Monitoring Shifts in the Conformation Equilibrium of the Membrane Protein Cytochrome P450 Reductase (POR) in Nanodiscs

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Background: Investigating the mechanism of NADPH-dependent conformational changes of POR in nanodiscs.

Results: The conformational equilibrium of compact and extended POR, shifts toward the compact form (from 30 to 60%) upon reduction by NADPH.

Conclusion: The NADPH-dependent conformational changes follow the “swinging model.”

Significance: This is the first time that the action of a membrane protein located in a lipid bilayer environment is probed by neutron reflectivity.

Nanodiscs are self-assembled 50-nm2 patches of lipid bilayers stabilized by amphipathic belt proteins. We demonstrate that a well ordered dense film of nanodiscs serves for non-destructive, label-free studies of isolated membrane proteins in a native like environment using neutron reflectometry (NR). This method exceeds studies of membrane proteins in vesicle or supported lipid bilayer because membrane proteins can be selectively adsorbed with controlled orientation. As a proof of concept, the mechanism of action of the membrane-anchored cytochrome P450 reductase (POR) is studied here. This enzyme is responsible for catalyzing the transfer of electrons from NADPH to cytochrome P450s and thus is a key enzyme in the biosynthesis of numerous primary and secondary metabolites in plants. Neutron reflectometry shows a coexistence of two different POR conformations, a compact and an extended form with a thickness of 44 and 79 Å, respectively. Upon complete reduction by NADPH, the conformational equilibrium shifts toward the compact form protecting the reduced FMN cofactor from engaging in unspecific electron transfer reaction.

In 2002, nanodisc technology was introduced for functional and structural studies of membrane proteins as an alternative to conventional biomimetic models of the cell membrane such as liposomes and supported lipid bilayers. Nanodiscs are monodisperse patches of bilayers confined by the amphipathic membrane scaffolding proteins (MSPs) that self-assemble from mixtures of MSPs and phospholipids at appropriate stoichiometries upon detergent removal (1, 2). So far, various membrane proteins were reconstituted into nanodiscs (3–5). Due to their monodispersity, small angle x-ray (SAXS) and neutron scattering (SANS) could be used to confirm, not only the size and the disc-like bilayer structure of the nanodiscs (2, 6) but also the physical properties of the bilayer patch (7, 8). However, attempts to structurally characterize membrane proteins in nanodiscs are currently limited to SAXS studies of bacteriorhodopsin (9) and a human cytochrome P450 (CYP3A4) (10). The main challenge in scattering bulk studies is their high sensitivity to the presence of even minute amounts of different types of aggregates in the solution, thus imposing high demands in monodispersity of the sample and absence of membrane protein oligomers in the solution. Reflection-based techniques constitute an alternative approach for structural studies of membrane proteins in nanodiscs, as the reflectivity is insensitive to the presence of bulk aggregates. In particular, neutron reflection (NR) presents enhanced sensitivity in the direction perpendicular to the interface enabling detection of conformation changes as a result of the reducing conditions in native like

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References:

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2. The abbreviations used are: MSP, membrane scaffold protein; POR, cytochrome P450 reductase; NR, neutron reflectivity; CYP, cytochrome P450; SAXS, small angle x-ray scattering; QCM-D, quartz crystal microbalance with dissipation monitoring; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine.
The effects of redox state and binding of NADP\(^+\) were studied in the two extremes of POR oxidation state, either as fully oxidized or in the four-electron reduced state in the presence of bound NADP\(^+\). Briefly, POR inserted into nanodiscs were phosphorylated onto a silicon oxide surface native like conditions. The adsorption was first monitored by quartz crystal microbalance with dissipation (QCM-D) and the structure of the film in the oxidized and four-electron reduced state was probed with NR. This is to our knowledge the first time the action of a membrane protein is studied in a lipid bilayer model system by NR, here in terms of changes in conformational equilibrium. So far only the insertion, orientation and conformations of viral, bacterial, and antibacterial proteins in Langmuir monolayers (30–32), supported lipid bilayers (33), or tethered lipid bilayers (34, 35) has been used probing with NR.

**MATERIALS AND METHODS**

**Materials**—1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) obtained from AVANTI was dissolved in chloroform and used without further purification. Cyanine dye 5 (Cy5) (GE Healthcare) and 1,1′-dioctadecyl-3,3′,3′-tetramethyldiiodo-carbocyanine perchlorate, DiIC\(_{18}(5)\) oil were obtained from Invitrogen. Tris-HCl buffer and sodium chloride were purchased from Sigma Aldrich. Milli-Q purified water was used in all experiments. D\(_2\)O was provided from the Institute Laue Langevin.

**Protein Expression and Purification of Both Native and Cys Mutant POR**—Codon optimized full-length *Sorghum bicolor* CPR2b (accession: XP_002444097.1) for maximum expression and double mutant C536N/Q680C were cloned into the BamHI/SalI site of a pET52b vector and expressed in *Escherichia coli* strain BL21(DE3) cells. A starter culture (50 ml) of BL21(DE3) containing the pET52b POR plasmid was grown in Terrific Broth with ampicillin (50 \(\mu\)g/ml) overnight (37 °C, 220 rpm). Fresh Terrific Broth (4 liters) with ampicillin (50 \(\mu\)g/ml) adjusted to 37 °C was inoculated with starter culture (50 ml). Cells were grown in a fermentor (Biostat\(\text{®}\) B plus, Satorius Stedim Biotech) for 3 h at constant settings (37 °C, 5 liters/min airflow, pH 7.2, 800 rpm) to reach a cell density giving an \(A_{660}\) of \(\sim\)10. Temperature was adjusted to 28 °C, and POR protein expression was induced by addition of isopropyl 3-D-thiogalactopyranoside (1 m\(\text{mol}\)) followed by continued growth for 16 h. Cells were harvested by centrifugation (1000 \(\times\) g, 15 min), resuspended in buffer (50 mM Tris-HCl, 100 mM NaCl, and Complete protease inhibitor mixture) and broken using a French pressure cell (Constant Systems Ltd, Ts2 Series). Cell debris was removed by centrifugation (10,000 \(\times\) g, 15 min), and the membranes were collected from the supernatant by ultracentrifugation (100,000 \(\times\) g, 1 h, Beckman Coulter Ultracentrifuge). Solubilization of the membrane pellet was accomplished by homogenization in a Potter-Elvehjem homogenizer (50 m\(\text{mol}\) Tris-HCl, 100 m\(\text{mol}\) NaCl, 20 m\(\text{mol}\) sodium cholate, pH 7.9) and the expressed POR purified by affinity chromatography on a 2′5′-ADP-Sepharose column (elution buffer (50 mM Tris-HCl, 100 mM NaCl, 20 mM sodium cholate, 5 mM NADP\(^+\) (Sigma-Aldrich)) and by anion exchange chromatography (Q-Sepharose column, diameter, 10 mm; height, 70 mm) and elution buffer (50 mM Tris-HCl, 400 mM NaCl, 20 mM sodium cholate).
Cytochrome P450 Reductase Characterization—The concentration of the isolated POR was determined based on analysis of the amino acid content following acid hydrolysis and by analysis of the flavin content. Flavin content was determined at ε_{425nm} = 9200 M\(^{-1}\) cm\(^{-1}\) (36). The POR sample was diluted to 50 μM. The purity of the sample was analyzed both by SDS-PAGE and Coomassie staining. A partial proteolytic cleavage, resulting in the loss of the ~6-kDa N-terminal peptide segment, including the membrane anchor, was evidenced by the presence of an additional staining band with reduced molecular mass in comparison to intact POR. Visible absorption spectra (425–700 nm) of the isolated full-length POR were recorded using a PerkinElmer spectrophotometer. Isolated POR was incubated with 4 Molar excess potassium ferricyanide or with 10 mM NADPH for 10 min to obtain the fully oxidized or reduced form of POR, respectively.

Preparation of Empty and POR Containing Nanodiscs—All nanodisc assemblies were performed using DMPC lipids (Avanti Polar Lipids) and the MSP1D1 scaffolding protein in buffer (50 mM Tris-HCl, pH 7.9, 100 mM NaCl). Empty nanodiscs was prepared as reported previously (11). POR nanodiscs were assembled in buffer with a lipid:MSP ratio of 80:1, and purified POR was added to reach a 10-fold molar excess of nanodiscs to POR. Detergent was removed by dialysis overnight, and the reaction mixture was fractionated by gel filtration (flow rate, 0.5 ml/min) on a preparative HPLC (Shimadzu type) equipped with a Superdex™ 200 HR 10/30 column (Amersham Biosciences; diameter, 10 mm; height, 300 mm). Elution of proteins and POR was continuously monitored by absorbance at 280 and 450 nm, respectively. POR nanodiscs were collected and further purified by a second gel filtration chromatography step to remove empty nanodiscs (supplemental data). For microscopy, the POR nanodiscs contained a ratio of 96:4 for DMPC and DiIC\(_{18}\)(5) oil.

Physisorption of Nanodiscs onto the Solid-Liquid Interface—Si/SiO\(_2\) surfaces were soaked in Piranha solution to obtain hydrophilic properties. Empty or POR containing nanodiscs solubilized in buffer (20 mM Tris, 100 mM NaCl, pH 7.4) were injected into the NR cell (15 °C). Following an incubation period (15 min) that ensured saturation of nanodisc adsorption to the SiO\(_2\) surface, extensive rinsing with buffer was performed. In the single molecule studies using confocal microscopy, the POR nanodiscs sample was diluted ~1000 times compared with the concentration used in the neutron reflectivity experiment. The sample was applied to a glass slide and left (15 min) to ensure adsorption to take place (see supplemental data for more experimental details).

Neutron Reflectivity and the Optical Models—The NR experiments were performed at the FIGARO beamline (37) at Institute Laue-Langevin, France. NR allows determination of the averaged composition and structure of an adsorbed thin film along an axis perpendicular to the interface. The specular (mirror-like) neutron reflectivity (R) of a collimated neutron beam is a function of the scattering vector Q, perpendicular to the interface Q_x = (4π/λ) sinθ, where θ is the angle of reflection and λ is the neutron wavelength). The reflectivity is related to the scattering length density (ρ) of the material at the surface ρ = Σ_i(ρ_i/V), where b is the coherent scattering length of each nuclei (i) in a given volume (V), via an inverse Fourier transformation (38). Neutron scattering is isotope-dependent, and its utility in biology arises particularly from the different scattering power of hydrogen \(^1\)H and deuterium \(^2\)H (39). This enables for isotopic substitution (in particular deuteration) to create contrasts between the different components in a sample. The NR profiles obtained in this study were analyzed by fitting a simulated reflectivity curve of a model structure of the system to the experimental data via the software Motofit (40), which uses the Abeles optical matrix method (41) to calculate the reflectivity of thin layers and enables simultaneous fitting of data sets of different isotopic compositions. A detailed description of the optical model used is given in the supplemental data. Briefly, the analysis of the POR nanodiscs were accomplished using either one (POR\(_{\text{in}}\) in Model 1) or two (POR\(_{\text{in}}\) and POR\(_{\text{out}}\) in Model 2) layers to represent the POR. The nanodiscs were in both cases modeled as a single layer (termed ND) having an averaged scattering length density of the lipids and protein belts of known composition. The fixed volumes and scattering length densities used for the phospholipids, MSP1D1 and POR are listed in supplemental Table S1.

Confocal Microscopy—All samples were examined with a Leica TCS SP5 inverted confocal microscope using an oil immersion objective HCX PL APO CS×100 (numerical aperture, 1.46) and equipped with two Avalanche photodiode detectors. In all cases, sequential scanning was used when acquiring images to avoid cross-excitation. The 512 × 512 pixels images had a resolution of 100 nm and a bit depth of 8 bit. POR and nanodiscs were detected by using 633 and 543 nm laser lines, respectively. Signal splitting was accomplished using an 625 nm beam splitter. Detection of nanodiscs was accomplished using an ET 575/25 filter and POR detection through a 647/75 filter. A typical set of images for the nanodisc channel and the POR channel is shown in supplemental Fig. S3. The microscope was maintained at 22 ± 1 °C.

RESULTS

Formation of a Nanodisc Film at the Silica-aqueous Interface—Using NR, we showed that nanodiscs can be aligned at the solid-liquid interface. Physisorption of DMPC nanodiscs to the silica-liquid interface results in a relatively dense (62 (v/v) %) monolayer of nanodiscs separated from the solid substrate by a thin (4–5 Å) layer of solvent, as probed by NR. The nanodiscs adsorb with their lipid bilayer parallel to the interface and are well aligned with respect to the axis perpendicular to the surface, in agreement with earlier atomic force microscopy images (42). The adsorption of DMPC nanodiscs with and without POR as monitored by QCM-D (supplemental Fig. S2) shows a quick (~1 min) saturation both for the empty and POR-containing nanodiscs. The NR profiles (Fig. 1) differ for empty and POR-containing DMPC nanodiscs adsorbed to the silica-liquid interface upon saturation conditions. The difference is particularly evident in the D\(_2\)O isotropic contrast, whereas only small differences are observed for 0.02 < Qz < 0.05 Å\(^{-1}\) in H\(_2\)O contrast. This is consistent with the larger difference in scattering length density between a protein layer and D\(_2\)O (∆ρ ~ 3.5×10\(^{-6}\) Å\(^{-2}\)) than a protein layer and H\(_2\)O (∆ρ ~ 2.3×10\(^{-6}\) Å\(^{-2}\)). The measured reflectivities for the POR nanodisc film are shown in Fig. 2A together with simulated
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Composition of POR in Nanodisc Film—Due to their surface area, the lipid core of the nanodisc could theoretically accommodate multiple POR. To avoid the reconstitution of more than one POR/nanodisc, we used a low ratio of POR/nanodisc (1:10, see “Materials and Methods”). SDS-PAGE analysis of isolated POR showed a Coomassie staining band with a mass of ~78 kDa and a minor band with a mass of ~72 kDa (supplemental Fig. S1D). Mass spectrometric analysis demonstrated that the latter represents a truncated POR form that lost the N-terminal membrane anchor. Analysis of the POR nanodisc preparation by blue native-PAGE (Fig. 3, A and B) revealed that the sample contained a mixture of free POR oligomers, POR nanodiscs, and empty nanodiscs. SDS-PAGE analysis of the POR nanodisc band excised from the blue native gel suggested a single POR per nanodisc as judged from the relative intensity of the Coomassie-stained POR and MSP1D1 band (Fig. 3B). The nanodisc films at the SiO2 surface were formed by adsorption from a sample containing both free oligomers of POR and POR nanodiscs. The neutron reflection experiment gives a POR coverage of 9% (v/v), which compared with the nanodisc coverage (58 ± 2% (v/v)) corresponds to 0.9 ± 0.2 POR/nanodisc (N_POR/ND). Thus, there is selective and specific adsorption of the POR nanodiscs because the sample preparation also contained free POR oligomers. Indeed, both empty and POR containing nanodiscs align with the lipid bilayer parallel to the surface (Fig. 2B, iv) demonstrating the preference for interactions between the lipid heads and the SiO2 surface rather than interactions between the proteins (POR or MSP1D1) and the SiO2 surface. Additionally, at the high concentration used in the reflectivity experiments, we expect the film to be more markedly populated by POR nanodiscs due to mass transport conditions: saturation is reached within a minute, and the POR nanodiscs will, due to their smaller size, diffuse faster to the interface than the large POR oligomers. However, the error of the best fit could indeed accept up to 6% (v/v) POR oligomers coexisting with 50% (v/v) POR nanodiscs. Considerable differences in surface and bulk composition were previously reported in mixtures of palmitoyloleyl phosphatidylcholine and dipalmitoyl phosphatidylcholine, for example (43).

Confocal scanning fluorescence microscopy with single molecule resolution was carried out to verify that the film is indeed composed of nanodiscs containing a single POR, rather than a mixture of empty nanodiscs and nanodiscs with multiple PORs that on average could add up to a 1:1 stoichiometry. Labeled POR nanodiscs were prepared under identical conditions as those used in the NR experiments except for the inclusion of a membrane dye, DiI (4%), and an engineered single cysteine POR variant (C536N/Q680C) fluorescently labeled with Cy5 (see supplemental data for details in POR labeling). Dilute samples of the fluorescently labeled POR nanodiscs were allowed to physisorb on the glass microscope surface before confocal scanning fluorescence microscopy imaging. This methodology enabled direct visualization of single nanodiscs containing POR (see Fig. 3, C and D, and supplemental Fig. S3 for more detail) and offered the option to record single POR bleaching steps.

Neutron reflectivity data of empty nanodiscs in D2O (blue) and H2O (black) and POR nanodiscs in D2O (red) and H2O (green) adsorbed to silica.

FIGURE 1. Neutron reflectivity data of empty nanodiscs in D2O (blue) and H2O (black) and POR nanodiscs in D2O (red) and H2O (green) adsorbed to silica.

FIGURE 2. A, neutron reflectivity data of POR nanodiscs (green markers) and simulated reflectivity curves (lines) of the POR nanodiscs adsorbed to the silica-liquid interface in D2O (upper data sets) and H2O (lower data sets). The simulated reflectivity curves in A represent nanodiscs adsorbed in the different orientations schematically shown in B: (i) without any particular orientation represented by a single homogenous layer with scattering length density resulting from the weighted average of DMPC, MSP1D1, and POR (black). This model fails to represent the experimental data and the film must therefore possess an ordered structure. A variety of possible orientations were then envisioned: (ii) strong POR-silica interactions, which cause the protein to face the surface (blue), (iii) or POR nanodiscs aligned perpendicular to the interface (pink), and (iv) POR nanodiscs aligned at the interface in the same manner as empty nanodiscs, with the lipid bilayer parallel to the interface and with the water soluble part of the POR protein facing the aqueous bulk solution (green). The POR was modeled as a cylinder with constant volume, whereas the height (protrusion from the bilayer) was allowed to vary.

reflectivities for a range of possible orientations of the POR nanodiscs as shown in Fig. 2B (i–iv). The ρ profiles perpendicular to the interface for the different models are shown in Fig. 2C. This figure clearly demonstrates that POR nanodiscs align with their lipid bilayer parallel to the interface having the soluble domain part of POR directed away from the solid substrate, in agreement with earlier atomic force microscopy studies (23). This orientation is a prerequisite to study structurally unperturbed, functional membrane proteins. Interaction of the soluble part of the protein with the solid substrate (as for Fig. 2B, ii) may result in impaired structural dynamics or possible functional inactivation. However due to the uncertainty of the measurement, the best model (Fig. 2B, iv) accepts maximally 3% of the nanodiscs at the surface in an upside down orientation with POR facing the substrate. The optical model that gave the best fit to the POR-nanodisc film data in Fig. 2A gave a nanodisc coverage of approximately ~60% (v/v) in agreement with that found for empty nanodiscs on smooth surfaces.2 Thus, the high dissipation (1.7·10^−10) measured by QCM-D upon adsorption of POR nanodiscs (supplemental Fig. S1) must be related to the presence of POR, which increases the water coupled to the nanodisc film.
that support the existence of a single POR per nanodisc (see supplemental Fig. S4). To quantify the exact POR/nanodisc ratio, the distribution of normalized integrated POR intensities for ~150 POR molecules found in nanodiscs was fitted with double Gaussians. The data in Fig. 3E show that indeed ~83% of the POR nanodiscs contain a single POR enzyme and ~17% a second POR. The small number of nanodiscs that appear to contain more than two POR molecules is primarily but not exclusively originating from protein aggregation (as seen from strong DiI signal and nonspherical point spread function intensity, see supplemental data) and should not significantly bias our modeling. At the low sample concentration used in confocal scanning fluorescent microscopy (~1000 times lower than in the NR experiments), only a few percentage of the surface is coated with nanodiscs (see supplemental Fig. S3) and the adsorption of POR oligomers cannot be disregarded. Finally, the area integration in the microscopy images still shows that the average number of POR/nanodisc is 1.17 ± 0.4, in good agreement with the NR result (0.9 ± 0.2). The fact that an independent, single molecule technique, directly recorded the existence of a single POR/nanodiscs strongly validates our modeling.

Equilibrium Conformation of POR in the Nanodisc Film in Presence and Absence of NADPH—Fig. 4A gives the NR profiles for POR nanodiscs both fully oxidized and after reduction with 10 mM NADPH. The figure also includes data for empty nanodiscs before and after addition of 10 mM NADPH. Reference curves obtained using empty nanodiscs in the absence (black) and presence (green) of NADPH are also shown using D2O and 9:1 D2O:H2O as solvents, respectively. B, schematic representation of the two POR layers with different density corresponding to coexisting compact and extended POR (Model 2).

sent the POR molecule. In Model 1, the high roughness (18 Å) found for $r_{POR/bulk}$ indicates that the POR layer is highly uneven. Thus, the POR protein may exist in different conformations in the film. To differentiate between any distinct populations of POR thicknesses (Model 2), this layer was split in two isotropic layers, POR$_{in}$ and POR$_{out}$, each with different density (coverage and thickness) as schematically represented in Fig. 4B. The overall quality of the fit of Model 2 is as good as that of Model 1 (Fig. 4A). However, the high roughness in Model 1 is avoided in Model 2, for which low values of $r_{POR/bulk}$ and $r_{POR_{in}/POR_{out}}$ satisfactorily fit the data. Using Model 2, the inner layer (POR$_{in}$) is in direct contact with the lipid bilayer of the nanodiscs and is 44 Å thick with 10% (v/v) of protein. The second and outer layer (POR$_{out}$) was 35 Å thick with 6% (v/v) POR.

FIGURE 3. Analysis of the POR preparation by blue native-PAGE and by SDS-PAGE. A, molecular mass marker (M) and analysis of the following: 1) free POR, 2) free MSP1D1, 3) POR-nanodisc assembly start material, 4) empty nanodiscs, and 5) purified POR nanodiscs. Bands (i–iv) marked with an arrow were excised from the blue native-PAGE and re-electrophoresed on a second dimension SDS-PAGE for the purified POR sample only. B, analysis of the content of POR and MSP1D1 in each of the excised bands (i–iv) from blue native-PAGE compared with the molecular mass markers (M). C–E, fluorescence microscopy results confirming that the vast majority of POR nanodiscs contain a single POR enzyme. Zoomed in fluorescent micrographs of the DiI-labeled nanodiscs (C) and the Cy5-labeled POR (D). For typical full images, see supplemental Fig. S3. E, distribution of POR molecule reconstituted in nanodiscs fitted with a double Gaussian. Integration of the area of each Gaussian allowed us to determine that 83% of the POR nanodiscs contain a single POR enzyme confirming our modeling results. SLD, scattering length density.

FIGURE 4. A, neutron reflectivity curves and scattering length density profiles (inset) of POR nanodiscs in the absence (blue) and presence (red) of 10 mM NADPH. Reference curves obtained using empty nanodiscs in the absence (black) and presence (green) of NADPH are also shown using D2O and 9:1 D2O:H2O as solvents, respectively. B, schematic representation of the two POR layers with different density corresponding to coexisting compact and extended POR (Model 2).
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The thickness, coverage, and roughness of the different layers in Models 1 and 2 in the absence and presence of 10 mM NADPH

SiO₂ was precharacterized before adsorption of the POR nanodiscs. The thickness, solvent penetration, and roughness of the layer were 6 Å, 10% (v/v), and 3 Å, respectively. The solvent layer represents the H₂O/D₂O trapped between the SiO₂ surface and the nanodisc film.

### DISCUSSION

The presence of two POR layers with different thickness and coverages is explained by two coexisting conformations of the protein at the interface: an extended POR conformation with a total thickness of 79 ± 8 Å and a compact conformation with a total thickness of 44 ± 10 Å. The extended conformation is smaller than the maximum distance of 110 Å found for the truncated solubilized POR as measured by SAXS (22). The difference in maximum distance most likely reflects POR-lipid bilayer interactions that prevent complete extension of the POR. Alternatively, the extended POR molecules in the adsorbed nanodiscs may not arrange themselves completely perpendicular but rather tilted in relation to the lipid bilayer. This will give rise to an underestimation of the maximum distance within the POR soluble part by NR as compared with SAXS.

The denser POR<sub>in</sub> layer represents a mixture of the compact and extended POR, whereas the less dense POR<sub>out</sub> layer corresponds solely to the extended POR. From the volume coverage of each fitted layer (nanodisc, POR<sub>in</sub>, and POR<sub>out</sub>), the average number of POR/nanodiscs on the surface was calculated from the fit of Model 2 and was found to be 0.9 ± 0.3 consistent with Model 1 and the confocal scanning fluorescence microscopy data. The roughness in the protein layers, <i>r</i><sub>NAND</sub>, <i>r</i><sub>PORin</sub>, and <i>r</i><sub>PORout</sub>, are relatively low, suggesting that the protein is uniformly inserted to the bilayer and the majority of the proteins are either extended or compacted and not present as intermediates of these. The rather large errors in <i>t</i><sub>PORin</sub> and <i>t</i><sub>PORout</sub> are due to the relatively low coverage in these layers. From <i>ϕ</i><sub>PORin</sub> and <i>ϕ</i><sub>PORout</sub>, the relative amount of POR present in the compact and extended conformation was extracted and found to be 30 and 70%, respectively.

The reflectivity profile of the POR nanodisc film changed upon addition of 10 mM NADPH, whereas it remained unchanged for empty nanodiscs (Fig. 4A). In the presence of large excess of NADPH, POR becomes fully reduced while the oxidized NADP⁺ remains bound (supplemental Fig. S5) (22). The NADPH molecule is rather small (744 g/mol), and the binding alone to the protein cannot explain the observed change in reflectivity. Thus, the change in reflectivity must be related to a change in the conformation equilibrium of the POR.

Model 1 (Table 1) shows that the POR layer turns significantly thinner (39 Å) and denser (13% (v/v)) in the presence of NADPH. This indicates that the POR is centered closer to the bilayer, whereas the high roughness still suggests a coexistence of different protein conformations. Thus, the change in reflectivity is likely due to changes in the relative distribution within the two layers as represented by Model 2. The best fit of Model 2 (Fig. 4) suggests that POR<sub>in</sub> becomes slightly more dense, whereas POR<sub>out</sub> turns less dense in the presence of 10 mM NADPH. Moreover, the number of POR/nanodisc is not affected by the addition of NADPH in Model 2, further supporting our hypothesis on equilibrium between these two conformations. Taken together, the addition of NADPH causes a redistribution of the conformation equilibrium toward the compact structure of the POR. From the fitted coverage in the POR layers in presence of 10 mM NADPH (Table 1), the relative amount of POR present in the compact and extended conformations was calculated to be 60 and 40%, respectively. The presence of two coexisting conformations for POR and the shift of the conformational equilibrium toward the compact structure upon addition of NADPH, support the swinging model as a mode of action of the POR. Indeed, the favored compact structure should protect the reduced FMN cofactor from engaging in unspecific electron transfer reaction.

The existence of a discrete number of conformational states that are redistributed upon ligand binding was proposed as early as in the 1960s, commonly known as the conformational selection model for multimeric enzymes and proteins (44, 45). The prevalence of the conformational selection over the induced fit model (where ligand binding induces new conformational states) has been highly debated over the past few years (45). To date, a very limited number of studies have directly validated conformational selection, including monomeric enzymes (46). Herein, our data not only decipher the underlying conformational motions of POR, but also, this turnover cycle provides validation of the conformational selection hypothesis.

Interestingly, the net equilibrium values for the truncated POR differed from those of the full-length POR reconstituted in nanodiscs. (The fraction of the compact form was 15 and 50% in oxidized and reduced state, respectively, in the truncated form compared with 30 and 60% in the nanodiscs.) Such discrepancy
may arise from lipid-protein interactions in the nanodiscs not accounted for in the SAXS data (22). Currently, the majority of in vitro studies are performed with proteins in solution. Over the past few years, however, an increasing amount of data highlights the immense importance of the membrane for proper protein function (47). Recent studies explicitly showed that the reconstitution of POR in native membrane systems significantly altered the behavior of this protein, where the membrane charge was found to have a marked effect (48). The methodology hereby presented allows future studies of the effect of lipid composition on the conformation equilibrium of this POR.

Finally, the dimension of the nanodiscs used in the present study permits the incorporation of several membrane proteins within the same disc. The present study thus provides the foundation for future neutron reflectivity-based structural studies on protein complexes introduced into nanodiscs. The POR used in this study is derived from sorghum (CPR2b) (19). In sorghum, it catalyzes the transfer of reducing equivalents to cytochrome P450 mono-oxygenases situated in the endoplasmic reticulum. These P450s catalyze a range of reactions in primary and secondary metabolism. One of the most prominent functions is electron transfer to the two multifunctional membrane-bound cytochrome P450s, CYP79A1 (49) and CYP71E1 (50) which, in combination with a soluble UDP-glucosyl transferase (UGT85B1) (12) catalyzes the biosynthesis of dhurrin. Knowledge on the structure of the entire complex of membrane bound enzymes in dependence of the interactions with CPR2b and of the presence of different substrates and cofactors and the possible binding of UGT85B1 may now be addressed following reconstitution of the complex in nanodiscs and the use of neutron reflection.

In summary, the changes in the conformational equilibrium associated with the POR redox state were studied in this work. This is, to our knowledge, the first time the action of a membrane protein, reconstituted into a lipid bilayer model system, has been probed by NR. The data show that the nanodiscs align with the lipid bilayer parallel to the surface and with the POR soluble domain exposed toward the aqueous solution. Confocal fluorescent microscopy validates NR modeling, which shows equilibrium between two different POR conformations: A compact of 44 ± 8 Å, and a more extended protruding 79 ± 8 Å above the lipid bilayer. Upon addition of 10 mM NADPH, the conformational equilibrium shifts toward the compact conformation. Thus, our data supports the swinging model (19) of the conformational changes accompanied with the POR catalyzed electron transfer from NADPH to cytochrome P450s.

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