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Effect of lipid bilayer properties on the photocycle of green proteorhodopsin

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ABSTRACT

The significance of specific lipids for proton pumping by the bacterial rhodopsin proteorhodopsin (pR) was studied. To this end, it was examined whether pR preferentially binds certain lipids and whether molecular properties of the lipid environment affect the photocycle. pR's photocycle was followed by microsecond flash-photolysis in the visible spectral range. It was fastest in phosphatidylcholine liposomes (soy bean lipid), intermediate in 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonate (CHAPS): 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) bicelles and in Triton X-100, and slowest when pR was solubilized in CHAPS. In bicelles with different lipid compositions, the nature of the head groups, the unsaturation level and the fatty acid chain length had small effects on the photocycle. The specific affinity of pR for lipids of the expression host *Escherichia coli* was investigated by an optimized method of lipid isolation from purified membrane protein using two different concentrations of the detergent *N*-dodecyl-β-D-maltoside (DDM). We found that 11 lipids were copurified per pR molecule at 0.1% DDM, whereas essentially all lipids were stripped off from pR by 1% DDM. The relative amounts of copurified phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin did not correlate with the molar percentages normally present in *E. coli* cells. The results indicate a predominance of phosphatidylethanolamine species in the lipid annulus around recombinant pR that are less polar than the dominant species in the cell membrane of the expression host *E. coli*.

1. Introduction

Proteorhodopsin (pR) is a natural photoactive protein embedded in the lipid bilayer of many bacteria found in the sea and salt lakes. First discovered in the gammaproteobacteria 'SAR86' group [1], it was subsequently also found in alphaproteobacteria [2], archaea [3], bacteroidetes [4], eukaryotes [5] and viruses which are thought to have acquired the pR gene from bacteria [6].

Like the archaeal bacteriorhodopsin (bR), pR contains the retinal chromophore bound via a Schiff base linkage to a lysine residue in the seventh transmembrane segment (Lys231 in pR) and adopts several intermediates after light excitation. The photocycle of pR is shown in Fig. 1. It is similar to that of bR in which light excitation of the retinal induces conformational changes in the protein, resulting in the translocation of a proton across the membrane. Since the chromophore retinal exhibits a high spectral sensitivity towards its environment, the photocycle can be monitored by the transient absorbance changes at different wavelengths [7]. In the first step of the photocycle, the retinal isomerizes from all-*trans* to 13-*cis* upon transition from the pR ground state (named pR 520 in Fig. 1) to the K intermediate. This is followed by deprotonation of the Schiff base and protonation of the primary proton acceptor Asp-97 upon formation of the M state ($\lambda = 410$ nm), which may consist of two substrates M_1 and M_2 [8]. The decay of the M-intermediate reflects reprotonation of the Schiff base and gives rise to the formation of late intermediates. The first of them is observed near $\lambda = 560$–580 nm and is termed O [7] or N [8]. The following intermediate absorbs near the pR absorption maximum and is termed N by Friedrich et al. [7] who observe it near 530 nm, but is named pR ′(O) by Varo et al. [8] who consider its absorption as indistinguishable from that of pR. Eventually, the retinal isomerizes back to the all-*trans* configuration by thermal relaxation and the proton gradient created by pR is used for ATP synthesis. In Fig. 1 and in the following, we name the intermediates according to Friedrich et al. [7].

Abbreviations: bR, bacteriorhodopsin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; CL, cardiolipin; DDM, *N*-dodecyl-β-D-maltoside; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMoPC, 1,2 dimyristoyl-*sn*-glycero-3-phosphocholine; DOPA, 1,2-dioleoyl-*sn*-glycero-3-phosphate; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; pR, proteorhodopsin; TOCL, tetraoleoyl cardiolipin; TLC, thin layer chromatography.

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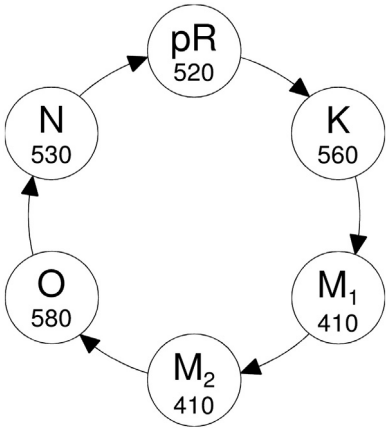

Fig. 1. Simplified photocycle of pR according to [8] in the terminology of [7]. Numbers denote the absorbance maximum of the respective state. The photocycle starts with excitation of the ground state of pR which is named pR 520 in the above scheme.

The native lipid environment of green absorbing proteorhodopsin (Uniprot accession code: Q9F7P4) is not precisely known as pR studied so far has been recombinantly overexpressed in *Escherichia coli* (*E. coli*). The *E. coli* membrane is a valid starting point for testing the lipid effect on pR's photocycle because the marine bacteria, in which pR was originally discovered, have a similar phospholipid composition as *E. coli* [2,9–14]. The head group composition of phospholipids in *E. coli* remains constant over a broad spectrum of growth conditions [15]. The aminophospholipid phosphatidylethanolamine (PE) is the major phospholipid in *E. coli* constituting 70–80% of the total phospholipid content. It is zwitter-ionic at physiological pH due to the protonated amino group and the negatively charged phosphate group. PE is accompanied in the membrane by the two anionic lipids, phosphatidylglycerol (PG) and cardiolipin (CL), accounting for 15–20% and ≤5% of the membrane phospholipids, respectively [16].

In order to understand more about the conditions affecting pR's photocycle, detailed knowledge about the interactions of wild type green pR with membrane lipids is important. It is well known that lipids can affect the activity of membrane proteins through stable or dynamic interactions. As an example, charged protein residues can readily interact with lipids through electrostatic interactions and form hydrogen bonds (H-bonds) with lipid head groups [17]. It has been observed that the hydrophobic thickness of the lipid bilayer is an important factor for the correct topology and insertion of a membrane protein and consequently for its optimal function [18,19]. Many membrane proteins are known to prefer specific lipids, for example the Ca^{2+} ATPase prefers phosphatidylcholine (PC) over PE for optimum activity [20], the respiratory complexes II–IV are CL dependent [21], and KcsA requires anionic lipids for optimum activity [22]. The activity of bR, is reported to be influenced by several lipids [23,24], and a brief treatment of the purple membrane with low concentrated detergent causes changes of the bR photocycle without disrupting the trimer structure of bR [24] indicating a strong dependence of bR activity on native membrane lipids. This assertion is verified by the recovery of normal photocycle behavior by incubating the disrupted membranes with a total extract of the native lipids from the purple membrane [24].

pR has earlier been studied in different lipid and detergent environments [7,25]. However, no systematic investigation has been done regarding the effects of lipids on the photocycle. Therefore, the aim of our research was to identify and understand how the lipophilic and amphiphilic environments shape pR activity. In this study, we have mainly used bicelles, which are also known as mixed micelles, consisting of a mixture between CHAPS and a phospholipid. Specifically, we tested the influences of different types of lipid head groups, various fatty acid chain-lengths and the extent of saturation in the fatty acid chain on the pR photocycle. Furthermore, we analyzed lipids that are copurified with pR using two different detergent concentrations in order to detect whether pR specifically binds certain lipids. Taken together, we observed that an intact membrane is beneficial for optimum function but that particular lipid properties have only small effects.

2. Materials and methods

2.1. Chemicals and buffers

Most lipids used in our experiments were purchased from Avanti Polar Lipids (Alabaster, AL). Soy bean type II-S lipids and CHAPS were purchased from Sigma Aldrich and *N*-dodecyl-β-D-maltoside (DDM) from Affymetrix. Other chemicals were all obtained from Sigma Aldrich.

2.2. Cloning, growth, purification and reconstitution of unlabelled pR

The wild type green pR gene was synthesized by Eurofins MWG Operon (Ebersberg, Germany) and cloned into a pET27b+ (Invitrogen) vector using NdeI and XhoI as cloning sites [7]. A C-terminal His_6-tag was included in this construct to facilitate pR purification with Ni-NTA affinity purification. C43 (DE3) [26] was used as an expression host for pR. pR was expressed and purified as described by Pfleger et al. [27] with the exception that DDM was exchanged for CHAPS during the Ni-NTA purification step for pR intended for bicelle studies.

Proteoliposomes from soy bean phosphatidylcholine were prepared by a freeze–thaw procedure. In brief, 30 mg lipids dissolved in chloroform were dried in a round bottomed flask by a stream of nitrogen and 2 h of vacuum. The lipids were then resuspended in 3 ml reconstitution buffer (50 mM MOPS, pH 7.5, 50 mM NaCl) and vortexed for 10 min at room temperature under nitrogen atmosphere. Liposomes were made unilamellar and homogeneous in size by a tip sonicator on ice (Sonics, VCX130PB, 6 cycles, 30 s pulse, 30 s pause, 40% output). For reconstitution, 100 μl of 1 mg/ml pR was added to 1 ml of liposome suspension and frozen in liquid nitrogen and thawed in water. The freeze–thaw cycle was repeated once. For flash photolysis measurements, 100 μl of liposomes was diluted with 400 μl reconstitution buffer.

We checked our reconstitution of pR into liposomes using a pH sensitive dye on the inside of the liposomes. The light induced proton pumping experiments indicate that pR is functionally incorporated and that the majority of pR molecules are oriented such that they pump protons to the inside of the liposomes.

2.3. Overexpression and purification of pR for the analysis of tightly bound lipids

In order to analyze lipids that bind to pR with high affinity, phospholipids were radioactively labeled during pR expression by supplying the growth medium with [1-^{14}C] sodium acetate (Perkin-Elmer). Two ml of overnight culture was used to inoculate 200 ml of 2× Luria Bertani (LB) medium supplemented with 50 μg/ml kanamycin and 1 μCi/ml ^{14}C acetate (Large culture). The culture was grown at 37 °C at 200 rpm. When OD_{600} reached 0.8, isopropyl-β-D-1-thiogalactopyranoside (IPTG) and all-*trans* retinal (Sigma-Aldrich) in ethanol were added to final concentrations of 1 mM and 0.44 mM, respectively. The cells were kept at 37 °C, 200 rpm for 4 h and harvested by centrifugation (3000 ×*g*) for 20 min at 4 °C. The cells were suspended in 50 mM MES buffer pH 6.0, containing 300 mM NaCl, and disrupted mechanically with a glass homogenizer. The cell lysate was centrifuged for 1 h at 3700 ×*g* in a top bench centrifuge at 4 °C and the membrane pellet was further solubilized for 48 h in 50 ml of 50 mM MES pH 6.0 buffer, containing 300 mM NaCl, 5 mM imidazole and either 1% or 0.1% DDM.

Detergent solubilized protein was obtained by centrifugation at 150,000 ×*g* for 45 min at 4 °C and the supernatant was incubated with 4 ml of Ni-NTA beads overnight at 4 °C. After binding of pR to the resin, the resin beads were washed with wash buffer containing 0.1% Triton and 50 mM imidazole, followed by a second wash with 900 ml

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