Experimental Report

Proposal: **8-05-413** **Council:** 10/2012

Title: Metabolon Investigation: The Binding of UDP-glucosyltransferase

This proposal is a new proposal

Researh Area: Other...

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Samples: Nanodisc
Nanodisc with cytochrome P450 Reductase
Nanodisc with CYP79A1
Nanodisc with CYP71E1

Instrument	Req. Days	All. Days	From	To
D17	3	3	30/04/2013	03/05/2013

Abstract:

Membrane proteins (MPs) continue being an elusive target for research due to the challenges regarding isolation and preservation of their native states. These problems can be overcome by immobilizing MPs in so-called nanodics, which can be perceived as stabilized pathces of lipids. In our experiment proposal we seek to clarify the exact binding position of UDP-gluosyltransferase that participates in a metabolon comprised of multiple enzymes of the cytochrome P450 (CYP) superfamily in Sorghum Bicolor: cytochrome P450 reductase (CPR) and two cytochromes P450, CYP79A1 and CYP71E1. UGT85B1 is believed to bind to CYP71E1, hence the key element in our proposal is to investigate whether and where the UGT85B1 displays affinity. Furthermore, the effect of lipid phase is to be examined by varying temperature from 15 to 30 degrees celsius. In total, the overall strategy revolves around CPR, CYP79A1 and CYP71E1 captured in nanodics and utilizing neutron reflectometry to detect changes in the density of the different sublayers in the adsorbed layer.

Experimental Report for Experiment Nr 8-05-413

Metabolon Investigation: The Binding of UDP-glucosyltransferase

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In this experiment we studied a series of proteins that continue to be the most elusive in nature: membrane bound proteins. We aim at understanding the functional and structural characteristics behind association of multiple enzymes of the cytochrome P450 (CYP) superfamily from the subtropical crop plant Sorghum bicolor. The dhurrin pathway includes four enzymes, which convert tyrosine to an aldoxime, mediated by CYP79A1. The aldoxime is further converted into a hydroxynitrile, mediated by CYP71E1, which is finally glycosylated by UGT85B1 to form the product dhurrin. These enzymes are believed to form an enzymatic complex (metabolon), where the substrate, tyrosine, is highly channeled towards the product, dhurrin, a cyanogenic glucoside[1]. This model system includes a membrane bound cy- tochrome P450 reductase (CPR), two cytochromes P450 (CYPs) CYP79A1 and CYP71E1, and a soluble UDP-glucosyltransferase (UGT85B1). CPR acts as an electron donor in a NADPH-dependent manner to all microsomal CYPs and therefore has to navigate in the membrane in order associate with specific partner enzymes. Organization of pathways is believed to be a common feature in the production of bio-active natural products in all plants. Therefore, insight into the organization of this model system is of great interest, but has proved challenging as the associations are transient in nature.

Neutron reflectometry was used to characterize the averaged properties of an adsorbed membrane bound protein integrated in nanodiscs films. In this way, we have shown earlier that we can extract the conformational equilibrium between the open and closed conformations of CPR in nanodiscs[2]. In this experiment we continued to characterize the factors that affect the equilibrium conformation of CPR. We know that NADPH changes the conformation equilibrium from ”open” to ”closed” states. In order to clarify whether these changes are due to actual binding of NADPH or to changes in the redox state of the protein, we performed experiments in which we used $NADP^+$, which binds to CPR without inducing a change in oxidation state at all, or DTT, a reducing agent that does not bind to CPR. We also investigated the effect of temperature-induced phase transitions of the nanodisc lipid bilayer on the CPR conformation by increasing the temperature from 15 to 38 $^\circ$C the lipid bilayer (DMPC) undergoes the gel to fluid phase transition[3]. Finally, we also used two so far unexplored membrane bound proteins the CYP partners of the dhurrin metabolon. We note that in these experiments we have shifted from a small to a larger belt, since the incorportation of the membrane proteins is more efficient in the latter ones.

We found that increasing the temperature from 15 to 38 $^\circ$C induced irreversible changes in the structure of the film, suggesting a change in conformation of the nanodiscs structure regardless of the presence of the CPR (Fig 1A,B). However, in this case we did not see a change in equilibrium conformation for the CPR as observed earlier (Fig 1C). The sample preparation for CPR nanodiscs is quite cumbersome and time consuming, and requires the proper ratio of cofactors per CPR protein to ensure full functionality. This particular sample contained a particularly low cofactor/CPR ratio, but we decided to use the protein as we did not have time to prepare new samples on time for this beam time. We performed however the controls for DTT (Fig 1D), which shows no effect on the empty nanodiscs at all. From previous experiments at ISIS, we know that NADPH does not bind to empty nanodiscs and that NADP+ binds unspecifically both to empty and CPR loaded nanodiscs. NADP+ does not bind to the silica surface so this effect comes from binding with the either

the lipids or the belt protein. Thus, we will need to apply for new beam time with fully functional CPR nanodiscs to complete the measurements with DTT and the full characterization of the effect of NADPH.

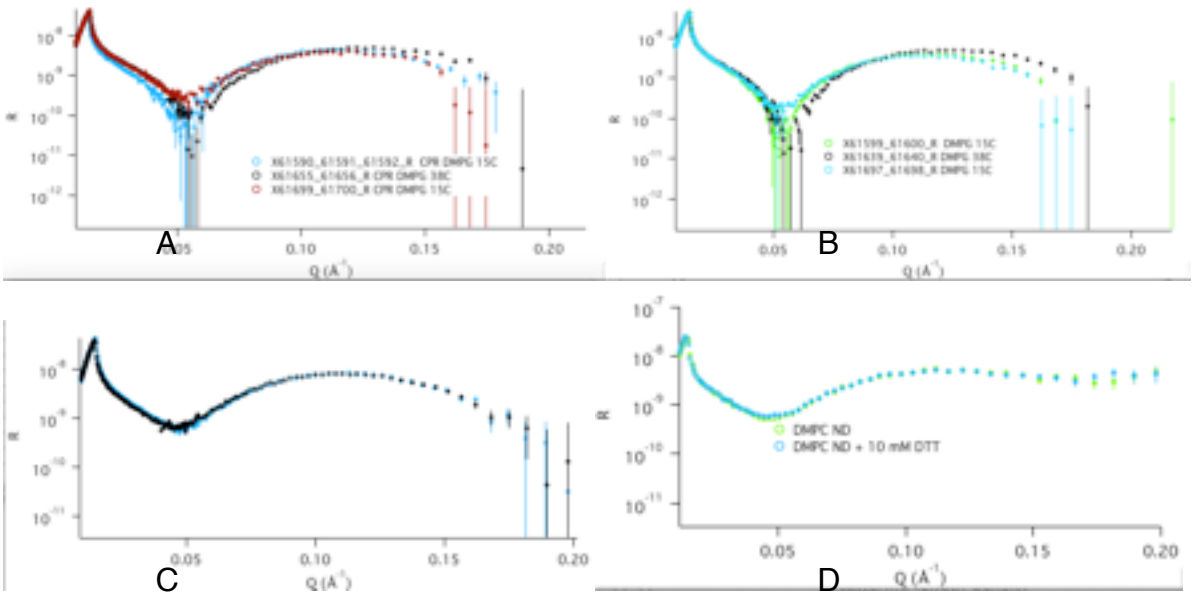

Figure 1. NR profiles for nanodiscs films made of DMPG lipids with (A) and without (B) CPR below and above the melting of the DMPG lipids. The figure also shows the effect of NADPH on the CPR in DMPC nanodiscs (C) and the effect of DTT presence of DMPC nanodiscs (D).

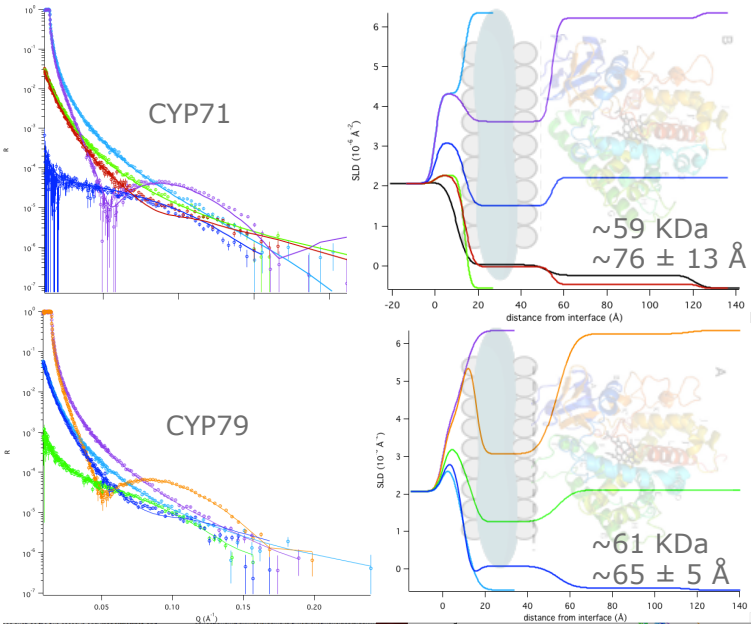

Figure 2. NR and SLD profiles for CYP71 and CYP79 nanodiscs films.

We then turned our attention to the other partners of the Dhurrin metabolon, the CYP71 and CYP79 reconstituted in DMPC nanodiscs adsorbed at 15 °C and using the large protein belt. Figure 2 shows that the protein layer thickness is different for these two proteins, even though they have similar molecular weights. Their predicted structure, based on homology modeling as compared to human P450, suggests that CYP71 lacks a random hydrophobic loop between the F' and G helixes (at the interface with the lipid bilayer), that is otherwise present for CYP79. Moreover, CYP71 contains an extra alpha helix in the C terminal. QCM-D studies indicate significantly higher frequency and dissipation changes for CYP71 nanodiscs films than CYP79. Together QCMD and NR strongly suggest that the CYP71 is less strongly bound to the lipid membrane and this correlates well with its less specificity for substrates. We are currently preparing a manuscript to cover these latter results.

References: 1) Phytochemistry, 72(17):2113–2123, 2011; 2) JBC 2012, **287**, 34596-34603; 3) Soft Matter 2013, **9**, 2329 – 2337