Membrane Proteins

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Reconstitution of the Cytb_5–CytP450 Complex in Nanodiscs for Structural Studies using NMR Spectroscopy

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Abstract: *Cytochrome P450s (P450s) are a superfamily of enzymes responsible for the catalysis of a wide range of substrates. Dynamic interactions between full-length membrane-bound P450 and its redox partner cytochrome b_5 (cytb_5) have been found to be important for the enzymatic activity of P450. However, the stability of the circa 70 kDa membrane-bound complex in model membranes renders high-resolution structural NMR studies particularly difficult. To overcome these challenges, reconstitution of the P450–cytb_5 complex in peptide-based nanodiscs, containing no detergents, has been demonstrated, which are characterized by size exclusion chromatography and NMR spectroscopy. In addition, NMR experiments are used to identify the binding interface of the P450–cytb_5 complex in the nanodisc. This is the first successful demonstration of a protein–protein complex in a nanodisc using NMR structural studies and should be useful to obtain valuable structural information on membrane-bound protein complexes.*

Cytochrome P450s (P450s) are a ubiquitous superfamily of monooxygenases found in all living kingdoms, including plants, animals, bacteria, and fungi.[1] P450s are responsible for the metabolism of a wide variety of endogenous and exogenous substrates, including over 70% of the pharmaceuticals in the current market.[1] For P450s to carry out their function, two electrons are required to be sequentially delivered to the heme of P450s from their redox partners, namely cytochrome b_5 (cytb_5) and cytochrome P450 reductase (CPR).[2] Mammalian P450s and redox partners (CPR and cytb_5), consisting of a soluble domain and a single α-helical transmembrane (TM) domain, are primarily found on the cytoplasmic side of the endoplasmic reticulum (ER) membrane of hepatic cells.[1] Cytb_5 has been reported to stimulate, inhibit, or have no effect on the activities of P450s depending on various factors, including the relative amount of cytb_5 versus CPR, the particular substrate under investigation, and other experimental conditions.[3] Even though it is known that only the full-length membrane-bound P450 and cytb_5 form a productive complex, the vast majority of crystallographic studies have focused only on the structures of the soluble domains of P450s. Although it is necessary to characterize the dynamic interactions between cytb_5 and P450 at high resolution in a membrane environment, the size and stability of the systems pose tremendous challenges.

Our previous study suggested that bicelles composed of lipids and detergents better facilitate interactions between P450 and cytb_5, as compared to detergent micelles or a non-membrane environment.[4] Additionally, detergents were found to partially unfold the α-helices of P450 2B4 (CYP2B4), as well as convert P450 into an inactive form (P420).[4] Therefore, the elimination of detergents from the membrane mimetic used for structural and functional studies of P450, and its interactions with cytb_5, becomes necessary to provide a more physiological environment for P450.

Nanodiscs, composed of a planar lipid bilayer and membrane scaffold proteins (MSPs) forming the rim, have been widely recognized as a promising membrane mimetic for studying membrane proteins including P450s.[5,6] This disc-shaped nanoscale membrane model is well-defined in size and demonstrates a high stability for reconstituted membrane proteins whose activities are deteriorated by the detergents present in other membrane mimetics, such as micelles.[7] However, the use of nanodiscs to investigate protein–protein interactions is limited by the sample preparation process and difficulties in the reconstitution of protein–protein complexes with both proteins properly oriented with respect to each other. Only a few examples of studies on membrane protein complexes in nanodiscs have been reported to date.[8–15]

In this study, we demonstrate the first successful incorporation of the CYP2B4–cytb_5 complex into a peptide-based nanodisc consisting of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) lipids. In this system, a 22 residue peptide is used in place of large MSPs to form the rim of the nanodiscs. The procedure to prepare peptide-based nanodiscs is simple and it was easy to control the size of resultant nanodiscs.[16,17] Using size exclusion chromatography (SEC) and NMR experiments, we have characterized the effect of the lipid bilayer on the aggregation of full-length cytb_5 and the reconstitution of productive CYP2B4–cytb_5 complexes into nanodiscs. We believe that this study paves the way to a better understanding of the structure and function of membrane

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protein complexes in a more physiologically relevant lipid membrane environment.

Lipid-bilayer-containing nanodiscs were prepared using an amphipathic 22-residue peptide (PVLDLFRELLNELLEALKQKLK).[16–18] This peptide was designed based on the helical structure and the consensus amino acid sequence of the amphipathic α-helical segments in the apolipoprotein A-I (Apo A-I);[17] previous studies have used Apo A-I mutants as membrane scaffold proteins for nanodisc reconstitution.[5] Application of this 22-residue peptide (referred to as 22A hereafter) allows a greatly simplified preparation protocol for nanodiscs and easy control of the size of the nanodiscs simply through the variation of 22A:lipid molar ratio.[16,19,20] Incorporation of a lipid bilayer in the 22A-nanodisc resulted in a single peak on the SEC elution profile (Figure 1, dotted line), suggesting high homogeneity in the size distribution of the nanodiscs. Dissociation of cytb_5 aggregates, as revealed by SEC (see Figure S1 in the Supporting Information) and NMR spectroscopy (Figure S2), was achieved through successful reconstitution of cytb_5 into 22A-nanodiscs (Figure 1, dashed line).

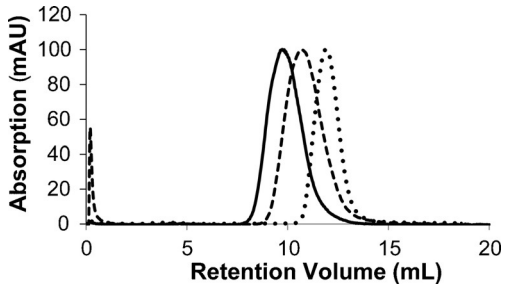

Figure 1. Size exclusion chromatography. Elution profiles of empty 22A-nanodiscs (dotted line), nanodiscs containing cytb_5 after purification (dashed line), and nanodiscs containing CYP2B4–cytb_5 after purification (solid line).

In an effort to incorporate the CYP2B4–cytb_5 complex into the 22A-nanodisc, a variety of sample preparation methods were screened, among which two procedures (method 1 and method 2, reported in the Supporting Information) led to the successful incorporation of both proteins into nanodiscs, as suggested by their SEC elution profiles (Figures 1 (solid line); Figure S3). The nanodiscs containing both proteins were stable for more than a week at room temperature, as revealed by no precipitation and a reproducible SEC profile after 10 days (Figure S3 B). In contrast, CYP2B4–cytb_5 complexes incorporated into isotropic bicelles, which consist of lipids and detergents at a 1:4 molar ratio, are only stable for around 3 days (Figure S3 C), after which the sample starts to precipitate. The higher stability of the protein complex achieved by the use of nanodiscs compared to isotropic bicelles is likely the result of the complete elimination of detergents throughout the sample preparation process. The presence of detergents not only disturbs the overall folding of CYP2B4 but also hampers the activity of the protein.[4] Although both sample preparation methods resulted in identical SEC elution profiles suggesting successful reconstitution of both proteins into the nanodiscs, it is important to distinguish between the following two potential protein arrangements: 1) both CYP2B4 and cytb_5 are anchored on the same side of the nanodisc, and a productive complex is formed between the two proteins; 2) CYP2B4 and cytb_5 are reconstituted to opposite sides of the nanodiscs, which hampers the formation of the CYP2B4–cytb_5 complex.

To verify the nature of the CYP2B4–cytb_5 complex formed in a nanodisc, 2D ^{15}N/^{1}H TROSY HSQC spectra were recorded for the samples prepared by both methods but using ^{15}N-labelled cytb_5 (^{15}N-cytb_5). Significant differential line broadening and modest chemical shift perturbation (CSP; <0.01 ppm) were detected only for the sample prepared using method 1, implying productive CYP2B4–cytb_5 complex formation in the nanodiscs (Figure 2 A, B). This result agrees with our previous studies on the interaction between CYP2B4 and cytb_5 in bicelles.[4] In contrast, in detergent micelles, the interaction between the two proteins is very weak and almost negligible compared to that obtained in nanodiscs (Figure S4 A).[4]

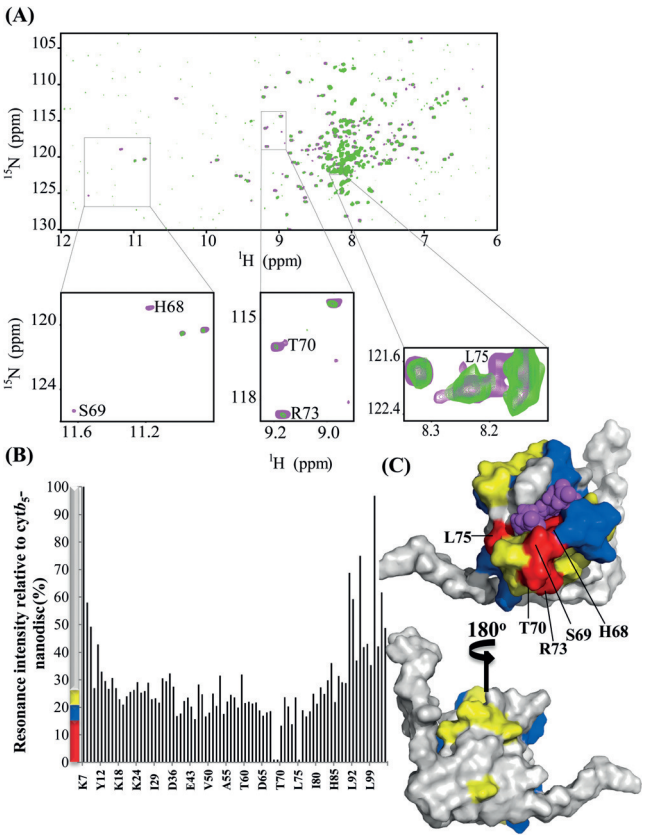

Figure 2. 2D ^{15}N/^{1}H TROSY HSQC spectrum of cytb_5. A) Overlay of the 2D ^{15}N/^{1}H TROSY HSQC spectra of cytb_5 alone in 22A-nanodiscs (magenta) and cytb_5 in complex with CYP2B4 reconstituted in 22A-nanodiscs (green). B) A histogram of the resonance intensities of cytb_5 in complex with CYP2B4 incorporated in 22A-nanodiscs normalized to free cytb_5 in 22A-nanodiscs, which exhibits a differential line-broadening pattern. The line broadening is categorized as significant (red), medium (blue), not significant (yellow), and negligible (gray), as shown by the vertical color strip, and is also mapped onto the structure of cytb_5 in (C).

The small magnitude of the CSP could be attributable to the formation of an ensemble of encounter complexes and/or protein–protein interactions on a fast-to-intermediate timescale.[3] Mapping of differential line broadening on the cytb_5 (Figure 2C) reveals a potential binding interface for the CYP2B4–cytb_5 complex in nanodiscs, including residues H68, S69, T70, R73, and L75, which are located on the lower cleft of the front face of cytb_5 around the heme edge. These residues, with the exception of L75, have been found on the binding interface of the CYP2B4–cytb_5 complex structure in bicelles.[3] In addition, previous studies have shown that T70 is involved in stereospecific complex formation between CYP2B4 and cytb_5.[21]

For the first time, L75 is reported to be likely involved in CYP2B4–cytb_5 interactions. This could be attributed to potential changes in membrane topology of the two proteins in terms of their orientations in lipid bilayers as well as the depth of insertion into the membrane; as nanodiscs, unlike bicelles, completely devoid of detergents, the membrane interaction of CYP2B4 could be enhanced and its folding may be slightly different.[22,23] Above all, identification of this binding interface, which matches previous findings, implies successful formation of productive complexes between CYP2B4 and cytb_5. On the other hand, the sample prepared using method 2, although resulting in identical SEC profiles to those obtained using method 1, failed to produce a differential broadening pattern of cytb_5 resonance signals. Instead, uniform broadening was detected as expected for the overall increase in the total correlation time of the entire particle when CYP2B4 was anchored to the opposite side of the disc (data not shown). The addition of substrates resulted in enhanced protein–protein interactions for samples prepared using method 1 (Figure S5) but not method 2, which further supports productive complex formation with method 1.

It is worth pointing out that our results cannot fully rule out the possible existence of a fraction of nanodiscs with CYP2B4 and cytb_5 located on opposite sides in method 1. However, given the fact that the method 1 sample predominantly displays characteristics typical of CYP2B4–cytb_5 productive complexes as revealed by the NMR experiments, it is highly likely that the situation where both proteins are on the same side of the nanodiscs dominates in method 1.

In summary, we have successfully demonstrated reconstitution of CYP2B4–cytb_5 complexes into peptide-based nanodiscs. NMR experiments have provided the evidence for productive complex formation in nanodiscs, implying that both proteins are reconstituted to the same side of the nanodiscs. The use of the 22A peptide as the belt protein enables a simplified preparation procedure for the nanodiscs. Additionally, the complete elimination of detergents from the samples renders membrane proteins such as CYP2B4, whose structure and function are sensitive to the presence of detergents, highly stable. In addition, the high spectral resolution and sample stability demonstrated by the use of this approach should enable high-resolution structure and dynamics studies using NMR techniques.

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