We have incorporated CYP3A4 (cytochrome P450 3A4) and CPR (NADPH-cytochrome P450 reductase) into liposomes with a high lipid/protein ratio by an improved method. In the purified proteoliposomes, CYP3A4 binds testosterone with $K_a$ (app) = 36 ± 6 μM and Hill coefficient $= 1.5 ± 0.3$, and 75 ± 4% of the CYP3A4 can be reduced by NADPH in the presence of testosterone. Transfer of the first electron from CPR to CYP3A4 was measured by stopped-flow, trapping the reduced CYP3A4 as its Fe(II)–CO complex and measuring the characteristic absorbance change. Rapid electron transfer is observed in the presence of testosterone, with the fast phase, representing 90% of the total absorbance change, having a rate of $14 ± 2$ s$^{-1}$. Measurements of the first electron transfer were performed at various molar ratios of CPR/CYP3A4 in proteoliposomes; the rate was unaffected, consistent with a model in which first electron transfer takes place within a relatively stable CPR–CYP3A4 complex. Steady-state rates of NADPH oxidation and of 6β-hydroxytestosterone formation were also measured as a function of the molar ratio of CPR/CYP3A4 in the proteoliposomes. These rates increased with increasing CPR/CYP3A4 ratio, showing a hyperbolic dependency indicating a $K_a$ (app) of $∼0.4$ μM. This suggests that the CPR–CYP3A4 complex can dissociate and reform between the first and second electron transfers.

Key words: artificial liposome, cytochrome P450, cytochrome P450 3A4 (CYP3A4), electron transfer, kinetics, lateral diffusion, reductase.

INTRODUCTION

The cytochrome P450 mono-oxygenase system in the endoplasmic reticulum, particularly in the liver, lung and small intestine, plays a central role in the metabolism of drugs and other xenobiotics. The members of the cytochrome P450 superfamily share a common overall fold and a common catalytic mechanism, but differ markedly in their active-site architecture, leading to very diverse substrate specificity. In humans, some 15 cytochrome P450s are involved in drug metabolism, although a group of only six to seven cytochrome P450s can account for the metabolism of 90–95% of drugs [1]. A broad specificity coupled with high levels of expression in the liver means that CYP3A4 (cytochrome P450 3A4) is responsible for the metabolism of $∼50$% of drugs in current use; the crystal structure of CYP3A4 indeed reveals a large active site capable of accommodating substrates with a wide range of structures [2–4].

Cytochrome P450s catalyse the insertion of one atom of molecular oxygen into their substrates, with the concomitant reduction of the other atom to water (Figure 1). This reaction requires two electrons, which in the case of the drug-metabolizing mono-oxygenase system are most commonly derived from NADPH, although the second electron can in some circumstances be supplied by cytochrome $b_5$ [5]. The electron transfer from NADPH is mediated by the flavoprotein CPR (NADPH-cytochrome P450 reductase) [5,6] which accepts electrons from the obligatory two-electron donor NADPH and donates them one at a time to two distinct steps in the reaction cycle of cytochrome P450 (Figure 1, steps 2 and 4). The mechanism by which this is achieved, particularly the interflavin electron transfers involved, have been studied in detail [5,7,8] and evidence has been obtained indicating that the internal electron transfer in CPR is limited by domain motion [9–11].

Both cytochrome P450 and CPR are attached to the endoplasmic reticulum membrane through hydrophobic sequences at their N-termini, although the available crystal structures are of truncated proteins in which these sequences have been removed to aid crystallization. In the liver endoplasmic reticulum, cytochrome P450s are present in excess over CPR, with a molar ratio of cytochrome P450/CPR as high as 20:1 [12]. This stoichiometry and the mutual arrangement of the two enzymes in the membrane clearly have important implications for the electron transfer from CPR to cytochrome P450. The first electron transfer step, labelled 2 in Figure 1, which can readily be isolated by carrying out the reaction in a carbon monoxide atmosphere and trapping the cytochrome P450 in the Fe(II)–CO complex, has been studied extensively, largely in reconstituted systems. However, consensus has not yet been reached concerning the mechanism by which CPR and cytochrome P450 interact functionally in the membrane [13]; there are significant differences in the results obtained with different cytochrome P450s and with different reconstitution systems. Evidence has been presented both in favour of the view that the electron transfer is achieved by a collisional mechanism involving lateral diffusion of CPR and cytochrome P450 in the plane of membrane and in favour of the idea that it takes place in relatively stable functional complexes between CPR and one or more cytochrome P450 molecules.

In the present study we report studies of the electron transfer between CPR and CYP3A4 in a purified reconstituted proteoliposome system. The reconstitution of the catalytic activity of cytochrome P450 mono-oxygenase systems from purified components has proved challenging ([14,15] and references therein), particularly for cytochrome P450s of the 3A family, but Guengerich and Johnson [16] have reported successful reconstitution of CYP3A4 with complex mixtures of phospholipid, Mg$^{2+}$, glutathione, cytochrome $b_5$ and substrate.
We have now measured the rates of the first electron transfer, of NADPH consumption and of testosterone 6β hydroxylation in a simple system consisting only of CYP3A4, CPR, phospholipid and substrate. Rapid transfer of the first electron from CPR to CYP3A4 is observed, showing that in our system there is no requirement for cytochrome b₅ for this step. The dependence of the rates on the ratio of CPR to CYP3A4 is interpreted in terms of a model in which the first electron transfer takes place within a relatively stable CPR–cytochrome P₄₅₀ complex, which can dissociate and reform before the much slower second electron transfer takes place.

**EXPERIMENTAL**

**Materials**

NADPH, horse cytochrome c, CHAPS, testosterone, 6β-hydroxytestosterone and erythromycin were purchased from Sigma–Aldrich. POPC (1-palmitoyl-2-oleoyl-sn-glycero-phosphocholine) and POPA (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate) were obtained from Avanti Polar Lipids. Complete Protease™ inhibitors were from Roche. All other chemicals were of analytical grade.

**Expression and purification of recombinant proteins**

CYP3A4 with a 6-histidine affinity tag was expressed in Escherichia coli JM109 from an ompA+2–3A4 construct in the pCW-Ori+ vector ([17]; kindly provided by Professor Roland Wolf, Biomedical Research Institute, Ninewells Hospital and Medical School, University of Dundee, Dundee, U.K.). During protein expression, the 21-amino-acid ompA+2 leader sequence is cleaved by bacterial proteases to leave only the full-length unmodified CYP3A4 [18]. Protein was expressed and purified by a method described previously [17]. Cells were harvested by centrifugation, resuspended in buffer A [50 mM potassium phosphate, 0.5 M potassium chloride and 20 % (v/v) glycerol, pH 7.40], supplemented with Complete Protease™ inhibitors, and stored at −80°C until processed. Cells were lysed by sonication and solubilized by stirring in the presence of 10 mM CHAPS. The cell suspension was then centrifuged and the soluble material was loaded on to a Hi-Trap chelating column (2.6 cm × 5 cm) which had been charged with nickel ions and pre-equilibrated with buffer A. The column was washed with 2 vol. of buffer A and weakly binding proteins were removed by washing with 3 vol. of 50 mM potassium phosphate buffer, 5 mM CHAPS and 10 % (v/v) glycerol, pH 7.40, containing 100 mM imidazole. CYP3A4 was eluted by a linear gradient of 0.1–0.5 M imidazole in the same buffer. The protein sample was dialysed overnight at 4°C against 0.1 M potassium phosphate, 5 mM CHAPS, 0.1 mM DTT (dithiothreitol), 0.1 mM EDTA and 20 % (v/v) glycerol, pH 7.40. The purified protein was judged to be >95 % pure by SDS/PAGE; it was stored at −80°C until required. The concentration of CYP3A4 was determined by the method of Omura and Sato [19] using a molar absorption coefficient of 91 mM⁻¹·cm⁻¹ at 448 nm for the reduced CO complex.

Human CPR was expressed in E. coli BL21 (DE3) pLysS cells using the expression vector pB209 ([20]; provided by Professor Roland Wolf) and purified using a combination of anion-exchange chromatography and 2,5-ADP Sepharose affinity chromatography [21]. The cells were grown at 30°C to a D₅₀₀ of 0.8–1, expression was induced by adding IPTG (isopropyl β-D-thiogalactoside; 0.5 mM) and the cells were grown for a further 8–12 h at 28°C. Cells were then harvested by centrifugation and resuspended in buffer B [50 mM Tris/HCl, 0.5 mM EDTA, 0.5 mM DTT and 10 % (v/v) glycerol, pH 7.80], supplemented with Complete Protease™ inhibitors and stored at −80°C until needed. Cells were lysed by sonication (six 20 s bursts, Soniprep ISO apparatus, 19 mm tip diameter probe, amplitude 10 µm), solubilized by stirring in the presence of 10 mM CHAPS and the insoluble material was removed by centrifugation at 25000 rev./min for 50 min at 4°C using a Beckman JA-30.5 rotor. The supernatant was then loaded on to a DEAE anion-exchange column (2.6 cm × 10 cm) that had been pre-equilibrated with buffer C (30 mM Tris/HCl, 0.15 M NaCl, 0.1 mM EDTA, 0.1 mM DTT and 10 % (v/v) glycerol, pH 7.60), and the protein was eluted in a linear gradient of 0.15–0.5 M NaCl in buffer C. Eluted fractions were pooled and loaded on to a 2.5'-ADP Sepharose affinity column (2.6 cm × 5 cm) pre-equilibrated with buffer B. Impurities were removed by washing the column with 2 vol. of buffer C and protein was oxidized by 0.5 vol. of buffer C.
containing 1 mM potassium hexacyanoferrate. Excess potassium hexacyanoferrate was removed by washing the column with 2 vol. of buffer C. Finally, protein was eluted using buffer C containing 5 mM 2,3′-AMP. The protein sample was then dialysed overnight at 4°C against 0.1 M potassium phosphate, 2 mM CHAPS and 10% (v/v) glycerol, pH 7.40 and kept frozen at −80°C until needed. As judged by SDS/PAGE the protein was ≥90% pure, the only significant impurity being ~10% truncated CPR which arose during the purification in spite of the presence of protease inhibitors. The CPR concentration was determined based on the flavin content using a molar absorption coefficient of 21 mM⁻¹ cm⁻¹ at 456 nm.

Preparation of liposomes

The procedure for preparation of liposomes was adapted from that described by Kim et al. [22]. The phospholipids, 9 mg/ml POPA and 10 mg/ml POPC (50:50 mol %), were dissolved in dichloromethane and the solvent was removed by evaporation under a stream of nitrogen followed by subsequent drying under vacuum for 2 h. The dry lipids were then suspended in buffer solution (25 mM Tris/HCl, 100 mM NaCl and 0.5 mM EDTA, pH 7.40) by vortex-mixing and subsequent gentle sonication (30 s, Soniprep IS0 apparatus, 3 mm tip diameter probe, amplitude 10 µm). The suspension was frozen and thawed five times and extruded 25 times through a polycarbonate filter (Whatman) to obtain homogeneous LUVs (large unilamellar vesicles).

Reconstitution of CPR and CYP3A4 in liposomes

The SRS (simple reconstitution system) consisted of mixing 2 nmol of CPR, 2 nmol of CYP3A4 and 2 µmol of LUVs (POPC/POPA 50:50 mol %) in 1 ml of 0.1 M potassium phosphate buffer and 1.6 mM CHAPS, pH 7.40. The solution was incubated for 10 min at room temperature (~20°C) before the activities were measured. Since only a limited proportion of the proteins were incorporated into the vesicles in the SRS, the proteoliposomes were purified by gel filtration. The SRS was prepared as described above with the desired amount of proteins and liposomes, and was then loaded on to a Superose 6 column which was eluted at a flow rate of 0.4 ml/min with 0.1 M potassium phosphate buffer, pH 7.40, in the absence of detergent. The elution of the column was followed at 280 nm, 420 nm and 450 nm. A broad peak in the void volume and subsequent smaller peaks were collected in fractions of 2 ml and were then analysed by SDS/PAGE, and assayed for cytochrome c reductase activity [21] and for phospholipids by the ammonium ferriothiocyanate method [23]. Proteins that were eluted in the void volume were considered to be physically incorporated into liposomes. The desired molar ratios of CYP3A4 and CPR in the proteoliposomes could be obtained by adjusting the concentrations in the initial mixture before gel filtration.

Electron microscopy

For negative staining, a 5 µl sample of proteoliposomes was applied to the surface of a carbon-coated copper grid and the grid was suspended in the neck of a bottle of 25% glutaraldehyde for 3 min to fix the sample on to the carbon surface. Excess sample was removed by touching the grid with filter paper and a droplet of 2% (w/v) uranyl acetate was immediately applied. The stain was removed with filter paper and air dried. After drying, the material was analysed by using a Jeol 1220 transmission electron microscope (Jeol UK Ltd).

Stopped-flow experiments

All reduction measurements were made using an Applied Photophysics SX-18 MV instrument at 25°C in an anaerobic CO-saturated environment. Reduction of ferric CYP3A4 to the ferrous–CO complex was monitored at 448 nm upon mixing the proteoliposome system in 0.1 M potassium phosphate buffer, pH 7.40, in one syringe with NADPH in the same buffer in the other. The instrument was not maintained within an anaerobic glove box; prior to the stopped-flow experiment, the optical cell was made anaerobic by first flushing with concentrated sodium dithionite solution (~15 mM) from gas-tight syringes connected to the cell block of the instrument via a series of Luer connectors, followed by extensive flushing with anaerobic 0.1 M potassium phosphate buffer, pH 7.40. Proteoliposomes and NADPH solutions (with or without substrate) were prepared in gastight syringes in an anaerobic glove box, bubbled with CO gas under a fume hood (1 ml of sample bubbled for at least 1 min) and transferred anaerobically using gas-tight syringes to the sample-handling unit of the stopped-flow instrument. Data were collected using a ‘logarithmic mode’ in which a minimum of 1000 data points per trace are collected as a logarithmic function of time after mixing, so that both fast and slow phases are adequately sampled; traces shown are the average of three to six individual reactions. Averaged traces were fitted by either single or double exponential equations using the Applied Photophysics software.

Substrate binding

Changes in the optical spectrum of CYP3A4 in the vesicles resulting from testosterone or erythromycin binding were recorded on a Cary 300Bio UV–visible spectrophotometer with a Peltier temperature control unit maintaining the temperature at 25°C. A 1 ml sample was divided equally between two quartz cuvettes (10 mm path length) and a baseline was scanned between 350 and 500 nm. Testosterone or erythromycin were dissolved in methanol to a final concentration of 40 mM and added in 0.25 µl increments into the sample cuvette and the same volume of methanol was added to the reference cuvette; the final methanol concentration was always less than 1.5%. The difference spectrum was recorded after each incremental addition and the difference in absorbance change between 390 nm and 420 nm (ΔA390–A420), was plotted against substrate concentration. Dissociation constants were estimated by non-linear regression analysis with Microcal Origin software using the Hill equation: Δ(A390–A420) = AmaxS/n [Kd (app)+S], where Amax is the value of Δ(A390–A420) at saturating substrate concentration, S is the substrate concentration, Kd (app) is the apparent dissociation constant (the substrate concentration giving an absorbance change of 50% Amax) and n is the Hill coefficient. The percentage of the enzyme–substrate complex in the high-spin state was calculated using a Δ(A390–A420) difference molar absorption coefficient of 126 mM⁻¹ cm⁻¹ [24].

NADPH consumption assay

The rate of NADPH oxidation was determined at 25°C by monitoring absorption changes at 340 nm using the Cary Bio300 spectrophotometer. A 480 µl sample of the proteoliposomes in 0.1 M phosphate buffer, pH 7.40, was pre-incubated for 3 min at 25°C in the presence or absence of testosterone (500 µM). The reaction was initiated by the addition of 20 µl of NADPH (final concentration 100 µM) and the decrease in absorbance at
340 nm was monitored for 30 min, the reaction being linear over this time period. Rates were calculated using a molar absorption coefficient of 6.22 mM$^{-1}$ cm$^{-1}$ for NADPH at 340 nm; values are the average of a minimum of three experiments.

**Testosterone 6β-hydroxylation**

A 480 μl suspension of proteoliposomes in 0.1 M potassium phosphate buffer, pH 7.40, was equilibrated with 500 μM testosterone at 25°C for 3 min and the reaction was initiated by the addition of 20 μl of NADPH (final concentration 100 μM). The sample was incubated for 30 min at 25°C, the reaction being linear over this time period, after which 0.25 ml of ice-cold methanol was added to stop the reaction. The sample was placed on ice for 10 min and was spiked with 0.1 μM 2β-hydroxytestosterone as an internal standard; it was then clarified by centrifugation and 250 μl of the supernatant was removed for analysis. A 10 μl aliquot was loaded on to a Thermo-Hypersil 5 μm ODS (octadecylsilane) C18 column (4.0 mm × 250 mm) on an Agilent 1100 series HPLC instrument equipped with diode array UV–visible detection. The sample was eluted by a gradient of methanol/water/acetonitrile from 42.5:55:2.5 to 72.5:22:2.5 at a flow rate of 1 ml/min over 19 min. The peak corresponding to 6β-hydroxytestosterone was identified by co-elution with an authentic standard and quantified by comparison of UV peak area integrations with known concentrations of the authentic standard. The data presented are the average of three experiments.

**RESULTS AND DISCUSSION**

**Characterization of the reconstitution system**

The SRS, which consists of mixing purified cytochrome P450 and CPR with preformed liposomes (LUVs) in the presence of a small amount of detergent, has been widely used (e.g. [16,22] and references therein). We chose to use a mixture of phosphatidylcholine and phosphatic acid in view of the evidence that the presence of negatively charged phospholipids has a favourable effect on the activity of CYP3A4 (see [22]) in reconstituted systems. Anionic lipids have recently been shown to modulate the redox potential of CPR to make electron transfer from CPR to cytochrome P450 more efficient [25]. In the SRS, only 1.9 ± 0.2% (without substrate) or 6.3 ± 0.8% (in the presence of 500 μM testosterone) of the added CYP3A4 was reducible by NADPH, as measured by CO-difference spectroscopy. These results are similar to those obtained by Kim et al. [22], who reported 6.5–7.0% reduction of CYP3A4 in the presence of 50:50 mol% phosphatidylcholine/phosphatic acid vesicles and 100 μM testosterone.

It appears possible that, in the SRS, only a small proportion of the cytochrome P450 and/or the CPR is physically incorporated into the liposomes. We therefore purified the SRS by gel-filtration chromatography on a Superose 6 column. As shown in Figure 2, the chromatogram displayed three peaks. SDS/PAGE analysis confirmed that the broad peak eluting in the void volume, corresponding to proteoliposomes, contained CYP3A4 and full-length CPR. The second peak, eluting at 50 ml, consisted of CPR and CYP3A4 aggregates, whereas truncated CPR (lacking the N-terminal membrane-binding sequence) was found to elute at 70 ml (peak 3). Assays of the cytochrome P450 and CPR concentrations in the proteoliposome fractions eluting in the void volume showed that 20–30% of the total amount of each protein was incorporated into the purified proteoliposomes. Electron microscopy of the proteoliposomes showed a reasonably homogeneous dispersion of unilamellar vesicles with an average diameter of 200 ± 15 nm.

From the average surface area of the proteoliposomes and a measured lipid/protein ratio of 1000:1 for the samples from peak 1, the number of proteins per liposome was estimated to be approx. 350, corresponding to one protein molecule per ~360 nm$^2$ of the lipid bilayer.

In comparison with the SRS, CYP3A4 in the proteoliposomes purified by gel filtration was markedly more readily reducible by NADPH, to the extent of 10 ± 2% in the absence of substrate and as high as 75 ± 4% in the presence of testosterone, demonstrating efficient electron transfer between the two proteins in this system. This is consistent with previous reports [26,27] showing that CYP3A4 in solution forms oligomers which are incompletely reducible, but incorporation of the enzyme into nanodiscs (lipoprotein bilayer constructs in which the CYP3A4 is monomeric [28]) or into proteoliposomes with a high lipid-to-protein ratio markedly increases the degree of reducibility.

The use of CHAPS rather than cholate, which has been commonly used in cytochrome P450 reconstitution, led to reasonably efficient incorporation of both CPR and CYP3A4 into the phospholipid vesicles and, as judged by the CO-difference spectra, to minimal formation of inactive (P420) cytochrome. When we substituted 0.1% sodium cholate for 0.1% CHAPS, the amount of CYP3A4 reducible by NADPH was halved, and the other 50% of the CYP3A4 was observed to be in the inactive (P420) form. This suggests that sodium cholate was not wholly removed by the gel filtration and inhibited the CYP3A4 activity in the proteoliposomes. It is not known whether CHAPS remains associated with the proteoliposomes or is removed by the gel-filtration step. In the case of CYP1A2 and CYP2B4, the use of either CHAPS or glycocholate instead of cholate has also been reported to result in higher incorporation efficiencies and in decreased cytochrome inactivation [14].

**Substrate binding to CYP3A4 in proteoliposomes**

The binding of testosterone to CYP3A4 incorporated along with CPR in proteoliposomes produces characteristic type I spectral changes indicative of shifts in the spin equilibrium of the haem iron towards the high-spin state (Figure 3A), although the high turbidity of the sample limits the quality of the difference spectra. Figure 3(B) shows the amplitude of the difference spectrum, Δ(A390 – A450), as a function of testosterone...
Figure 3  Testosterone binding to CYP3A4 in proteoliposomes

(A) Difference spectra of 0.2 μM CYP3A4 in proteoliposomes on addition of testosterone (the numbers on the right show the testosterone concentration in μM for each of the colour-coded traces) at 25°C. (B) The absorbance difference Δ(A390 - A420) as a function of the testosterone concentration, fit by the Hill equation with Kd (app) of 36 ± 6 μM and Hill coefficient of 1.50 ± 0.30.

concentration; analysing the data by using the Hill equation yielded an apparent dissociation constant of 36 ± 6 μM and a Hill coefficient of 1.5 ± 0.3. These values were unaffected by the omission of CPR from the proteoliposomes [Kd (app) = 33 ± 2.6 μM, n = 1.6 ± 0.2]. The magnitude of the change in absorbance at saturating testosterone concentrations shows that the testosterone complex is >85% in the high-spin state in the proteoliposomes. The values of Kd (app) and Hill coefficient measured here are similar to those reported by Denisov et al. [29] [Kd (app) = 28 μM, n = 1.6] for testosterone binding to CYP3A4 in a nanodisc system containing a 1:1 molar ratio of CPR/CYP3A4. In the latter system, the CYP3A4 is of necessity monomeric and the detergent has been removed; this suggests that in our proteoliposomes, with a high lipid-to-protein ratio, the behaviour of CYP3A4 is not affected by aggregation or by interactions with any residual CHAPS.

We also observed type I spectral changes on binding of erythromycin to CYP3A4 in the proteoliposomes. The estimated Kd (app) was 125 ± 20 μM, but only ~10% of the CYP3A4 was converted from the low- into high-spin state on binding of erythromycin. The magnitude of this shift in the spin-state equilibrium on erythromycin binding is consistent with a report for CYP3A4 in nanodiscs [30].

Figure 4  Kinetics of reduction of ferric CYP3A4 in proteoliposomes

The CPR/CYP3A4 proteoliposome system was mixed with NADPH in a CO atmosphere in a stopped-flow spectrophotometer at 25°C. CYP3A4 reduction was monitored at 448 nm by formation of the Fe(II)–CO complex. (A) CPR/CYP3A4 proteoliposomes (0.1 μM of each protein) plus 0.5 mM testosterone were mixed with the same volume of 0.4 mM NADPH plus 0.5 mM testosterone. The fit by a double exponential with rates of kfast = 12.4 s⁻¹ and kslow = 0.67 s⁻¹ is shown as the smooth line.

Kinetics of CYP3A4 reduction in proteoliposomes

The transfer of the first electron from CPR to CYP3A4 in the proteoliposomes was investigated by stopped-flow experiments carried out in CO-saturated solutions so as to trap the cytochrome P450 after the first electron transfer in the form of the Fe(II)–CO complex. Figure 4(A) shows a typical time course for the reduction of CYP3A4, followed at 448 nm, in the presence of testosterone, when the reaction was initiated by rapidly mixing the purified proteoliposome system (containing equimolar amounts of CYP3A4 and CPR) with an NADPH solution. There is an initial drop in absorbance followed by an increase. Control experiments with liposomes alone and with proteoliposomes containing only CPR showed that the rapid absorbance decrease was made up of two components: a mixing artefact due to light-scattering changes resulting from the rapid dilution of the liposomes, and the reduction of the flavins of CPR [31].
The subsequent increase in absorbance at 448 nm, corresponding to the reduction of CYP3A4, is almost absolutely dependent on the presence of substrate; without substrate there is only a very slow increase in absorbance, some 300-fold slower than in the presence of testosterone (Figure 4B; $k = 0.03 \pm 0.008$ s$^{-1}$). Type I substrates have been shown to increase the redox potential of CYP3A4 by modulation of the ferric haem iron spin state [30]. The increase in absorbance in the presence of testosterone was best fitted by the sum of two first-order reactions, the fast phase accounting for 90 ± 3 % of the absorbance change. The rates of CYP3A4 reduction in the proteoliposomes in the presence of testosterone were $k_{\text{fast}} = 12.4$ s$^{-1}$ and $k_{\text{slow}} = 0.7$ s$^{-1}$ at a 1:1 ratio of CPR/CYP3A4.

Several groups have noted that cytochrome $b_5$ can increase the catalytic rate of cytochrome P450s [16,32], and Guengerich and Johnson [16], using a SRS containing phosphatidylcholine, phosphatidylserine and cholate, reported that the first electron transfer from CPR to CYP3A4 in the presence of testosterone is essentially absolutely dependent on the presence of cytochrome $b_5$ or apo-$b_5$, with a rate of 12–20 s$^{-1}$ in the presence of cytochrome $b_5$ but only <0.03 s$^{-1}$ in its absence [16]. Our proteoliposome system differs from that used by Guengerich and Johnson [16] in the phospholipid mixture used, in the absence of Mg$^{2+}$, glutathione and cytochrome $b_5$, in the use of CHAPS rather than cholate and in the use of gel filtration to purify the proteoliposomes. It is not completely clear which of these differences can account for the fact that we observe rapid and reproducible reduction of CYP3A4 by CPR in the absence of cytochrome $b_5$ (comparable with the rate reported by Guengerich and Johnson [16] in the presence of $b_5$), but, as noted above, the use of CHAPS rather than cholate appears to have a beneficial effect (see also [14]). Cytochrome $b_5$ may increase the stability of the cytochrome P450 or the CPR–cytochrome P450 complex [33,34], perhaps by protecting them from the effects of detergents, and the observation that in our proteoliposomes the first electron transfer is rapid in the absence of cytochrome $b_5$ might be explained by a different stability of the complex in the two systems.

In general, the reduction of cytochrome P450s has been observed to be biphasic, with the fast phase representing 50–90% of the absorbance change and having a rate of 2–18 s$^{-1}$. In some cases, the fast phase was shown to represent reduction of the high-spin cytochrome P450 (e.g. [35]), and this has led to a model in which the two phases represent reduction of high- and low-spin haem, although it is difficult to reconcile this with the generally very rapid rate of spin transitions (see [26]). We examined the kinetics of CYP3A4 reduction in the presence of erythromycin, whose binding led to a much smaller shift toward high-spin state for the haem iron (≈10% high spin) than that seen on testosterone binding (≫85% high spin). Erythromycin did significantly increase the rate of reduction of CYP3A4 over that seen in the absence of substrate, although both the amplitude and the rate were much less than those in the presence of testosterone (Table 1). The data could be satisfactorily fit by a single exponential, with a rate of reduction ≈125-fold lower than the fast phase in the presence of testosterone, but also 6-fold lower than the slow phase. Thus the biphasic kinetics in the presence of testosterone cannot simply be described as the sum of a ‘high-spin rate’ and ‘low-spin rate’.

**Effect of the CPR/CYP3A4 ratio on CYP3A4 reduction**

By empirically altering the amounts of CPR and CYP3A4 in the reconstitution mix and the incubation time before gel filtration, we were able to vary the mole ratio of CPR/CYP3A4 in the purified proteoliposomes over a 36-fold range and to determine the effects of this on the electron transfer. Qualitatively, the initial rate of reduction, corrected for differences in CYP3A4 concentration in the different samples, increased with the CPR/CYP3A4 ratio, reaching a maximum at a ratio of ≈1; however, the initial absorbance changes due to CPR reduction and to light scattering make it impossible to measure the initial rate accurately. Fitting the whole time-course, as shown for a 1:1 ratio in Figure 4(A) and for the other ratios in Supplementary Figure S1 (see http://www.BiochemJ.org/bj/432/bj4320485add.htm), provides a more accurate and mechanistically more informative analysis. At all CPR/CYP3A4 ratios, the data were best fitted by a double exponential, with the percentage of the absorbance change in the fast phase remaining constant within experimental error at 90 ± 3%; the derived rate constants and amplitudes are shown in Table 1. The rate constants of both the fast and slow phases were essentially unaffected by the molar ratio of CPR to cytochrome P450 in the proteoliposomes, with average values of 14 ± 2 s$^{-1}$ and 0.6 ± 0.06 s$^{-1}$ respectively. By contrast, the relative amplitudes (corrected for differences in CYP3A4 concentration in the different samples) of both the fast and the slow phases of reduction initially increase with increasing CPR/CYP3A4 ratios and reach an approximate plateau when the CPR is in excess over CYP3A4 (Table 1). Because of the initial decrease in absorbance due to the mixing artefact and the reduction of the flavins of CPR, the absolute amplitudes of the absorbance change due to cytochrome P450 reduction cannot be measured accurately. However, they are qualitatively consistent with the equilibrium measurements (see above) in indicating >75% reduction at [CPR]/[cytochrome P450] ≥ 1.

If the CPR–CYP3A4 complexes in the proteoliposomes are stable on the timescale of the electron transfer, then the rate would be independent of the CPR/CYP3A4 ratio, but the extent of reduction (the amplitude of the change in absorbance) would be expected to increase to a maximum as the CPR/CYP3A4 ratio increases, depending on the stoichiometry of the complex. This is indeed what we observe, and our results thus strongly suggest that, in these proteoliposomes, CPR and CYP3A4 exist in complexes with a 1:1 stoichiometry and with a lifetime of the order of 100 ms (≥1/k$_{\text{fast}}$).

We are not aware of published studies on the effects of changing the CPR/cytochrome P450 ratio on the rate of reduction of CYP3A4, but a number of reports have shown that with other cytochrome P450s, notably CYP2B4, in simple reconstituted systems the rate of the fast phase of reduction increases with increasing CPR/cytochrome P450 ratio, tending to approach a constant rate at CPR/cytochrome P450 ratios >1 [36,37]. There have, however, been other reports that changing the CPR/cytochrome P450 ratio changes the amplitude but not the rate of reduction [38]. The observations of increasing rates with increasing CPR/cytochrome P450 ratio led Taniguchi et al. [37] to propose that electron transfer took place by a lateral diffusion–collision mechanism and this has been widely accepted. By contrast, the behaviour reported here for purified proteoliposomes, where the rate of the first electron transfer is independent of the CPR/CYP3A4 ratio, is most easily interpreted in terms of a model in which electron transfer takes place within a relatively stable complex between the two proteins. However, the difference between our observations with CYP3A4 and those reported for CYP2B4 may simply reflect a higher affinity of CYP3A4 than CYP2B4 for CPR. Estimates of the dissociation constants of different cytochrome P450–CYP complexes cover a wide range [39], those for CYP2B4 and CYP3A4 being in the range 14–120 nM [14,38–40]. Not all of these estimates correspond to proteoliposomes of the kind studied here, but

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all are clearly consistent with relatively long lifetimes for the complex.

The results do not allow us to reach a firm conclusion regarding the nature of the small slow phase of reduction. As discussed above, this does not seem to represent reduction of low-spin haem, whereas the fact that its amplitude depends on the CPR/CYP3A4 ratio in just the same way as that of the fast phase would argue against a model in which the slow phase represented reduction of cytochrome P450 molecules outside the complex with CPR. Perhaps the most likely explanation is the existence of conformational heterogeneity in the cytochrome P450 [38,41–43].

**Effect of the CPR/CYP3A4 ratio on the rate of 6β-hydroxytestosterone formation**

The steady-state rates of NADPH consumption and of 6β-hydroxytestosterone formation were measured as a function of the CPR/CYP3A4 ratio in the proteoliposomes (Figure 5). At a 6:1 molar ratio of CPR/CYP3A4, the NADPH oxidation rate in the absence of substrate is 0.3 s⁻¹ and this increases by almost 4-fold, to 1.1 s⁻¹, in the presence of testosterone. This is consistent with the general observation that the spin-state shift induced by substrate binding is an important factor in the consumption of reducing equivalents by cytochrome P450s. The rate of 6β-hydroxytestosterone formation (0.01 s⁻¹ at 6:1 CPR/CYP3A4) is much less than the rate of NADPH consumption, reflecting the poor coupling of product formation to NADPH consumption which is characteristic of many mammalian cytochrome P450s. Overall, the rates of NADPH oxidation in the presence of testosterone and of testosterone turnover measured in the proteoliposomes are broadly similar to values reported for CYP3A4 in a variety of systems, including yeast membranes and SRs, where NADPH consumption ranges from 0.7 to 0.9 s⁻¹ and 6β-hydroxytestosterone formation ranges from 0.03 to 0.2 s⁻¹ [22,44,45].

In contrast with the observations on the first electron transfer described above, it is clear that the rate of NADPH consumption and the rate of 6β-hydroxytestosterone formation both increase with increasing CPR/CYP3A4 ratio (Figure 5). The increase can in each case be described adequately by a rectangular hyperbola, consistent with a Michaelis mechanism involving a fast equilibrium binding step followed by a slower electron transfer in the complex. Fitting the data leads to estimates (only approximate, because of the limited CPR concentration achieved in the proteoliposomes) of the apparent dissociation constant for the CPR–CYP3A4 complex of 0.37 ± 0.1 μM from NADPH consumption and 0.41 ± 0.3 μM from testosterone hydroxylation.

### Table 1 The effect of the CPR/CYP3A4 ratio in the proteoliposomes on the rate of reduction of CYP3A4

<table>
<thead>
<tr>
<th>CPR (μM)</th>
<th>CYP3A4 (μM)</th>
<th>CPR/P450 molar ratio</th>
<th>Fast-phase amplitude (ΔA/[P450])</th>
<th>Slow-phase amplitude (ΔA/[P450])</th>
<th>Percentage of P450 reduced in the fast phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.48</td>
<td>0.08</td>
<td>6.0</td>
<td>11.5 ± 0.6</td>
<td>0.14 ± 0.01</td>
<td>88</td>
</tr>
<tr>
<td>0.31</td>
<td>0.1</td>
<td>3.1</td>
<td>16.7 ± 0.4</td>
<td>0.10 ± 0.005</td>
<td>87</td>
</tr>
<tr>
<td>0.1</td>
<td>0.1</td>
<td>1.0</td>
<td>12.4 ± 0.3</td>
<td>0.10 ± 0.01</td>
<td>77</td>
</tr>
<tr>
<td>0.08</td>
<td>0.24</td>
<td>0.33</td>
<td>12.9 ± 0.3</td>
<td>0.05 ± 0.003</td>
<td>95</td>
</tr>
<tr>
<td>0.09</td>
<td>0.55</td>
<td>0.16</td>
<td>14.9 ± 0.4</td>
<td>0.03 ± 0.008</td>
<td>88</td>
</tr>
<tr>
<td>Without testosterone</td>
<td></td>
<td></td>
<td>0.01 ± 0.008</td>
<td>0.0015</td>
<td>100</td>
</tr>
<tr>
<td>Erythromycin in place of testosterone</td>
<td>0.08</td>
<td>1:1</td>
<td>0.10 ± 0.003</td>
<td>0.0013</td>
<td>100</td>
</tr>
</tbody>
</table>

### Figure 5 Effect of the CPR/CYP3A4 ratio on the rates of NADPH consumption and 6β-hydroxytestosterone formation

The rates of (A) NADPH oxidation and (B) 6β-hydroxytestosterone formation are shown as a function of the CPR/CYP3A4 ratio in the proteoliposomes. In each case the lines are the best (least-squares) fit by a rectangular hyperbola, yielding $K_d$ (app) values of 0.37 ± 0.1 μM (A) and 0.41 ± 0.3 μM (B) respectively.

The hydroxylation reaction requires the transfer of both electrons from CPR to CYP3A4 (Figure 1, steps 2 and 4), and of course the consumption of NADPH will also reflect both electron transfers. The ferrous dioxygen complex of CYP3A4 is very unstable, even in the presence of substrate [46,47], making it difficult to measure the rate of the second electron transfer directly, although Zhang et al. [47] have been able to measure the rate of electron transfer to oxyferrous CYP2B4 using 5-deazaFAD T491V CPR. For CYP3A4, the relative rates of the first electron
transfer and of testosterone hydroxylation indicate that it must be a step after the first electron transfer whose rate increases with increasing CPR/CYP3A4 ratio, and their very different dependence on the CPR/CYP3A4 ratio (Table 1 and Figure 5) suggest that it is the second electron transfer, step 4. This in turn suggests that the second electron transfer may indeed, as often assumed, take place by a collisional mechanism involving lateral diffusion of CPR and CYP3A4 in the plane of membrane.

The different observations for the first and second electron transfer can be reconciled by considering the different timescales of the two processes. In a nanodisc system containing a homogeneous 1:1 complex of CPR and CYP3A4, where the bilayer disks are very small so that the possibility for the two proteins to diffuse apart is very limited, a rate of testosterone hydroxylation of 0.36 s$^{-1}$ was observed [29], which may be taken as an approximate estimate for the rate of the second electron transfer in the complex. This is significantly lower than the rate of the first electron transfer, measured here as 14 s$^{-1}$. Thus, if the lifetime of the CPR–CYP3A4 complex was of the order of 200 ms, the fast first electron transfer would take place within the complex, but on the timescale of the very slow second electron transfer, the complex would be able to dissociate. In contrast with the nanodiscic system, the proteins are ‘dilute’ in our proteoliposome system (a measured lipid/protein ratio of 1000:1 in the proteoliposomes compared with 100:1 in the nanodiscs [29]), and on dissociation of the complex the two proteins could diffuse laterally in the membrane, thus showing the characteristics of a collisional mechanism for the second electron transfer. It is important to emphasize that our results do not imply that dissociation of the complex is required for the second electron transfer, simply that dissociation would be possible before the second electron transfer occurs.

If, as our results indicate, in the proteoliposomes the first electron transfer takes place within the CPR–CYP3A4 complex, what determines the rate of this process? The structure of the complex, and hence the distance between the FMN of CPR and the haem of CYP3A4, is unknown, but the rate of 14 s$^{-1}$ is orders of magnitude lower than would be expected for electron transfer in a complex where the electron donor and acceptor are in close proximity (as discussed for the FAD–FMN electron transfer in CPR [9,10,31]). There is good evidence from a wide range of methods for the existence of domain motion in CPR and for the involvement of this in interflavin electron transfer [8,9,11,48,49]. Estimates of the rate of this motion [9,10] are similar to the rate of the first electron transfer reported here (although the experimental conditions are different), suggesting that domain motions in CPR, involving changes in domain positions from that appropriate for interflavin electron transfer to that appropriate for FMN to haem electron transfer, may contribute to the rate-limiting step in the first CPR–CYP3A4 electron transfer.

**AUTHOR CONTRIBUTION**
Gordon Roberts and Yassar Farooq designed the experiments and Yassar Farooq carried out the experiments. Gordon Roberts and Yassar Farooq wrote the paper.

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1 Stewart, J. C. (1980) Colorimetric determination of phospholipids with ammonium

0 Stewart, J. C. (1980) Colorimetric determination of phospholipids with ammonium
SUPPLEMENTARY ONLINE DATA
Kinetics of electron transfer between NADPH-cytochrome P450 reductase and cytochrome P450 3A4

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Figure S1  Kinetics of reduction of ferric CYP3A4 in proteoliposomes at different ratios of [CPR]/[P450]

Proteoliposomes containing CPR and CYP3A4 at the concentrations indicated plus 0.5 mM testosterone were mixed with the same volume of 0.4 mM NADPH plus 0.5 mM testosterone in a CO atmosphere in a stopped-flow spectrophotometer at 25 °C. CYP3A4 reduction was monitored at 448 nm by formation of the Fe(II)–CO complex. The fit by a double exponential is shown as the smooth line. The concentrations and the parameters of the double exponential fits are given in Table 1 of the main text. (A) CPR, 0.09 μM; CYP3A4, 0.55 μM. (B) CPR, 0.08 μM; CYP3A4, 0.24 μM. (C) CPR, 0.31 μM; CYP3A4, 0.1 μM. (D) CPR, 0.48 μM; CYP3A4, 0.08 μM.

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