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Improvisations in phototrophy. Protein engineering and functional investigation of rhodopsin proton-pumps

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Chapter 5

The effect of a detergent or lipid microenvironment on the properties of PR and GR

The insoluble nature of membrane proteins presents a major drawback in their structural and functional investigation, since they require excessive solubilization by detergents and subsequent purification steps. The choice of detergent is important, as the surfactant environment often affects the stability, oligomeric assembly, and activity of the resulting protein. We compared the efficacy of several commonly used detergent systems on the solubilization and stabilization of wild type PR and GR. We further investigated the spectral properties, stability and oligomeric distribution of the resulting pigment-detergent complexes. The pressure and membrane potential has an impact both on the structure and dynamics of intrinsic membrane proteins. For this reason, various membrane mimetic systems have been put forward, which allow these pigments to be studied in a near-native-like lipid environment. We investigate the properties of PR and GR in different open and closed bilayer systems, namely nanodiscs and liposomes. Finally, we present examples of the structural and functional studies made possible by using such native-like membrane systems.

5.1 Introduction

Membrane proteins account for roughly 30% of all gene transcripts, yet they make up less than 0.5% of the solved protein structures in the protein data bank. This presents a serious bottleneck, given that these proteins are integral to cellular life and are responsible for a wide range of functions ranging from cell to cell communication to signal transduction and ionic transport. The scientific community has invested much effort to overcome the difficulties inherent in the isolation and characterization of these insoluble proteins. Due to multiple membrane spanning domains, these proteins usually require amphipathic molecules for their extraction from the membrane. Solubilization by detergent micelles is the most popular method of choice, and is particularly useful for their initial biophysical characterization (Figure 5.1). The detergent DDM is among the most widely used detergent for the solubilization of membrane proteins, since it has relatively minor deleterious effects on structure and stability of most membrane proteins, thereby behaving as a very mild detergent. In the previous chapters, we have primarily used DDM micelles to solubilize and purify the pigment complexes that were generated. However, some novel variants like GR-FS:MMAR have low thermal stability even in DDM, confirming that protein and ligand engineering affect the structure and stability of the pigment-micelle complex. Hence, it is usually preferable to transfer purified membrane proteins in detergent solution back into a native-like membrane mimicking environment. Towards this end, various phospholipid bilayer systems such as proteoliposomes and nanodiscs (Figure 5.1) have been developed to stabilize these pigments, allowing their properties to be studied in a native-like membrane environment.

Proteoliposomes are commonly used for vectorial studies, and in the solid-state NMR investigation of protein structure [1, 2]. However, due to the light scattering induced by their large size (200 nm-10 μ m diameter), they are less suitable for optical studies.

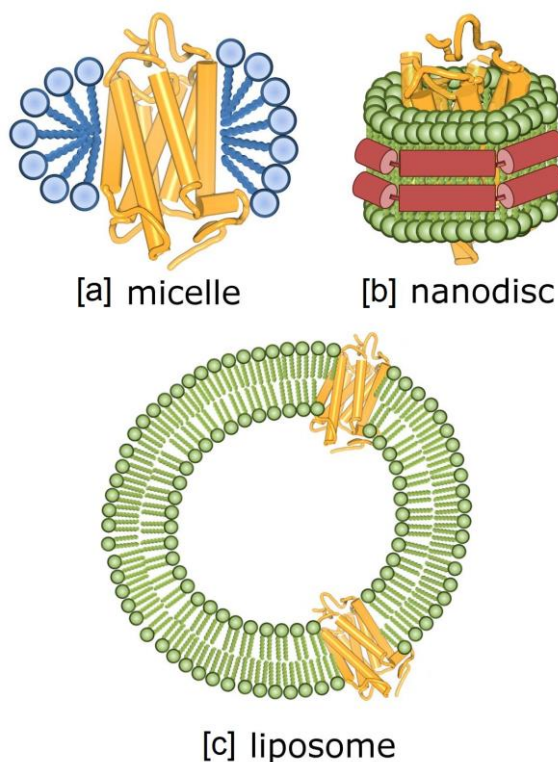


Figure 5.1. Schematic representation of the microenvironments for membrane proteins, which have been investigated in this chapter, after their extraction from their biological membranes [3]. Detergent molecules are represented in blue, lipids in green and membrane scaffold components in red. See the text for the difference in size range between the systems.

The much smaller discoid open nanodisc systems (10-30 nm diameter) are a suitable alternative. Nanodiscs consist of the protein of interest embedded in a phospholipid bilayer contained by a scaffolding amphipathic agent. Traditionally, nanodiscs are made using two molecules of the amphipathic membrane scaffold protein (MSP) [4]. However, these MSP-nanodiscs require prior detergent solubilization of the pigment, which then allows subsequent insertion into a non-native membrane environment. SMA-nanodiscs using styrene maleic acid copolymer as a scaffolding component, are an alternative emerging option which can allow detergent free insertion of membrane proteins into nanodisc structures [5].

SMA self-inserts into the cell membrane extracting native-nanodisc patches, which can be further purified to isolate the nanodiscs embedded with the protein of interest.

In this chapter, the effect of the local microenvironment on properties of wild type PR and GR was investigated. We compare a number of detergent and phospholipid bilayer systems, and study their influence on the spectral properties, stability, size and oligomeric state of the resulting pigment-complexes, in preparation for further extensive biophysical characterization.

5.2 Experimental section

Besides the methods described in Chapters 2 and 4, additional methods used for this chapter are described below. The source of special chemicals is given in the Appendix (A1.).

5.2.1 Cell lines, plasmids, and cell culturing

E. coli BL21 transformed with either plasmid pJBS1255 or pJBS1257, constructed as described elsewhere [6], was used to express 6x-His tagged PR or GR, driven by the inducible P_{trc} promoter. A main culture was grown from a 1:50 dilution of an overnight culture in LB at 37°C. Expression of the opsin was induced with 0.5 mM IPTG at an OD_{600} of 0.5-0.6. The cells were grown for an additional 14-16 h, and harvested by centrifugation at 3200xg for 20 minutes at RT. *E. coli* BL21 was also used to express the plasmid pET-28a containing the insert for the 6x-His tagged membrane scaffold protein MSP1E3D1 [4]. A main culture was grown from a 1:50 dilution of an overnight culture in Terrific broth at 37°C. Expression of MSPDE31 was induced using 0.5 mM IPTG at an OD_{600} of 0.6-0.7. The cells were grown for 2-4 h and harvested by centrifugation at 3200xg for 20 min at RT.

5.2.2 Hydrolysis of SMA

10 g of styrene maleic acid copolymer SMA2000 was refluxed in 100 mL 1M KOH for 3 h at 100°C. The solution was cooled to RT and kept at 4 °C overnight. The hydrolyzed SMA was precipitated with 1 M HCl, by lowering the pH to 7. SMA was spun down in a tabletop centrifuge at 6000xg for 20 min, and the pellet was further washed 4-5 times with 100 mM HCl. The supernatant was drained and the powder was lyophilized overnight. From the refluxed solution, 98 % of SMA was recovered. A stock solution of 10 % SMA (w/v) was made in 50 mM Tris, pH 8.

5.3.3 Extraction of PR and GR into SMA-nanodiscs

E. coli UT5600 cell pellets expressing PR or GR (c.f. chapter 2) were resuspended in 50 mM Tris, 0.6 M NaCl, pH 8 (3 mL per 100 mL culture), and were sonicated to generate membrane vesicles (4s on, 5s off, 30 % amplitude, 10 min, 4°C). 3 ml of 10 % SMA was added and the solution was sonicated again and left on the roller at RT for 2-3 days. The insoluble fraction was spun down at 147,000xg for 45 min at 4 °C. From the coloured supernatant, PR or GR containing SMA-nanodiscs were purified using nickel affinity chromatography (see below).

5.2.4 Purification of MSPDE31

The *E. coli* BL21 cell pellet was resuspended in 40 mM Tris, 0.3 M NaCl, 5 mM β -mercaptoethanol, pH 8 (10 mL per L culture) supplemented with one protease inhibitor tablet, 10 mg lysozyme and 6 μ L benzonase nuclease solution. After 30 min incubation on ice, 250 μ L of 10% Tergitol NP-40 was added and the pellet was sonicated 5 times, 45s, 30 % amplitude, 4°C. The cell debris was spun down at 16,000xg for 30 min at 4°C). The lysate was subjected to batch purification using 6 mL Ni²⁺ NTA resin, as described previously [7]. The fractions of pure MSPDE31 were combined and

concentrated using a 10 kDa centrifugal filter (Amicon). The purity was assessed using SDS-PAGE.

5.2.5 Formation of MSP-nanodiscs

PR and GR solubilized and purified in DDM, DPC or TritonX-100 were used to generate MSP-nanodiscs. A solution of 2 mg/mL asolectin or *E. coli* polar lipid was made in 40 mM nonylglucose, 50 mM HEPES, 100 mM NaCl, pH 7.8. The entire protocol was carried out at 4°C with continuous mixing during the incubation steps. Purified PR/GR was combined with the lipid solution using a molar ratio of 2:240 for PR/GR : asolectin/*E. coli* lipid. This mixture was incubated for 30 min. Then MSP1E3D1 was added in a molar ratio of 1:2; PR/GR : MSP. The solution was vortexed and incubated for 30 min. Subsequently, the detergent was complexed by the addition of β -cyclodextrin, which induces the formation of the MSP-nanodiscs [8]. β -cyclodextrin was added in three equal parts, with 15 min incubation steps for each addition, to achieve a final molar ratio of 1:1.5 for detergent: β -cyclodextrin. The insoluble material was then spun down at 14,000xg for 30 min at 4°C.

5.2.6 Purification of SMA- and MSP-nanodiscs

The SMA- and MSP-nanodiscs containing PR or GR were purified by nickel-affinity chromatography. 0.3 or 1 mL Ni²⁺ NTA-resin in 0.6 or 5 mL columns was spun down in a table top centrifuge at 2700xg for 1 min and washed once with Buffer A (20 mM bis-tris propane, 20 mM imidazole, 0.5 M NaCl, pH 8). The crude nanodisc solution was added to the column, incubated at RT for 20 min, and the unbound lysate was spun down. The columns were washed ten times with 0.6 mL of Buffer A. The bound nanodiscs were eluted using 600 μ L of 500 mM imidazole, 20 mM bis-tris propane, 0.5 M NaCl, pH 8. The absorbance spectra of the purified nanodiscs were recorded, and their purity was assessed by SDS-PAGE.

5.2.7 Insertion of purified PR/GR into liposomes

A 15 mg/mL *E. coli* lipid solution was made in 40 mM nonylglucoside, 50 mM HEPES, 100 mM NaCl, pH 7.8. Purified PR/GR was added to the lipid solution using a ratio of 1 protein monomer : 25 *E. coli* lipid molecules and left mixing continuously for 1 h, RT. β -cyclodextrin was then added in three equal parts as described above using a molar ratio for β -cyclodextrin : detergent of 1.5:1. The resulting liposomes were pelleted in a table top ultracentrifuge (21191xg, 30 min, 4°C). The resulting pellet was washed twice with 50 mM phosphate buffer, 150 mM NaCl, pH 8.

5.2.8 Size exclusion chromatography:

Size exclusion chromatography (SEC) of purified PR/GR in DDM or OGNG was done using a mobile phase containing 50 mM phosphate buffer, 150 mM NaCl, 0.2 % OGNG, pH 8 or 20 mM bis-tris propane, 150 mM NaCl, 0.1% DDM, pH 8 with a Amersham Pharmacia biotech äkta column at standard pressure and flow rate. The absorbance was measured at 215, 280 nm, 500 nm, 520 nm and 540 nm. Blue Dextran (Sigma-Aldrich) was used for molecular weight calibration.

5.2.9 Circular dichroism spectroscopy

Circular dichroism spectra were recorded using 2 mm quartz cuvettes on a J-815 spectrometer (Jasco, Gross-Umstadt, Germany) equipped with temperature control. The following parameters were used: wavelength range, 400-700/900 nm; data pitch, 1 nm; response time, 2 s; band width, 4 nm; scanning speed, 50 nm/min; temperature, 20°C. A sample absorbance of 0.5-1 OD units at the λ_{max} was used throughout.

5.2.10 Dynamic light scattering (DLS)

The size distribution of the purified nanodisc samples was analyzed using the Zetasizer Nano-S (Malvern Instruments Ltd, Malvern UK), equipped

with a 633 nm laser. The samples were spun down in a table-top centrifuge at 13,000xg for 10 min prior to the measurement. The signals were analyzed using the internal software of the equipment and converted into a size distribution with scatter intensity.

5.2.11 Kinetic spectroscopy

Transient absorption measurements were performed at the LaserLaB of the Vrije Universiteit Amsterdam, using a femtosecond to sub-millisecond pump-probe setup as described previously [9]. A 2-mm sapphire plate was used for supercontinuum white light generation, and a selected wavelength region was detected by the photodiode array. The data was acquired within a time window of -50 ps to 300 μ s, relative to time zero of the pump, with a minimum temporal step of 50 fs. The diameters of the pump and the probe beams at the sample position were $\sim$ 200 μ m and $\sim$ 70 μ m, respectively. The wavelength of the pump beam was centered at 520 or 620 nm. Global analysis was performed using Glotaran as described previously [9, 10].

5.2.12 Solid-state NMR measurements

ssNMR measurements were recorded using an Avance-I spectrometer operating at a field strength of 17.6 T, equipped with a 4 mm triple resonance MAS probe (Bruker, Karlsruhe, Germany). At this field, the ^1H resonates at 750 MHz while the ^{13}C resonates at 187.5 MHz. The optimum length of 90° proton and carbon pulse was determined on uniformly labelled ^{13}C tyrosine. For ^{13}C CP-MAS, a mixing time of 2ms with a recycle delay of 1s and total a number of 2048 scans were collected. 2D ^{13}C - ^{13}C correlation PARIS experiments were performed using a mixing time of 20 ms, 30 ms and 50 ms with a recycle delay of 1s, at a total number of 192 scans with indirect dimensions of 128 scans. ^{13}C CP-MAS experiments were measured at 10, 13 and 14 kHz spinning frequencies, to identify the

spinning side bands. All solid-state NMR measurements were performed at 236 K. The NMR data were processed in Topspin 3.2 and MestReNova.

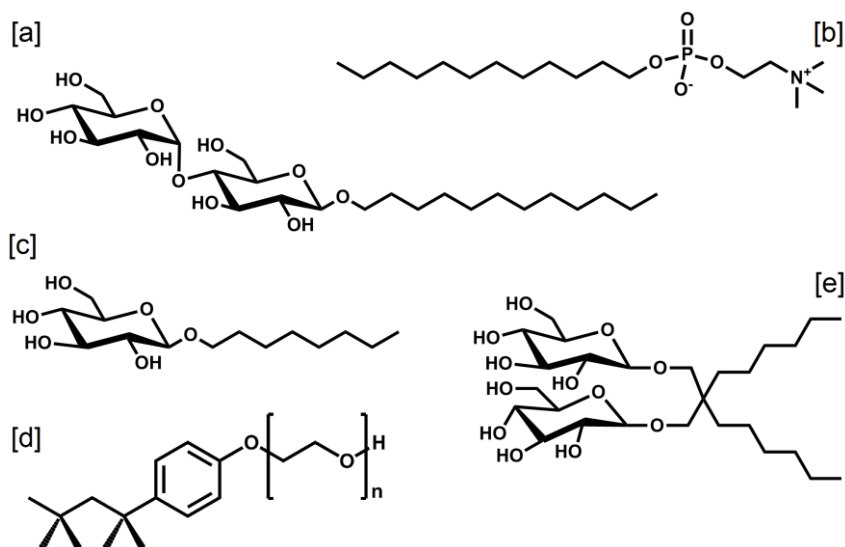


Figure 5.2 Chemical structures of detergents used in this study to solubilize and/or purify the pigments. DDM [a], DPC [b], OG [c], TritonX-100, n=9-10 [d] and OGNG [e].

5.3 Results

5.3.1 Effect of the detergent environment on properties of PR and GR

In the previous chapters, we used the mild nonionic detergent DDM for the solubilization and subsequent purification of all pigments generated. In this chapter, we compare the effect of several different detergent environments on the spectral properties and stability of wild type PR and GR (Figure 5.2). We included the popular nonionic detergents TritonX-100 and n-octyl- β -D-glucopyranoside (OG), and the recently synthesized glucose neopentyl glycol derivative OGNG, which produces smaller micelles, but has similar

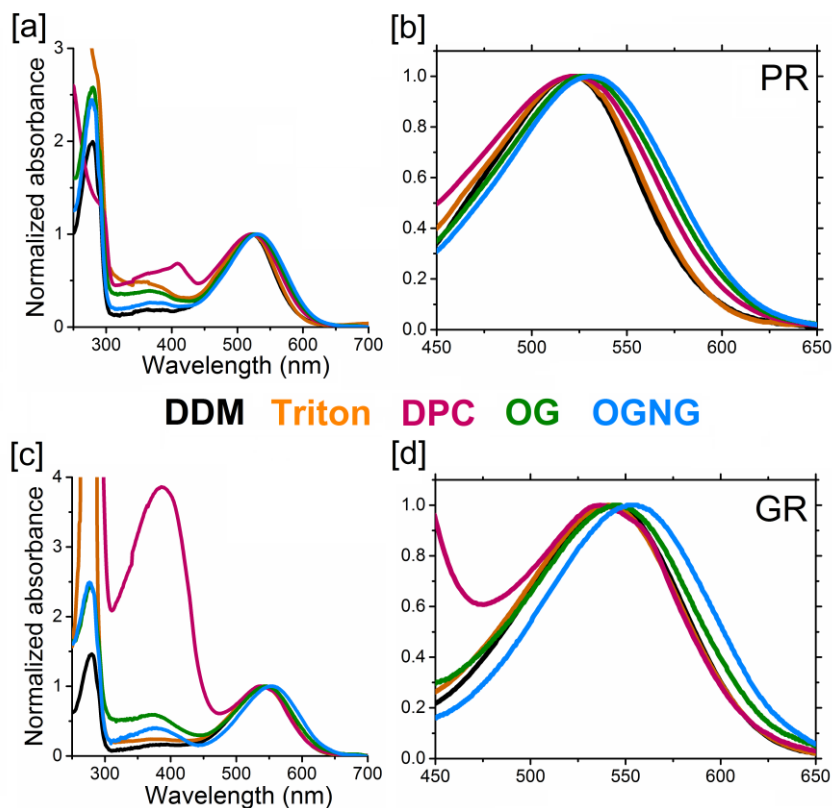


Figure 5.3 Absorbance spectra of purified PR [a,b] and GR [c,d] in various detergent environments, normalized at the main absorbance band. Color codes are as shown by the detergent abbreviations in the figure.

stabilization potential as DDM [11]. We further included the zwitterionic detergent dodecyl phosphocholine (DPC), which is commonly used to investigate membrane proteins with liquid-state NMR, since it generates relatively small mixed micelles [12].

The membrane vesicles containing PR or GR, isolated from the *E. coli* host cells, were solubilized using 2.5-5% of the detergent of choice and were subsequently purified and concentrated to a final detergent concentration of 2%. The exception to this was OGNG, which required solubilization of the pigments in 4% DDM, followed by several on-column washes and subsequent elution of purified pigment in 0.5% OGNG. The thermal

stability of the purified pigments was assessed by following the decay of the main absorbance band.

DPC was found to be the most destabilizing detergent. Even upon short solubilization for 1 h at 4°C followed by rapid purification, a strong peak was observed around 390 nm in the absorbance band of the purified protein (Figure 5.3). This band represents retinal released from denatured pigment. The destabilization was more pronounced for GR. The highest solubilization efficiency was obtained with TritonX-100 which required 1 hour solubilization at RT (or overnight at 4°C). The purified PR samples were stable at 4°C for several days (data not shown). However, GR purified in Triton did not survive RT incubation, long-term storage at 4°C, or repeated freeze-thaw cycles. OG required overnight solubilization at RT, but showed similar results to Triton in terms of stability of the pigments. Complete solubilization with DDM required 2-3 days at RT. The resulting pigments showed the highest stability and could be stored in DDM at RT for up to a week. The poorest solubilization efficiency was seen for OGNG, which achieved less than 10% solubilization of the pigments after a week of extraction at RT.

Detergent	^[a] cmc (mM)	^[b] conc (mM)	^[c] diameter (nm)	^[d] λ _{max} PR (nm)	^[d] λ _{max} GR (nm)
DDM	0.2	40	5.4	520	540
DPC	1.1	57	3.6	522	537
TritonX-100	~1	31	6.4	521	541
OG	20	65	4.3	527	545
OGNG	~1	35	nd	531	553

Table 5.1 Absorbance maximum of PR and GR in different detergent environments. ^[a] critical micelle concentration provided by the manufacturer, ^[b] detergent concentration used in this experiment, ^[c] average diameter of detergent micelle determined by DLS, accuracy ±1 nm ^[d] λ_{max} value of purified pigment at pH 8, accuracy ±2 nm. nd=not determined.

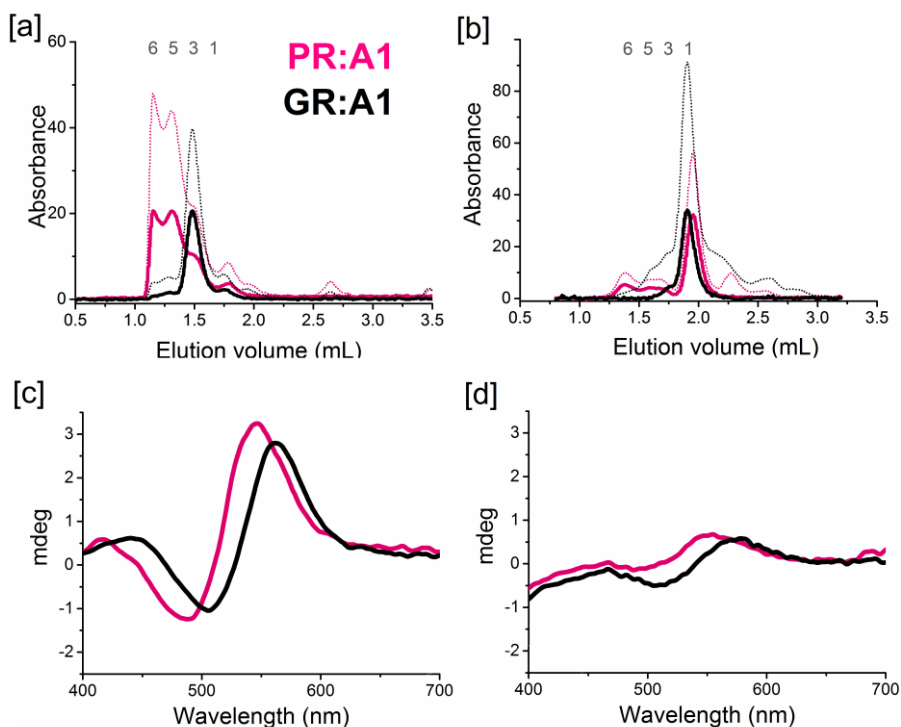


Figure 5.4 Top panel: SEC chromatograms of PR (pink) and GR (black) in 0.2% DDM [a] or 0.2% OGNG [b] dotted line, absorbance at 280 nm; solid line, absorbance ~500 nm. Bottom panel: Circular dichroism spectra in 2% DDM [c] or 2% OGNG [d].

However, the resulting pigments purified in OGNG were stable at RT for several days, similar to DDM. Solubilization with TritonX-100, DPC and DDM yield about the same absorbance maximum for the pigments (Table 5.1). However, both OG and OGNG cause a noticeable red-shift in the $\lambda_{\max}$ of PR as well as GR (5-13 nm). In view of the stability of the pigments in OGNG and their red shifted absorbance, we investigated their oligomeric status using SEC and CD. From the size exclusion pattern (Figure 5.4, top panels), we conclude that both DDM and OGNG micelles contain a relatively uniform population of PR and GR. However, while DDM maintains mainly a higher oligomeric state (hexamers and pentamers for PR, trimers for GR), OGNG micelles appear to predominantly contain smaller units, most likely dimers or monomers. The bilobal CD spectrum for PR and GR in DDM (Figure 5.4,

panel c) is in agreement with an ordered oligomeric state, since it implies close-range communication between the retinylidene ligands of the subunits. The CD spectrum of PR and GR in OGNG shows low intensity and has lost most of this bilobal character. (Figure 5.4, panel d).

5.3.2 Formation and characterization of nanodiscs

PR and GR purified in DPC, TritonX-100 and DDM were used to generate MSP-nanodiscs. The phospholipid mixture from soybean (asolectin), and *E. coli* (polar extract) were both tested as suitable lipid environments and yielded similar results. The purified pigment was mixed with lipid and MSPDE31 in a defined ratio, and the detergent was extracted with β -cyclodextrin [8]. This extraction step concomitantly cajoles the assembly of the MSP-nanodisc. The MSP-nanodisc was purified from the cyclodextrin mixture by nickel affinity chromatography exploiting the 6x-His tag on the opsin and MSP. The purified MSP-nanodiscs were characterized using absorbance spectroscopy, SDS-PAGE and DLS.

The yield of pigment MSP-nanodiscs generated with purified PR (~10% insertion of pigment) was consistently substantially lower than that obtained with GR (~95% insertion). Furthermore, the absorbance band of PR:MSP-nanodiscs at pH 8 was red-shifted by about 10 nm, relative to PR:DDM (Figure 5.5, Table 5.2). SDS-PAGE analysis of the purified PR MSP-nanodisc complexes suggests an assembly ratio of 2 MSPDE31 molecules per incorporated protein monomer (Figure 5.5). DLS measurements indicate a hydrodynamic diameter of 14-15 nm for both the PR- and GR-MSP-nanodiscs (Table 5.2). The pigments were extremely stable in the MSP-nanodisc environment and could be stored at 4°C for several months.

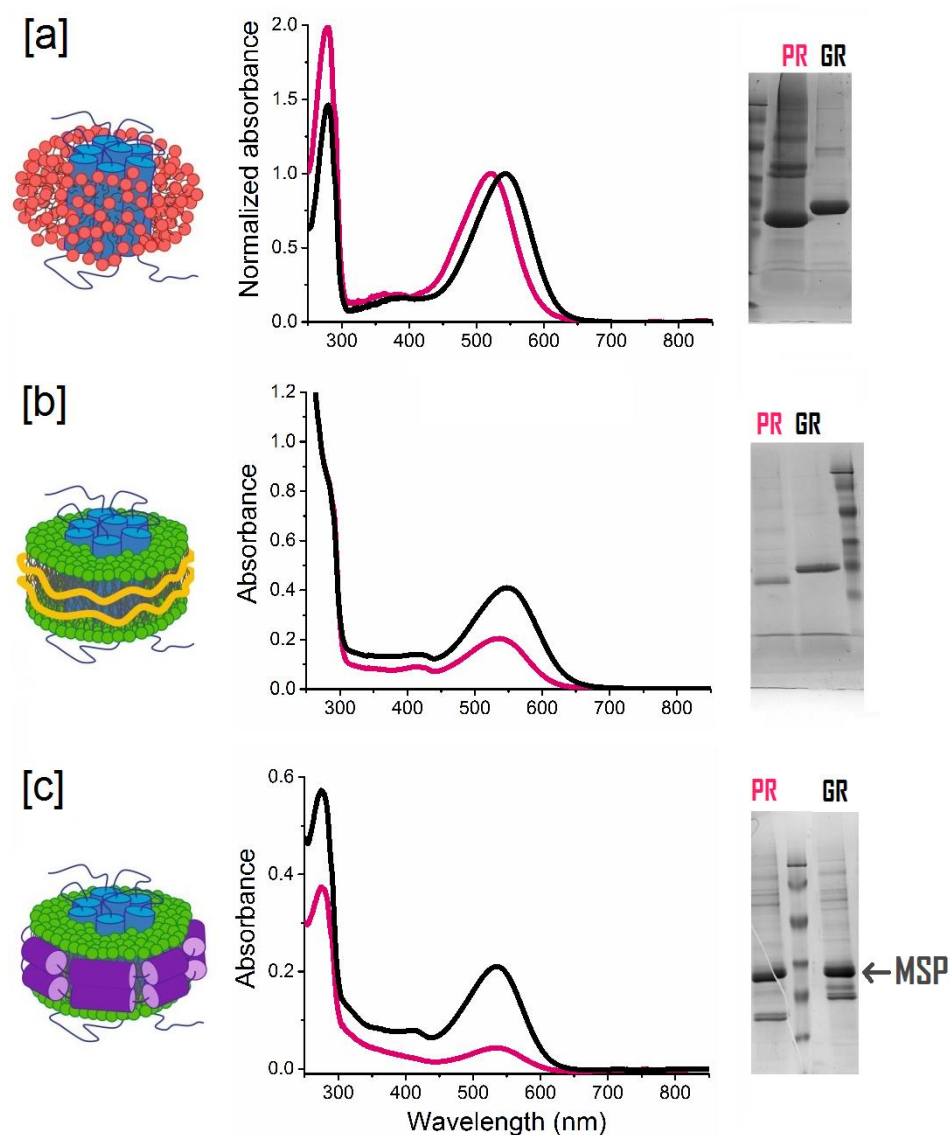


Figure 5.5 Comparison between the different soluble membrane-mimetic environments for PR (pink curves) and GR (black curves) in [a] DDM micelles, [b] SMA nanodiscs, or [c] MSP nanodiscs. Left panel: a schematic illustration of the environment with detergent molecules in red, lipids in green, SMA in yellow and MSP in purple, adapted from [13]. The middle panel shows absorbance spectra of the purified pigment-detergent/nanodisc complexes of PR (pink) and GR (black). Right panel: SDS-PAGE images of the purified fractions, lanes 1 and 2 representing PR and GR, respectively.

Effect of the microenvironment on properties of PR and GR

Environment	pigment	$\lambda_{\max}$ (nm) ^[a]	d (nm) ^[b]	yield (%) ^[c]
DDM	PR	520	nd	>90
	GR	540	nd	>95
SMA-nanodisc	PR	537	28	20
	GR	548	21	40
MSP-nanodisc	PR	531	15	10
	GR	538	14	95

Table 5.2 Comparison of purified pigment-detergent and nanodisc complexes. ^[a] $\lambda_{\max}$ determined from the absorbance bands of the pigments at pH 8, accuracy ± 2 nm. ^[b] average diameter of the nanodiscs determined by DLS, accuracy ± 1 nm. ^[c] % yield or insertion of purified pigment in-nanodisc complexes, accuracy $\pm 10\%$, nd=not determined.

To generate the SMA-nanodiscs, the cell pellets expressing the holo-protein were directly extracted with hydrolyzed SMA under high salt conditions at pH 8. The extraction required multiple rounds of sonication and incubation at RT for 5-7 days. About 60% of GR present in the *E.coli* membrane was extracted, in comparison to 30% extraction of PR. However, significant losses were incurred upon the subsequent purification steps, due to irreversible binding of these SMA-nanodisc complexes to the Ni²⁺ column. Nonetheless, the nanodiscs could be isolated with a high degree of purity, as determined from the absorbance bands of the pure fractions, and SDS-PAGE analysis (Figure 5.5). The absorbance bands of both PR and GR:SMA-nanodiscs are significantly red shifted, as compared to the absorbance of the pigments in DDM (Table 5.2). However, in contrast to the MSP-nanodiscs, the SMA-nanodiscs were not stable and did not survive long-term storage at -80 °C.

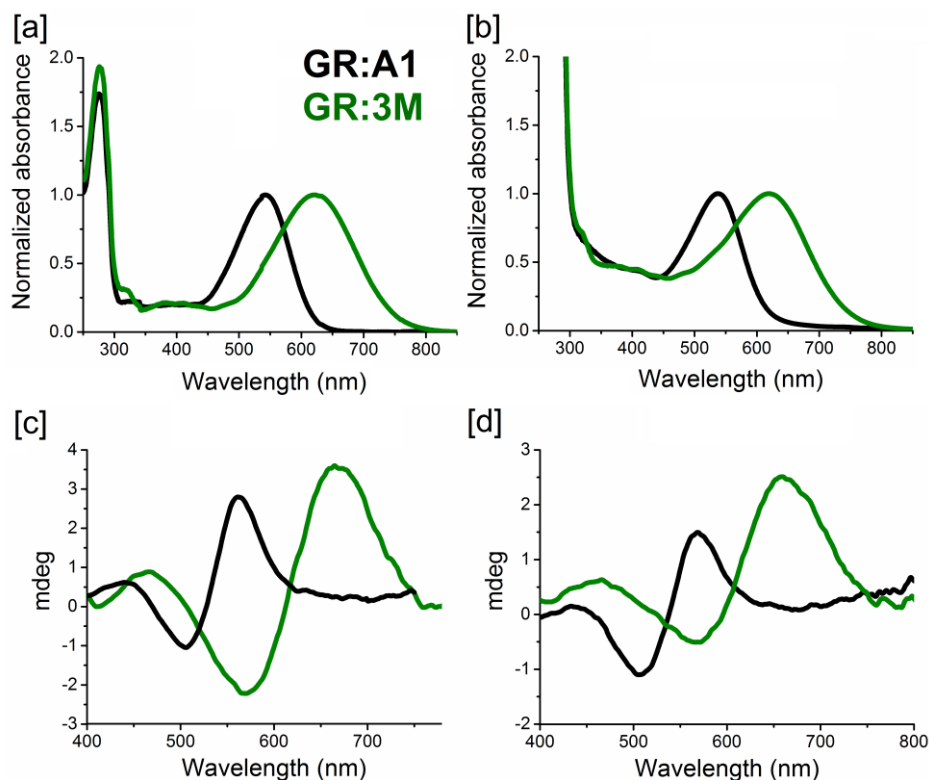


Figure 5.6 Top panel: Absorbance spectra of GR:A1 (black curve) and GR:3M (green curve) purified in 2% DDM [a] or in MSP-nanodisc complexes [b] at pH 8. Bottom panel: circular dichroism spectra of the above in 2% DDM [c] or MSP-nanodiscs [d].

5.3.3 Photocycle dynamics of GR in a nanodisc environment

The feasibility of generating and using MSP-nanodiscs to study the photocycle dynamics of the rhodopsin pigments was assessed using optical spectroscopy (Figure 5.7). GR:A1 and GR:MOA2 were taken as a first test, since the photocycle of GR:A1 has been investigated before [14] and GR:MOA2 is quite red-shifted with poor proton pumping (c.f. chapter 3). About 2-3 mg of each pigment was solubilized and purified in DDM, and subsequently inserted into MSP-nanodiscs as described above.

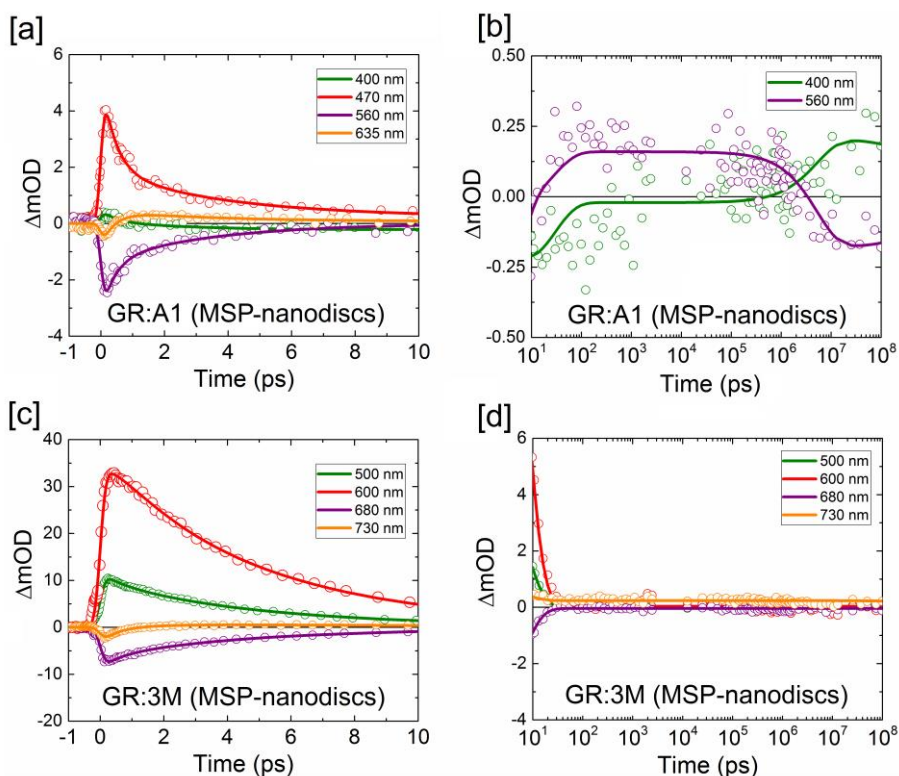


Figure 5.7 Time traces of different spectral intermediates extracted from a pump-probe transient absorption measurement of GR:A1 (top) and GR:MOA2 (bottom) in a MSP-nanodisc environment. The spectral intermediates evolve in the ps [a, c] and μ s [b, d] range. 3M stands for MOA2.

The MSP-nanodiscs were concentrated and their steady state absorbance and circular dichroism spectra were measured, which showed good agreement with results obtained with DDM (Figure 5.6). The nanodiscs were further used for pump-probe transient absorption spectroscopy, to identify early photocycle intermediates, upon excitation with 520 nm (GR:A1) or 620 nm (GR:MOA2). Figure 5.7 shows the spectral evolution of different photointermediates in the picosecond and microsecond range. For GR:A1, the characteristic photocycle intermediates of a rhodopsin proton-pump were observed. The excited state absorption was observed at 470 nm, and persisted for several picoseconds. An early 635 nm absorbance

was attributed to the vibrationally hot J intermediate, which blue shifts to 560 nm indicating the rise of a K-L equilibrium. The decay of this signal with a concomitant rise in absorbance at 400 nm (Figure 5.7, panel b) implies the deprotonation of the Schiff base and the rise of an M-state. Surprisingly, in the case of GR:MOA2, only an initial excited state absorption was observed at 730 nm, following which all the signals decayed homokinetically to the ground state.

5.3.4 Proteoliposomes as a membrane model for a solid-state NMR study of the retinal binding pocket

E. coli BL21 was used for large scale cultivation and expression of PR and GR. The cell pellets were lysed to generate the membrane vesicles containing the opsin. The opsin was regenerated using a retinal A1 labeled with ^{13}C at 10 carbons (8-15, 19 and 20; Figure 5.7), and subsequently solubilized and purified in DDM according to the protocols described in chapter 2. Proteoliposomes were generated using this purified PR and GR as a starting material. The pure protein was mixed with the *E. coli* lipid solution, following which the detergent was extracted using β -cyclodextrin as described in section 5.2.7. The resulting pink coloured proteoliposomes were pelleted and tightly packed into 4 mm NMR rotors.

1D CP-MAS and 2D ^{13}C - ^{13}C dipolar correlation spectroscopy using the RFDR and PARIS pulse programs were used to identify the chromophore resonances. The analysis of the resulting spectra (Figure 5.7) will not be explained further. They showed good resolution and relatively narrow resonances for the labelled carbons, which allowed the assignment of the chemical shifts of all ^{13}C carbons of the retinylidene chromophore in PR and GR (Table 5.3).

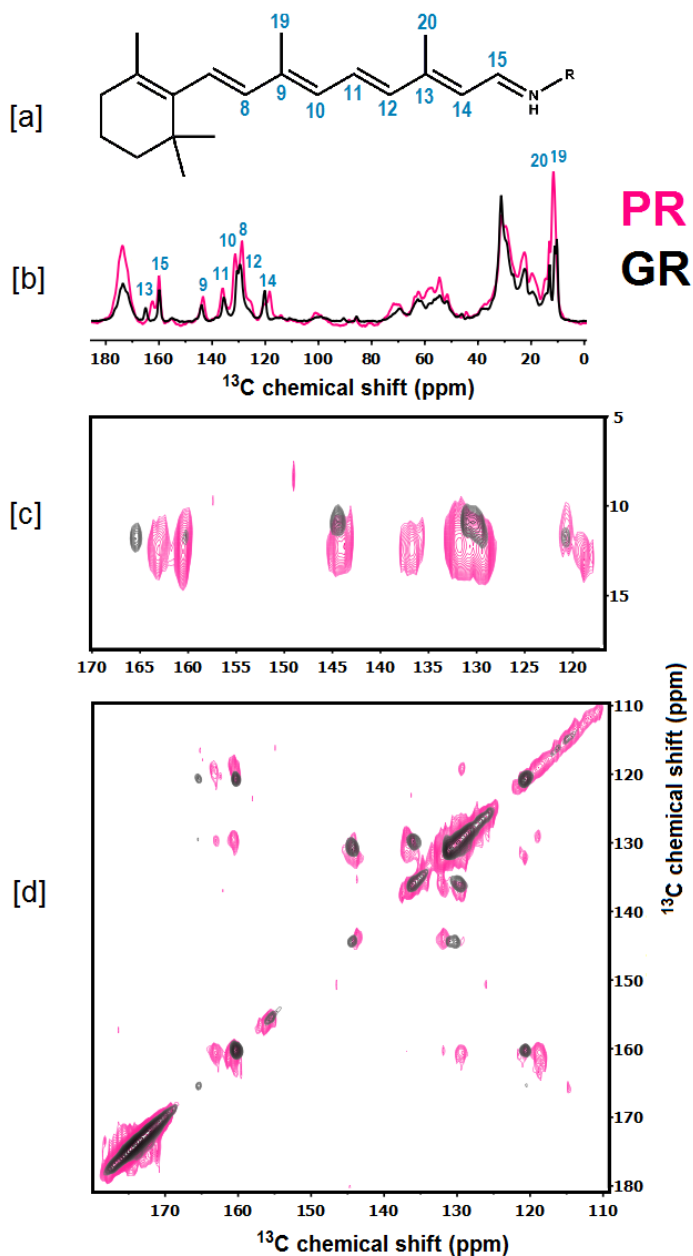


Figure 5.7 ssNMR spectroscopy of PR (pink) and GR (black) regenerated with a $^{13}\text{C}_{10}$ retinal. Chemical structure of the retinylidene group showing the positions of the ^{13}C atoms [a] 1D CP-MAS spectra [b] and 2D ^{13}C - ^{13}C RFDR [c, d] and PARIS (not shown) spectra were used to assign the chemical shifts of all the ten ^{13}C atoms of the retinylidene chromophore.

¹³ C	8	9	10	11	12	13	14	15	19	20
PR	131.7	143.9	131	136.5	129.2	162.8	118.3	160.4	12.0	12.0
GR	131.0	144.4	130	136.0	129.9	165.4	120.7	160.3	10.7	11.5

Table 5.3 Chemical shifts (ppm) of the ¹³C resonances of the retinylidene chromophore in PR and GR

5.4 Discussion

5.4.1 General properties of the studied microenvironments

Here we briefly describe the most relevant properties of the various systems used in this chapter to extract, purify and characterize PR and GR.

Detergents are amphipathic molecules, containing hydrophilic and hydrophobic segments, similar to lipids. When their concentration exceeds their individual single molecule solubility, they self-assemble in an aqueous medium by extruding water to form micelles. Detergent micelles contain a hydrophobic interior and hydrophilic exterior and associate with membrane proteins, which share this amphipathic nature, yielding soluble protein-detergent complexes (Figures 5.1, 5.5). The size of these complexes usually depends on the polar tails of the detergent. A wide variety of detergents exist with different combinations of head and tail groups [15]. Choosing a suitable detergent to solubilize and stabilize a particular membrane protein is a challenge, both because of difficulty of choice and ill-defined properties of pigment-detergent complexes.

Micellar systems are suitable for studying the photophysical properties of colored membrane proteins. However, proteins are dynamic structures, and usually undergo functional conformational changes like the opening and closing of channels. The loss of protein-lipid and lipid-lipid interactions makes these pigments vulnerable to destabilization in the micellar

environment. The lateral pressure induced by the membrane has a stabilizing influence on membrane proteins. Therefore a number of systems have been devised which simulate the membrane environment of these proteins.

MSP-nanodiscs are discoid monodisperse open complexes, which consist of the protein of interest embedded in a phospholipid bilayer, enclosed by two amphipathic MSP molecules (Figures 5.1, 5.5). These nanodiscs are usually 9-20 nm wide, with the diameter of the nanodisc controlled by the length of the MSP used [16]. SMA-nanodiscs should be an attractive alternative to MSP-nanodiscs, since they do not require prior solubilization of the protein by detergents. The small size of these nanodisc systems leads to optical near-transparency of the sample, due to which nanodiscs have been widely used in a variety of spectroscopic techniques.

While nanodiscs work well to simulate the fluidity and lateral pressure of a membrane, the impact of membrane curvature and protein (hetero)-oligomerization on structural and functional properties can become lost in this environment. Furthermore, vectorial properties of the proteins, such as proton transport, are not easily studied in these open systems [17]. For such studies, the closed proteoliposome system is much more suitable. Proteoliposomes are 10-100 times larger than nanodiscs and their light-scattering properties render them less suitable for optical studies. However, they are widely used for the structural determination of membrane proteins by ssNMR, in functional assays and in a variety of biotechnological applications.

5.4.2 Differences between the detergent systems investigated

The choice of surfactant has an important effect on the oligomeric distribution and stability of membrane proteins. The nonionic sugar based detergent DDM [18] has been extensively used in membrane protein

studies. DDM forms large well-structured micelles (56-71 kDa) thereby stabilizing protein dynamics and protein-protein contacts and minimizing denaturation [15]. Previous studies have shown that the native oligomeric assembly of PR is preserved in DDM micelles, which contain a predominant fraction of hexamers and a minor monomeric population [19]. This agrees with our analyses (Figure 5.4). However, the large protein-detergent complexes formed by DDM make it less suitable as a detergent of choice for crystallization trials, and for structural studies using solution-state NMR. Smaller protein-detergent complexes, which would stabilize a solubilized monomer of these proteins are highly desirable. Here, we compare a number of commonly used detergent environments, and their influence on the thermal stability, oligomeric structure and spectral properties of PR and GR.

A variant of the zwitterionic detergent DPC was recently used to solve the solution NMR structure of PR [20]. DPC forms much smaller micelles (18-21 kDa) and is hence more useful to isolate the monomeric form of these proteins. However, in our study, DPC was found to be the most destabilizing of all detergents tested. Despite rapid solubilization and purification steps in the cold, extensive denaturation of in particular GR was observed accompanied by release of retinal (Figure 5.3). We thereby decided to focus on nonionic detergents such as TritonX-100, OG and OGNG, which also form smaller protein-detergent complexes. Prior studies have shown that solubilization of PR with 13% TritonX-100 stabilizes a monomeric red-shifted fraction of PR [21]. However, we found that though the solubilization efficiency of TritonX-100 was high, at concentrations higher than 10% it did not stabilize the pigments sufficiently for long-term storage or further characterization. Despite its poor solubilization potential, OGNG came out as the most stabilizing detergent of this triad, with no significant loss of intact pigment, even upon incubation at RT for several days. We thereby decided to investigate the oligomeric distribution

of PR and GR in OGNG using SEC and CD, so as to compare it to the well-characterized DDM microenvironment. From the SEC distribution, we conclude that in OGNG micelles, both PR and GR most likely largely exist as a monomeric fraction.

The CD spectra of PR and GR in DDM exhibit a strong bilobal bandshape (Figure 5.4c), centered around the absorption maxima of the pigments. Such a bandshape was also observed in the CD spectrum of bR [22, 23] and XR [24]. It is thought to originate from an oligomeric complex of the pigment due to excitonic interactions between the retinal molecules, and an asymmetric conformation in the protein. Hence, such a strong bilobal bandshape is not expected for monomeric units of these pigments. Indeed, the CD spectra of the pigments purified in OGNG (Figure 5.4d), particularly PR, show a strong reduction in the bilobal structure. Previous CD spectra of the pigments in a high concentration of 15% Triton-X 100 have shown an unordered mono-peak absorbance band, indicating a purely monomeric fraction (data not shown). We interpret this as supporting the spectral and SEC data in that predominantly PR or GR monomers are present in OGNG micelles, which have low CD activity in their main absorbance band.

In OGNG micelles, a significant red-shift in the absorbance bands of both PR and GR was observed relative to DDM micelles (Table 5.1). Saccharide headgroups are likely to have minimal effect on the retinal-protein interaction. Besides, such a red-shift is also observed for PR in SMA- as well as MSP-nanodiscs and for GR in SMA nanodiscs (Table 5.2). Rather, the oligomeric state of PR and GR will have a predominant impact. It was shown that in the hexameric and pentameric complexes of PR, the pKa of D97 (6.5-6.7) is about one unit lower than in monomeric PR (7.4-7.8) [25]. Presumably this originates in the cross-protomer interaction between H75-W34, which reduces the weakening effect of H75 on the acidity of D97 [22]. The absence of this H75-W34 interaction in the monomer thus enhances

protonation of D97 in the pH range 6-9, thereby inducing a red-shift. This is further evidence for a monomeric state of PR in OGNG micelles. The red-shift in the absorbance band of GR however is still unexplained, since the equivalent counterion D121 in GR has a lower pKa (≈ 4.5) and its absorbance band shows little pH dependence in the range 6-9 (Table 2.1).

5.4.3 Differences between bilayer systems

We investigated the potential of different bilayer systems, namely the open soluble nanodiscs and closed insoluble proteoliposomes, and tested their application towards the structural and functional characterization of PR and GR in a membrane environment.

We generated two different types of nanodiscs, namely MSP and SMA-nanodiscs. Generation of SMA-nanodiscs requires longer incubation steps, and we noticed that the SMA-nanodiscs had poor thermal stability, despite the native membrane environment. The multiple sonication steps could have had an impact on the structure of the resulting complexes. We further observed that in general, GR was more readily incorporated into the nanodisc environment than PR. This is attributed to an oligomeric assembly of the pigments in the membrane.

Various studies have shown that PR exists predominantly as hexamers or pentamers in the membrane environment via crystallography of blue absorbing PR [26, 27], pulsed EPR [28], mass spectrometry [29] and atomic force microscopy in lipid bilayers [30]. Crosslinking studies have further shown that hexamers of PR can be found in the native *E. coli* membrane, indicating that this assembly is physiological [25]. A previous study showed that PR was reconstituted into MSP-nanodiscs as photoactive monomers after solubilization with TritonX-100 [21]. Considering the diameter of the MSP-nanodiscs, we generated, (14-15 nm) and that of the oligomeric PR complexes (ca 10 nm; [22, 25]), we conclude that the predominantly hexa-

meric population of PR is too large to be comfortably contained within a MSP-nanodisc. Thus, we primarily isolate nanodiscs containing the smaller monomeric fraction of PR, which explains the much lower yield of PR nanodiscs, as compared to GR nanodiscs. This is further corroborated by the red-shifted absorbance bands of the purified PR-nanodiscs relative to DDM micelles (c.f. previous section), due to the higher pKa of D97 in the monomers. On the other hand, GR is thought to exist as a trimer in DDM [31], which agrees with our data (Figure 5.4) and is likely to represent its physiological form in the membrane. Presumably, trimers of GR would easily incorporate within MSP-nanodiscs, since trimeric bR and LHCII, proteins of similar size, are well contained in these nanodiscs [7, 32].

The puzzling observation of the significant red-shift of PR (17 nm) and GR (8 nm) after incorporation into SMA-nanodiscs (Table 5.2) is still unexplained. The measured size of these nanodiscs (diameter of 28 and 21 nm, for PR and GR respectively), suggests that they could easily contain PR and GR in their oligomeric state. With better yields, analysis by CD spectroscopy would probably have brought more insight. Currently, we cannot exclude that the harsh conditions required to generate the SMA-nanodiscs has led to conformational deformation, affecting protein structure as well as protein-ligand and protomer-protomer interaction. Possibly, supplementing with extra lipid during protein extraction would alleviate these conditions, as recently reported for some G protein-coupled receptors [33].

5.4.4 Potential applications of bilayer systems

Due to the high thermal stability of MSP-nanodiscs, and the ease of incorporation of a physiologically relevant assembly of GR, we decided to test GR:MSP-nanodiscs as a biomimetic system for kinetic spectroscopy. Using pump-probe spectroscopy, we could observe the early photointermediates in the photocycle of GR:A1 up until the formation of

the M-intermediate that corresponds to the deprotonation of the Schiff-base. However, we observed little evidence of a photocycle for the red-shifted GR:MOA2, as the photointermediates could not be resolved (Figure 5.7c and d). This is in line with previous measurements in DDM (data not shown) which suggests that GR:MOA2 does not have an active photocycle under these experimental conditions, which in part could account for its low proton pumping activity reported in chapter 3. MSP-nanodiscs thus have potential in characterizing the photocycle dynamics of these proteins in a natural membrane environment.

Proteoliposomes are less suited for kinetic spectroscopy due to the light scattering induced by their large size. However, they are much more suited for structural studies using ssNMR. Here we employed proteoliposomes embossed with PR and GR containing a $^{13}\text{C}_{10}$ labeled retinal chromophore. Using high-resolution 1D CP-MAS and 2D correlational spectroscopy, we could assign the chemical shift values of all ^{13}C atoms of the retinylidene chromophore in the binding pocket of PR and GR. Further discussion of these data is outside the scope of this thesis. This first-stage effort will pave the way towards a full structural assignment of PR and GR in a natural lipid micro-environment.

5.5 Conclusion

We tested various detergent and membrane based biomimetic systems to assess their effect on the spectral properties, thermal stability and structural state of PR and GR. While there are advantages and drawbacks inherent in each biomimetic system, the ultimate decision of which system to use depends on the research question and technique of choice. While a micellar environment is usually preferred for crystallographic studies, selection of a proper detergent is critical. From our analysis, OGNG appears to be a suitable candidate for small protein complexes, however it is less suited for multimeric protein assemblies. As an emerging alternative, SMA-

and MSP-embedded membrane proteins may have strong potential in lipid-based crystallization screens utilizing cubic and sponge-phase systems. We further recommend the MSP-nanodisc system for more demanding spectroscopic studies of smaller protein complexes. These nanodiscs can also be primed for transport studies. For non-crystallographic structural studies proteoliposomes are the system of choice in combination with solid-state NMR.

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