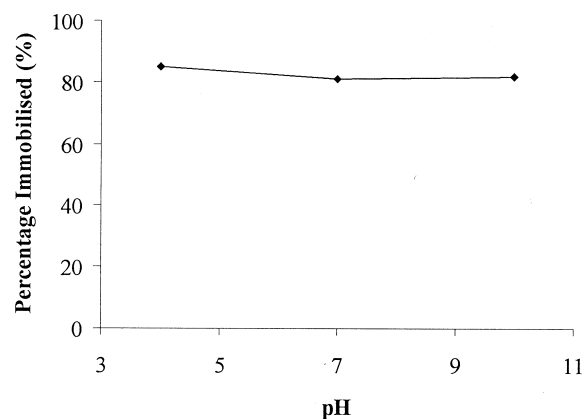


Fig. 1. Effect of continuous phase pH on the immobilisation of the cytochrome P450/NADPH cytochrome P450 reductase fusion protein (CYP71B1:CPR). Aphrons formulated from 1% (w/v) Softanol 30 in decane and 1% (w/v) SDS in water to PVR 4. Results are the average of three determinations.

3. Results

3.1. Genetic manipulation and protein expression

CYP71B1:CPR was constructed and expressed in *E. coli* as a modified protein where the native N-terminal sequence was replaced with that employed for the heterologous expression of CYP17A in *E. coli* [13], thus incorporating codons that facilitate expression in *E. coli*. CYP71B1:CPR levels were low (29 nmol/l), however, supplementing the growth medium with the haem precursor δ -aminoeluvinic acid increased the expression levels approximately twofold. The CPR activity of the fusion protein was calculated to be 650 nmol cytochrome *c* reduced/min per mg membrane protein reflecting an equivalence of CYP and CPR levels [12].

3.2. Formulation of CLAs containing CYP71B1:CPR

To study the activity and applications of CLA-immobilised CYP71B1:CPR, it was first necessary to formulate stable polyaphrons containing the fusion protein. With an initial concentration of the P450 of 0.4 mg/ml in the aqueous phase, the CYP71B1:CPR loading of the aphrons obtained was 0.068 mg protein/ml polyaphron at pH 7. The immobilisation of the fusion protein did not seem to be affected by the pH of the bulk aqueous phase into which it was dispersed (Fig. 1). The spectral properties of the free CYP71B1:CPR fusion

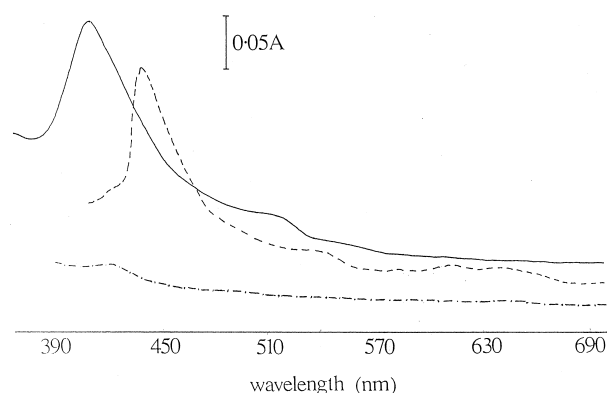


Fig. 2. Spectrophotometric absorption spectra of free, purified CYP71B1:reductase fusion. Solid line: oxidised form; dashed line: reduced carbon monoxide difference complex; and dashed/dotted line: aqueous phase after immobilisation on CLAs.

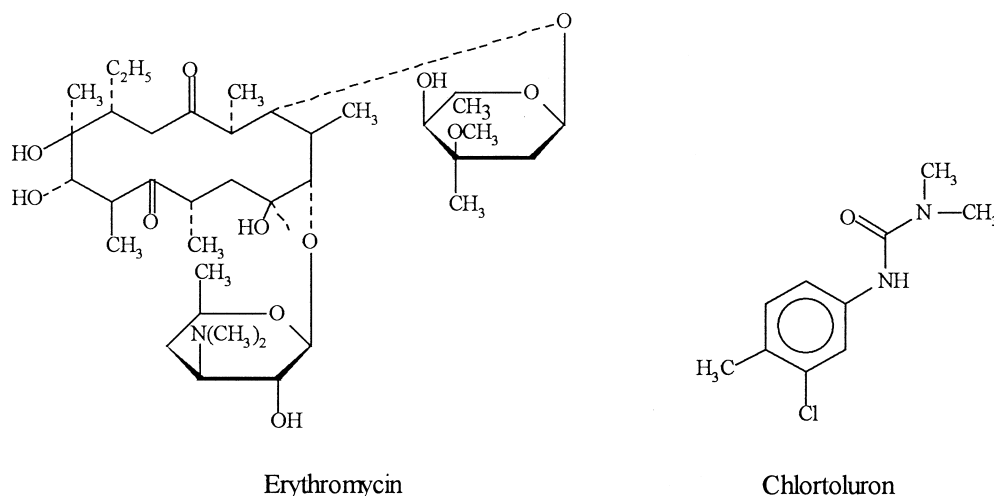


Fig. 3. Chemical formulae of erythromycin and chlortoluron.

showed the oxidised form exhibiting a spectral maximum at 417 nm corresponding to the protein haem, and the carbon monoxide reduced absorbance maximum located at 448 nm (Fig. 2). For the immobilised form, spectra were produced after immobilisation and subsequent dispersion for the remaining aqueous phase after flotation. The lack of a spectral maximum at either 417 or 448 nm indicated that the immobilisation of the CYP71B1:CPR fusion had been successful.

3.3. CYP71B1:CPR immobilised activity

To investigate the activity of CLA-immobilised CYP71B1:CPR, the *N*-demethylation of the model P450 substrate, erythromycin (Fig. 3), was measured. In addition, the

ability of immobilised CYP71B1:CPR to metabolise [^{14}C]chlortoluron (Fig. 3), a recalcitrant pollutant, was assessed and the major metabolite was shown to be *N*-monodemethylated chlortoluron. Immobilised CYP71B1:CPR could demethylate erythromycin with a turnover of 11 nmol product formed/min/nmol P450. This was an approximately 10-fold higher activity when compared with free enzyme prepared from heterologously expressing yeast and *E. coli*. Furthermore, chlortoluron was metabolised by the immobilised system with a turnover of 10.2 nmol *N*-monodemethylated chlortoluron formed/min/nmol P450 compared with 2.1 nmol *N*-monodemethylated chlortoluron formed/min/nmol P450 for the free enzyme (Table 1). These results show the

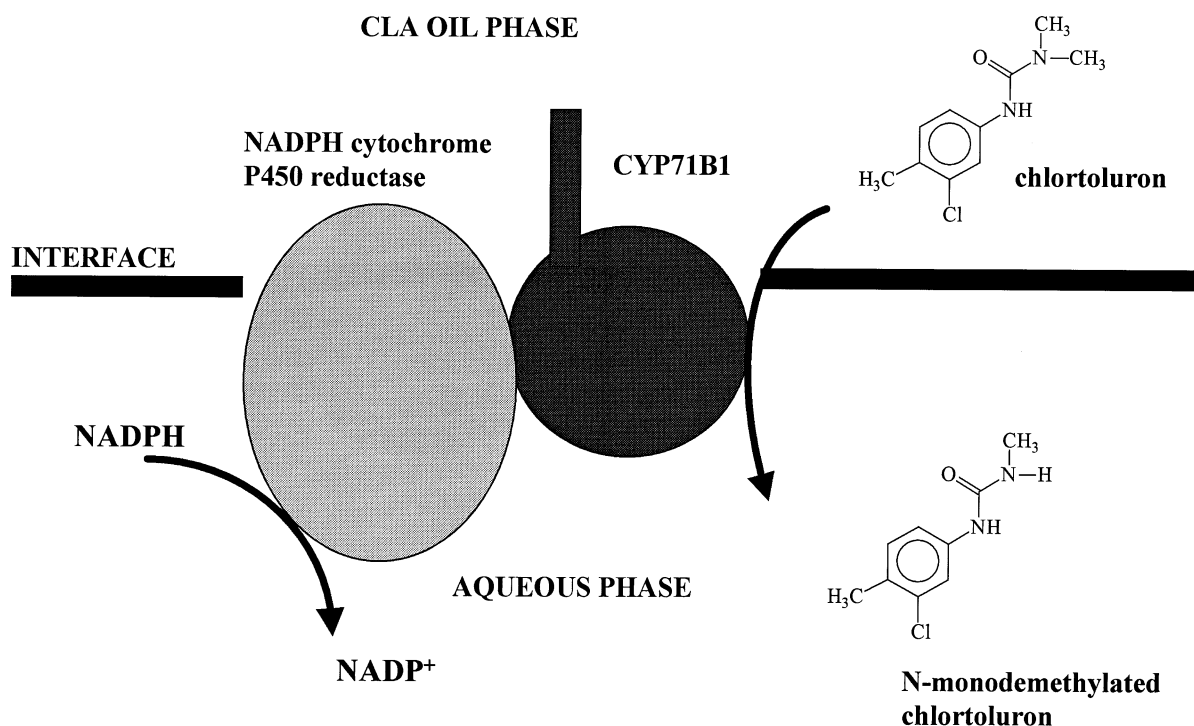


Fig. 4. Immobilised plant CYP71B1:reductase fusion catalytic cycle for chlortoluron *N*-monodemethylation. Chlortoluron partitions to the CLA oil phase where it has access and binds to the hydrophobic active site of the oxidised P450. Electrons are transferred via the fused plant NADPH cytochrome P450 reductase from NADPH. In the final step the complex decomposes to give the *N*-monodemethylated product, which partitions into the aqueous phase of the system, regenerating the oxidised P450 form.

CYP71B1:CPR becomes 'superactive' on immobilisation, a phenomenon shown to occur for the immobilisation of other enzymes [3,4].

4. Discussion

There has been a great deal of interest in the exploitation of the P450 enzymes for practical applications. To date the use of P450 enzymes in industrial processes has been restricted to in vivo biotechnology whereby an organism expressing a P450 form(s) has been manipulated to achieve commercial success. Examples include the manipulation of flower colour [15], the synthesis of progesterone and pregnenolone in yeast [16], and the use of P450sca in *Streptomyces carbophilus* in the production of the anti-cholesterol agent pravastatin [17]. Furthermore, the use of P450 enzymes in bioremediation is now becoming of increasing interest with experiments revealing that P450 from *Pleurotus* is needed for bioremediation and lignin degradation [18,19]. Thus it can be expected that rapid progress will be made in the biotechnological uses of P450 enzymes in the coming years.

Nevertheless, despite the applied potential of P450 enzymes, problems still exist in P450 biochemistry which need to be solved before they can be used industrially. Firstly, prior to monooxygenation, cytochrome P450 derives the electrons involved in the molecular splitting of atmospheric oxygen from another protein, namely NADPH-cytochrome P450 reductase. Consequently, immobilisation of P450 alone would not result in catalytic activity. In this study, we demonstrate the efficient immobilisation of plant P450 fused to its electron donor thus representing immobilisation of the complete eukaryotic P450 monooxygenase system. Previous studies using crude rat microsomal liver fractions were only immobilised with low efficiency and reduced activity using Romicon PM 10 polysulphone anisotropic hollow fibres [20]. It has been proposed that immobilisation on CLAs may be due to electrostatic or hydrophobic interactions [6]. The immobilisation of the fusion protein did not seem to be dependent on pH or the presence of non-ionic surfactants, and this tends to indicate that immobilisation may be predominantly due to hydrophobic interactions. The overall molecular weight of the fusion protein is 125 000 Da (MW of P450 ~ 53 000 Da and MW of reductase ~ 72 000 Da). This large molecular weight would indicate that the enzyme has a considerable hydrophobic core, which would be attracted to the hydrophobic surface of the CLAs. The P450 is a membrane bound enzyme and it is likely to be relatively non-polar. Hence, it would tend to associate itself more readily with the oil-water interface (Fig. 4).

In our study the enzyme system was active in its ability to *N*-demethylate erythromycin and chlortoluron. Furthermore, comparison of the immobilised enzyme with the free enzyme in chlortoluron metabolism revealed the immobilised enzyme to have greater activity. Similarly, immobilised CYP71B1:CPR had greater activity towards *N*-demethylation of erythromycin when compared to the free form. This may be due to structural activation of the CYP71B1:CPR complex brought about by its localisation at an oil/water interface with the CLAs, greater access or affinity of the substrate molecules to the active site of the P450, or more efficient electron transport. Many enzymes, including P450, are associated with non-polar cellular components such as membranes [21], and in these cases the natural microenvironment will be less polar

than bulk water and so this may lead to activation of the enzyme.

The present study demonstrates the feasibility of direct immobilisation of a cytochrome P450/NADPH cytochrome P450 reductase fusion protein for carrying out highly specific hydroxylation of a recalcitrant pollutant. Other studies for harnessing P450 enzymes for practical applications have involved driving the P450 by supplying electrons to the redox partners by the use of a platinum electrode [22,23]. However, current limitations to these systems include problems in separating the product from the bioreactor components, and direct reduction of the reductase partner proteins from eukaryotic systems which would require other electrode and/or bioreactor design strategies [23]. The development of expression systems allowing high level production of enzyme coupled with developments in the mechanical stability of immobilised supports may serve as an alternative route towards improving industrial processes. For the P450 studied here applications could include bioremediation strategies and predicting the metabolic fate of pesticides in the agrochemical industry. Other drug-metabolising P450 enzymes of humans may be used to produce metabolites of drugs/xenobiotics for toxicological evaluation as well as for biotransformations generally.

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