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Functional reconstitution of monomeric CYP3A4 with multiple cytochrome P450 reductase molecules in Nanodiscs

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Abstract

Traditional reconstitution of membrane cytochromes P450 monooxygenase system requires efficient solubilization of both P450 heme enzymes and redox partner NADPH dependent reductase, CPR, either in mixed micellar solution or by incorporation in liposomes. Here we describe a simple alternative approach to assembly of soluble complexes of monomeric human hepatic cytochrome P450 CYP3A4 with CPR by co-incorporation into nanoscale POPC bilayer Nanodiscs. Stable and fully functional complexes with different CPR:CYP3A4 stoichiometric ratios are formed within several minutes after addition of the full-length CPR to the solution of CYP3A4 preassembled into POPC Nanodiscs at 37 °C. We find that the steady state rates of NADPH oxidation and testosterone hydroxylation strongly depend on CPR:CYP3A4 ratio and reach maximum at tenfold molar excess of CPR. The binding of CPR to CYP3A4 in Nanodiscs is tight, such that complexes with different stoichiometry can be separated by size exclusion chromatography. Reconstitution systems based on the co-incorporation of CPR into preformed Nanodiscs with different human cytochromes P450 are suitable for high-throughput screening of substrates and inhibitors and for drug-drug interaction studies.

1. Introduction

The cytochromes P450 constitute a large superfamily of heme monooxygenases, which catalyze various chemical transformations using atmospheric oxygen and redox equivalents from NADH or NADPH. Two one-electron reductions of the heme iron of cytochrome P450 are performed by the protein redox partner. In most cases it is either a soluble iron-sulfur protein ferredoxin, or a membrane incorporated flavoprotein, NADPH dependent cytochrome P450 reductase (CPR) [1]. CPR isozymes from rabbit and rat are highly homologous to human CPR and can support multiple mammalian cytochromes P450 with similar efficiency [2–3]. Traditionally, the functional reconstitution of mammalian P450 monooxygenase systems is based on solubilization of purified heme enzymes and their associated reductases in the presence of detergents and lipids [3–4], or co-incorporation of the component membrane proteins into liposomes [5]. In both cases, the oligomerization state of the cytochrome P450 and its redox partner cannot be efficiently controlled [4]. Other problems with these systems may emerge. For instance, the presence of excess detergent can

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destabilize P450 heme proteins and inhibit substrate binding [4, 6], while in the absence of detergents the stoichiometry of a reductase-P450 complexes is difficult to evaluate. Even knowing the overall molar ratio does not allow one to easily distinguish between 1:1, 2:2, 3:3 complexes or mixtures of different stoichiometries such as 1:2 and 2:1. In addition, the reduction of monomeric cytochromes P450 is not complicated by heterogeneity of equilibrium and kinetic properties caused by oligomerization of the cytochromes [4, 7–9].

The recent development of Nanodiscs enabled the successful application of this technology to numerous biochemical and biophysical studies of membrane proteins, including cytochromes P450 [10–11]. Incorporation of monomeric cytochromes into POPC lipid bilayer results in highly active samples, which are stable for many weeks if stored at 4°C and can be safely frozen as 20% glycerol stock solutions. A self-assembled 1:1 complex of CPR and CYP3A4 incorporated into Nanodiscs has been prepared and used for the detailed analysis of functional cooperativity of CYP3A4 [12]. However, such reconstitution of two different proteins into Nanodisc at a precise 1:1 ratio requires the sequential application of two affinity columns which results in a concomitant loss of material during assembly and purification. In addition, the activity of the CPR-CYP3A4 complex in Nanodiscs gradually decreases over time, making impractical storage of an active complex. The 1:1 complex described in [12] also did not reach the levels of activity seen in some microsomal samples where the possibility of higher stoichiometric ratios of components exists.

In this communication we describe an alternative approach for the functional reconstitution of CPR-3A4 complexes in Nanodiscs, and document activity at higher CPR:CYP3A4 ratios. These results provide a means for easily establishing a functional bioreactor for the investigation of the rate and equilibrium processes of P450 metabolism. Full-length CPR with its intact N-terminal membrane-binding domain is added to preformed Nanodiscs with monomeric CYP3A4. As a result, a highly active reconstituted system can be quickly prepared with a high yield, as demonstrated by the rates of NADPH oxidation and testosterone hydroxylation. Using this system we document formation of the active complex between monomeric CYP3A4 and CPR at different stoichiometric ratios, and show that the steady state turnover in this system is significantly faster in the presence of 6 – 8 fold molar excess of reductase. This suggests an efficient intermolecular electron transfer between FAD and FMN in CPR oligomers, similar to the recently evaluated pathway in CYP102 dimers [13]. As shown by X-ray crystallography [14], such oligomers can be formed by two or more CPR monomers in an open conformation, where FAD and FMN domains from different reductase molecules come into contact to form the monomer-monomer interface.

2. Materials and methods

2.1 Protein expression and Nanodisc assembly

Expression and purification of MSP, CYP 3A4 and rat CPR, as well as preparation of CYP3A4 in POPC Nanodiscs were described previously [12, 15–16]. CYP3A4 was expressed from the NF-14 construct in the PCWori⁺ vector with a C-terminal pentahistidine tag generously provided by F.P. Guengerich and co-workers [17]. Expression, purification from *E. coli*, and incorporation of CYP3A4 in POPC Nanodiscs followed protocols described in detail in the Supplemental material of reference [18].

2.2 NADPH oxidation and TST metabolism

One ml of CYP3A4-ND solution in 0.1 M HEPES buffer pH 7.4 containing 7.5 mM MgCl_2 and 250 μM testosterone was brought 37 °C in a stirred quartz cuvette. After addition of CPR, the sample was incubated for 5–6 min before the reaction was initiated by addition of 13 μl of 20 mM NADPH. The absorption at 340 nm was monitored for 5 min, and the reaction was quenched by addition of 12 μl of 9 M sulfuric acid to bring the pH below 4.0. The sample was removed from the cuvette, frozen by immersion in liquid nitrogen, and stored at –80° C for product analysis. Optical measurements were performed on Hitachi U-3300 spectrophotometer supplied with temperature controller and built-in magnetic stirrer. In the experiments where CYP3A4 or CPR concentration exceeded 100 nM or 450 nM correspondingly, a three-fold higher concentration of NADPH was used with a path length of 0.4 cm rather than 1 cm. The rate of NADPH oxidation was determined from the slope of absorption at 340 nm during the first two minutes using an extinction coefficient of $6.22 \text{ cm}^{-1}\text{mM}^{-1}$.

Analysis of TST metabolites was performed as described elsewhere [16, 19] with minor modifications. Briefly, 1.5 ml of CH_2Cl_2 and 25 μl of 90 μM cortexolone solution in methanol were added as internal standard to 0.5 ml of the sample solution and thoroughly mixed. After separation, the organic phase was isolated and the solvent was removed under a stream of nitrogen. The dried sample was dissolved in 70 μl of methanol and 40 μl was injected onto C_{18} HPLC column, $2.1 \times 150 \text{ mm}$, 4 μm (Waters Nova-Pak) with the mobile phase of 30% acetonitrile and 10 mM ammonium acetate in water and a flow rate of 0.4 ml/min. Products of testosterone hydroxylation were separated in a linear gradient of acetonitrile from 30% to 70% over 25 min. Peak integration was performed with GRAMS/32 software (Thermo Fisher Scientific).

2.3 SEC and spectral analysis

Samples were prepared by mixing of 1 μM solution of CYP3A4-ND with various amounts of concentrated CPR to achieve the desired ratio. Mixtures were incubated at 37 °C for 10 minutes and cooled to ambient temperature before SEC. The injection volume was 430 μl for all the samples, with chromatography using a Superdex 200 column at a 0.5 ml/min flow rate on a Waters (Milford, MA) HPLC system equipped with a photodiode array detector. The column was calibrated with a mixture of four proteins with the following retention times: 18.9 min (thyroglobulin), 21.4 min (ferritin), 25.1 min (catalase), and 27.6 min (bovine serum albumin). CYP3A4-ND sample eluted at 24.5 min and CPR at 18.6 min. A hydrodynamic diameter of CPR oligomers of 17.5 nm was estimated from size exclusion chromatography and is in agreement with earlier reported sizes and molecular masses of CPR oligomers [4, 20–22].

3. Results and discussion

We have previously described a method for co-incorporating CYP3A4 and CPR into Nanodiscs at 1:1 ratio that results in a highly active complex of monomeric microsomal cytochrome P450 [12]. However, this approach requires generating a large excess of proteins incorporated in Nanodiscs, with low overall yield. A demanding multi-step

purification protocol is required to separate the Nanodiscs with the correct stoichiometry. As a result, the yield of CPR-CYP3A4 complex in Nanodiscs is relatively low, making high-throughput studies of turnover difficult. This is in contrast to the incorporation of monomeric CYP3A4 alone into Nanodisc, which is straightforward process with highly reproducible results and good overall yield [16, 23]. Here we describe a simple path to a functionally active reconstituted system by directly incorporating CPR into a preformed population of CYP3A4 Nanodiscs. This extends the previous discovery [22] that full-length CPR, with its intact N-terminal transmembrane helical fragment, can directly partition into the membrane bilayer.

In order to monitor the incorporation of CPR, we observed changes in the rate of steady-state NADPH oxidation upon mixing solutions of reductase and CYP3A4-ND in the presence of NADPH. The result of the typical experiment is shown in Figure 1a. Before addition of CYP3A4-ND, the NADPH is consumed relatively slowly, with a rate of 4 nmol/min/nmol CPR. Upon addition of CYP3A4-ND, the rate significantly increases with a lag period of about two minutes before the NADPH consumption becomes linear. If the mixture of the CYP3A4-ND and CPR is incubated for 4–8 min before addition of NADPH, the reaction progress is linear from the start (Fig 1b). This result indicates that CPR incorporation in the POPC bilayer is not instantaneous, but is completed in several minutes under the condition used in the experiment.

The analysis of testosterone metabolites revealed a high activity, with both product formation and NADPH consumption rates strongly depending on the amount of CPR added to the reaction (Figure 2). It has been shown previously [24] that the CPR membrane anchor is necessary for a high level of CYP450 activity in reconstituted systems. The same requirement is observed for CYP3A4 in Nanodiscs. When trypsin-treated CPR was used in control experiments, no CYP3A4 activity was detected, although CPR maintained its ability to reduce cytochrome c in solution.

When the ratio CPR:CYP3A4 is fixed, the activity measurements are reproducible with a typical standard deviation of ~4% in NADPH measurements and ~5% in product formation, even when different absolute concentrations of the two partners are used. As shown in Figure 2, the observed activity depends critically on the relative ratio of CPR to CYP3A4, indicating the tight binding of CPR to CYP3A4-ND with an estimated dissociation constant in the range of 10 – 50 nM. This is similar to those reported earlier for CYP3A4 [25] and other mammalian cytochromes P450 [26]. As the CPR:CYP3A4 molar ratio increases, the activity also increases, reaching a saturation at ~10:1 molar ratio. The overall coupling of reducing equivalents to product (i.e. the fraction of NADPH spent on a productive pathway) was estimated as ~4% at a CPR:CYP3A4 ratio of 1:10, gradually increasing to ~13 % at the highest ratio used in these experiments (10:1). Again, this is consistent with observations made in microsomal preparations.

In order to compare the composition of the complexes formed by monomeric CYP3A4 in Nanodiscs at different CPR:CYP3A4 ratios, a set of samples were prepared at higher concentrations and analyzed by size-exclusion chromatography. Spectral deconvolution of the chromatograms allowed calculating the separate elution profiles of CYP3A4 and CPR.

Figure 3 shows the concentration profiles of CYP3A4 (A) and CPR (B). At a 1:1 ratio, CYP3A4 is distributed between two bands, with about 60% corresponding to CYP3A4-ND with no reductase incorporated, and the remainder in a 1:1 complex of CYP3A4 and CPR. At a 2:1 overall ratio, most of the 3A4 is involved in the 1:1 complex, with smaller fractions of Nanodiscs with no reductase and or with more than one CPR.

As the ratio of CPR:CYP3A4 increases, the fraction of CYP3A4-ND without CPR disappears, and larger complexes with an increasing amount of CPR per CYP3A4-ND are formed. At the same time, it is clear that a small fraction of CPR remains in soluble aggregates and elutes significantly earlier than that associated with Nanodiscs, and is not involved in the complex even when low ratios 1:1 and 2:1 are used. In order to examine if this small amount of associated reductase changes the functionality of the complex, we repeated the experiment on a larger scale using CPR:3A4 ratios of 2:1 and 10:1, and purified the samples on an Ni-NTA column utilizing the penta-histidine tag on CYP3A4 to remove excess of CPR not incorporated in CYP3A4 Nanodiscs. The samples were analyzed by size exclusion chromatography, spectroscopically and for product forming activity. When a 2:1 ratio was used, the SEC result is similar to the experiment executed without additional purification. The CPR:CYP3A4 overall ratio determined from spectral deconvolution in the main band was ~1.5, with the absolute rates of NADPH oxidation and product formation found to be 220 and 8.5 nmol/min per nmol of CYP3A4, respectively. A second sample, prepared at 10:1 starting ratio, appeared remarkably constant in composition across the elution peak with a CPR to CYP3A4 ratio of ~7.6, NADPH oxidation rates of 415 nmol/min per nmol of CYP3A4 and testosterone hydroxylation of 30.2 nmol/min per nmol of CYP3A4.

In conclusion, we have described new results obtained using a functional reconstitution of monomeric CYP3A4 co-incorporated in Nanodiscs with its full-length redox partner CPR at different stoichiometric ratios. Simple mixing of two solutions followed by incubation for 5 – 10 minutes results in facile co-incorporation of CPR in Nanodiscs with CYP3A4 and formation of fully functional enzymatic complex with high activity as shown by steady-state kinetics of NADPH oxidation and testosterone hydroxylation. The interaction between CPR and CYP3A4 is tight, with an apparent dissociation constant of ~50 nM. Interestingly, catalytic activity of CYP3A4 strongly depends on the ratio of CPR and CYP3A4 concentrations, indicating the possibility of multiple pathways of reducing electron transfers within CPR oligomers interacting with one CYP3A4. Indeed, an electron transfer from FAD in one CPR molecule to FMN in different molecule may occur in dimers formed by CPR molecules [14, 27] although there is no indication of a biological significance of such mechanism for mammalian membrane bound reductases. Interestingly, the intermolecular electron transfer between FAD and FMN domains of two CYP102 molecules in the fully functional dimer have been described recently as a dominant pathway for this cytochrome P450 [13, 28]. Our results show a much faster testosterone hydroxylation by CYP3A in the presence of excess CPR, consistent with the same observations documented for Baculosomes and reconstituted systems in which CPR:CYP3A4 ratio is as high as 8:1 [3, 29]. Our system may offer significant advantages over the Invitrogen Baculosome system for the drug metabolism investigations. Although the concentration of CPR in hepatic microsomes is significantly lower than total P450 concentration, the system with higher

CPR stoichiometry described in this communication is useful for *in vitro* measurements, particularly in high-throughput screening which may involve multiple purified P450 enzymes. In such cases, the heme protein availability is often a limiting factor, and using excess CPR may be the only easy means to optimize the rates of product formation, and ultimately the sensitivity, of the assay.

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Abbreviations

CYP3A4-ND	cytochrome P450 CYP3A4 in Nanodiscs
CPR	cytochrome P450 reductase
POPC	palmitoylcholinephosphatidylcholine
MSP	membrane scaffold protein
TST	testosterone
SEC	size exclusion chromatography

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Figure 1.

(A) Kinetics of NADPH oxidation by CPR at T = 37°C before and after addition of CYP3A4-ND, shown by the arrow. Concentrations used: CPR, 0.35 μM; CYP3A4-ND, 0.072 μM; NADPH, 240 μM; MgCl₂, 7.5 mM; testosterone, 250 μM. (B) Kinetics of NADPH oxidation at different CPR concentrations, preincubated for 5 minutes with 0.075 μM CYP3A4-ND. Concentrations of CPR are: (1) 0.11 μM, (2) 0.29 μM, (3) 0.425 μM, (4) 0.64 μM, (5) 1.05 μM, (6) 1.6 μM. All other conditions are the same as above.

Figure 2.

Rates of NADPH oxidation (A) and testosterone hydroxylation (B) as a function of the CPR:CYP3A4 ratio. Closed symbols show the data obtained using 0.072 μM CYP3A4-ND and CPR concentrations from 0.11 μM to 2.77 μM . Open symbols represent the data obtained at a constant CPR concentration 0.43 μM , and various CYP3A4-ND concentrations from 0.01 μM to 0.42 μM .

Figure 3.

Size-exclusion chromatography of CPR-CYP3A4-ND complexes prepared at different CPR:CYP3A4 overall molar ratios. Panel A shows the distribution of CYP3A4 in elution profiles for pure CPR (dashed line) and for CPR:CYP3A4 at overall molar ratios 1, 2, 3, 4, 6, and 10. Panel B documents the distribution of CYP3A4 in elution profiles of CYP3A4-ND with no CPR (dashed line) and the same samples of CPR:CYP3A4 as in panel A. The distributions of CPR and CYP3A4 in the elution profiles were deconvoluted from experimental profiles monitored at multiple wavelengths using reference spectra of pure species as described in Materials and Methods.