Volume 263, number 2, 308-312 FEBS 08356 April 1990

# Oriented incorporation of bacteriorhodopsin into the lipid shell of phospholipid-coated polymer particles

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Received 5 March 1990

Phospholipid monolayers covalently fixed to spherical polymer carriers spontaneously form a stable bilayer-like structure by adsorption of further added phospholipids. Bacteriorhodopsin could be incorporated into the lipid bilayer growing on these particles. From the direction of proton pumping as well as from freeze-fracture electron micrographs and papain digestion, experimental evidence was obtained that the preferred orientation of bacteriorhodopsin within the lipid coat is 'inside-out' (more than 90%). A 'right-side-out' incorporation of bacteriorhodopsin could be demonstrated, if the negative charge density of the carrier surface was lowered by chemical modification.

Phospholipid-coated bead; Membrane model; Bacteriorhodopsin

## 1. INTRODUCTION

New methods have been developed to form stable lipid-membrane systems suitable for the incorporation of membrane proteins [1]. In contrast to the adsorptive assembly of phospholipids at solid surfaces, it was our approach to bind covalently a monolayer of phospholipids via their headgroups to the surface of spheric carrier particles. Such systems tend to form a stable bilayer arrangement, if further phospholipid is added. Membrane-bound proteins may be inserted with their hydrophobic anchors in this artificial system. Integral membrane proteins with membrane-spanning domains and bulky segments at both sides of the membrane should only be incorporated, if a hydrophilic spacer of appropriate length would be introduced between the carrier surface and the lipid headgroups.

The co- and post-translational insertions of proteins into biomembranes are directed by signal sequences, which determine both the target membrane and the protein orientation [2]. In contrast, incorporation of already processed and isolated proteins into artificial lipid systems normally follows a random distribution [3]. Because bacteriorhodopsin (BR) tends strongly to an oriented assembly, it could be a good example for the exception to this rule. BR, the light energy converting protein of the purple membrane of *Halobacterium halobium*, consists as a monomer of 7 membrane-spanning $\alpha$-helices connected by short loops which are exposed to the aqueous environment. When illuminated, light-adapted BR undergoes a photocycle with a number of intermediates (for a recent review see [4]) and proton translocation from the cytosolic side of the plasma membrane to its external side is linked to this cycle [5].

BR can be incorporated into liposomal membranes [6]. A preferred 'inside-out' orientation of BR into liposomes indeed was found together with a minor inversely pumping fraction [7]. This phenomenon was discussed in terms of the existence of two inversely pumping vesicle populations, but it was impossible to prove that all BR molecules of a discrete vesicle occur in the same orientation [8]. Due to the excess of negative charges at the cytosolic side of the purple membrane (PM) at neutral pH, the protein is incorporated in an orientation inverted with respect to that in vivo. Therefore BR-containing liposomes normally accumulate protons when illuminated. Happe et al. [9] demonstrated the formation of BR-containing liposomes in 'right-side-out' orientation if the pH was lowered to 2.5 and in this way the negative surface charge of the liposomes was eliminated. Ihara and Mukohata [10] in a similar way prepared 'right-side-out' BR-containing liposomes using blue membranes at pH values below 4.

Here we deal with specific incorporation of BR 'inside out' or 'right-side-out', into the lipid coat of

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*Abbreviations:* BR, bacteriorhodopsin; PE, phosphatidylethanolamine; EDC, 1-ethyl, 3-(3-dimethylaminopropyl), carbodiimide; PM, purple membrane



*Published by Elsevier Science Publishers B.V. (Biomedical Division)*
00145793/90/$3.50 

phosphatidylethanolamine-coated Callocryl particles by varying the negative surface-charge density.

## 2. MATERIALS AND METHODS

The carrier used, (Callocryl, VEB Chem. Komb. Piesteritz, GDR), was a polymerized linear poly(methylmethacrylate). The mean diameter of the spherical carrier beads was 7.1 ± 0.4 μm. The lipid used was PE purified from egg yolk according to [11]. EDC was purchased from Serva (Heidelberg, FRG) and the methylester of penta-alanine $(Ala)_5$-OMe was synthetized by Dr Jahreis from the Institute of Biochemistry in Halle. The lipidation of the particle surface and the introduction of a penta-alanine spacer was done as previously described [1]. In short, a stirred suspension of 20 g Callocryl was activated at pH 3.5 for 5 min with EDC (1:1 molar ratio, EDC to carrier-bound carboxylic groups). $(Ala)_5$-OMe (200 mg) was added to the washed and resuspended activated carrier beads and the suspension was shaken overnight at pH 8.5. The penta-alanyl methylester on the carrier beads was hydrolized with 1 M NaOH for 5 h. The free terminal carboxylic group of the spacer was activated again with EDC and reacted with a sonicated PE-dispersion for 2 days at pH 8.5. The negative surface charge density of the beads was lowered by incubation of the particles after lipidation with 0.1 M EDC at pH 8.5 for 12 h. Positive charges could be introduced, if the reaction was carried out under the same conditions, but in a solution containing 1 M ethylenediamine.

PMs were purified from the cell membrane fraction of *Halobacterium halobium* [12]. PM was treated with 1% β-D-octylglucoside for 24 h at 20°C to solubilize and monomerize the BR molecules. Their insertion into the PE-$(Ala)_5$Callocryl was done by incubating the particle suspension with monomerized BR at pH 5 for 2 min at 55°C.

Desorption of the incorporated BR together with the noncovalently fixed lipids from the carrier surface was accomplished either by washing with a 10-fold volume of 1% Triton X-100 or by successive incubation of the beads with methanol, butanol, methanol and water. It is possible to relipidate the carrier beads by dialyzing the delipidated particles with lipid solubilized in 1% octylglucoside for 3 days. BR could be incorporated into this regenerated matrix to the same extent as before.

Proton pumping activity of BR was measured after dilution of the particle suspension with basal salt solution (growth medium without pepton; pH 7.4). The suspension was illuminated with light from a 950 W projector filtered through an orange glass filter ($\lambda > 500$ nm; about $10^5$ lux). Light-induced pH changes were recorded using an assembly of a combination electrode with an amplifier and a recorder. After each experiment the system was calibrated by successive addition of 1 μl injections of 10 mM HCl and 10 mM NaOH.

The BR-coated particle suspension in 25 mM Tris/HCl buffer (pH 7) and 1 mM dithiothreitol was incubated with 5 μg/ml papain (Serva, Heidelberg, FRG) at 30°C [13]. At different times aliquots were taken and mixed with SDS solution (1% final concentration). The supernatants of the digested samples were analyzed by electrophoresis in 12.5% polyacrylamide gels containing 0.1% SDS [14]. The Coomassie stain of gels was recorded with an Elphor scanner.

Phospholipid concentrations at the particle's surface were determined by phosphorous analysis [15], and the BR concentration was measured spectroscopically using an extinction coefficient of 63 000 $M^{-1}cm^{-1}$

Freeze-fracturing of the lipidated BR-containing particles was carried out after pelleting and quenching by plunge freezing with a Balzers BA 360. The freeze-fracture was done at −100°C. The surface was decorated with a thin layer (1 nm) of Pt/C at an angle of 45°. Electron microscopy was performed using a Phillipps EM 420. Optical diffraction patterns (power spectra) from electron micrographs of the particle's surface were examined in order to select regions of the field showing good crystalline order. The selected fields were subjected to correlation averaging according to [16]. Image processing was performed using the Semper system [17,18] and the EM system [19].

## 3. RESULTS AND DISCUSSION

In a preceding paper [1] we demonstrated that BR could only be tightly fixed at lipidated polymeric carrier beads, if a hydrophilic 16 Å spacer was introduced between the carrier surface and lipid head groups. Nearly 20% of the surface carboxyl groups reacted with a penta-alanine spacer. During the lipidation procedure most of the spacer carboxyl groups coupled with PE, but the surface carboxyl groups remained unaltered and a net negative surface charge remained. Extending this work we now incorporated BR under a variation of the surface charge density on the PE-$(Ala)_5$-Callocryl beads. If the BR assembly in liposomes depends on the surface charge density [9,10], the elimination of unreacted methacrylate carboxyl groups at the particle surface after lipidation could influence the orientation of BR, although no direct contact between BR and the surface of the negative particle exists.

At alkaline pH not only amino but also carboxyl groups reacted with EDC and formed stable noncharged N-acylurea derivatives [20]. Titration curves of the particle suspension before and after charge neutralization are shown in Fig. 1. More than 75% of the carboxyl groups in the lipidated particles were neutralized by this procedure. An introduction of positive charges at the particle surface was possible by coupling of ethylenediamine to the EDC-activated carboxyl groups (Fig. 1) under the same conditions.

The results in Fig. 2 show that BR was incorporated into the lipid coat of the carrier beads in both directions. Without chemical modification of the residual surface carboxyl group, the incorporation of BR was

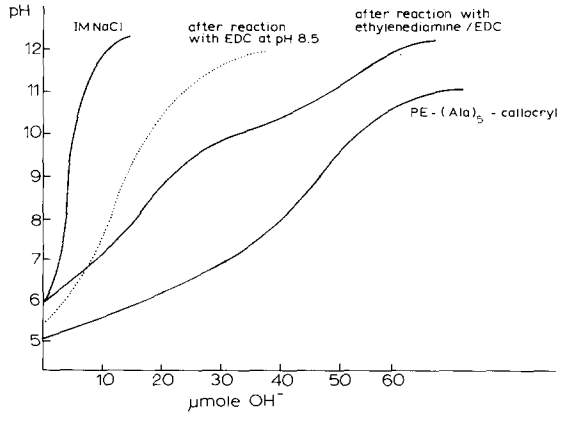


Fig. 1. Titration curve of modified PE-$(Ala)_5$-Callocryl beads. After lipidation the carrier beads were modified by incubation of 1 ml sedimented PE-$(Ala)_5$-Callocryl with 200 μmol EDC or with 200 μmol of both EDC and ethylenediamine for 12 h at pH 8.5. The carrier beads were converted into the COOH form, washed free of excess HCl, incubated in 6 ml 1 M NaCl and titrated with 0.2 N NaOH.

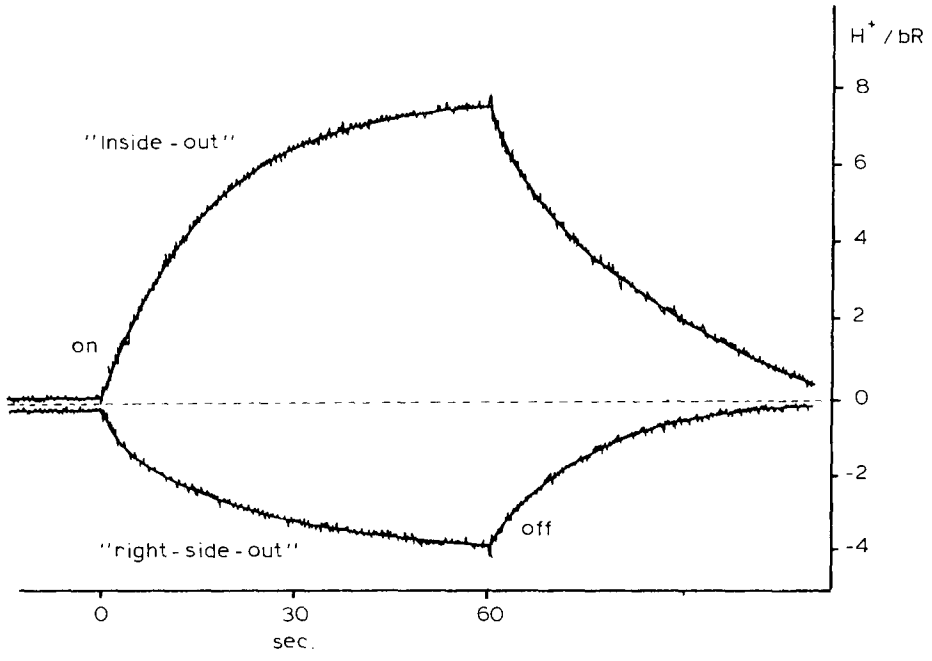


Fig. 2. Light-induced pH changes of PE-$(Ala)_5$-Callocryl beads containing BR in two orientations. Measurements were done with particles of maximal BR surface density (4.7 mg/m$^2$) for both 'inside-out' and 'right-side-out' orientation. The $H^+$/BR ratio is indicated as positive for alkalinization of the medium.

'inside-out' and alkalinization of the suspending medium at a level of 7.6 $H^+$/BR in the steady state was measured. When the surface carboxyl groups were charge-neutralized by chemical modification, the maximal insertion of BR into the lipid coat of the carrier beads was the same as for unmodified carrier beads (4.7 ± 0.5 mg/m$^2$). However, the incorporation was apparently 'right-side-out' as seen by the inversion of proton flow now acidifying the medium to a stationary level of 3.7 $H^+$/BR.

The inversion of the proton flow does neither strictly prove the homogeneity of the BR coat, nor a preferred 'right-side-out' orientation. To analyze this problem, both types of BR-coated particles were treated with papain. Proteolysis of BR by papain cleaves off the membrane-protruding C-terminal sequence if exposed to the suspending medium [12,21]. Proteolytically altered BR molecules can be detected and distinguished from unaltered BR by SDS-gel electrophoresis. The results are shown in Fig. 3. The SDS-PAGE patterns of papain-digested 'right-side-out' particles showed no decrease of material in the BR main band up to incubation times of 9 h, whereas a band with smaller molecular weight appeared with time in material from 'inside-out' pumping particles.

In order to demonstrate additionally the regularity and orientation of BR in the lipid coat at the particle surface, we used freeze-fracture electron microscopy. Fig. 4 shows the result of image processing of freeze-fracture replicas after Pt/C decoration of 'inside-out'- and 'right-side-out'-oriented BR at the surface of PE-$(Ala)_5$-Callocryl. The decoration pattern of thin Pt films (< 1 nm) yields structural information which does not critically depend on the thickness of the objects protruding from the surface. This is an explanation for the visibility of the regular pattern of the BR molecules although they are embedded to more than 80% in the membrane. Optical diffraction patterns of micrographs

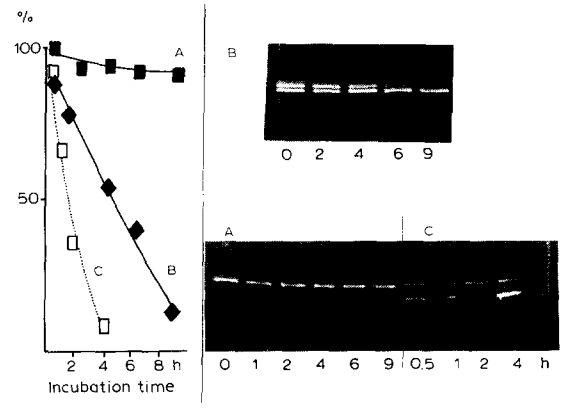


Fig. 3. Gel electrophoretic analysis of papain 'right-side-out' (A)- and 'inside-out' (B)-treated BR layers at the surface of lipid-coated PE-$(Ala)_5$-Callocryl beads. In a control experiment (C) a PM suspension was subjected to papain digestion. The lower band in the gels corresponds to BR without a carboxy terminus. To compare the separate experiments in a densimetric analysis (on the left) only the relative intensity change of the upper BR main band has been followed (intensity at $t$) corresponds to 100%. BR in sample B had already lost about 40% of its carboxy terminus when the first aliquot was taken.

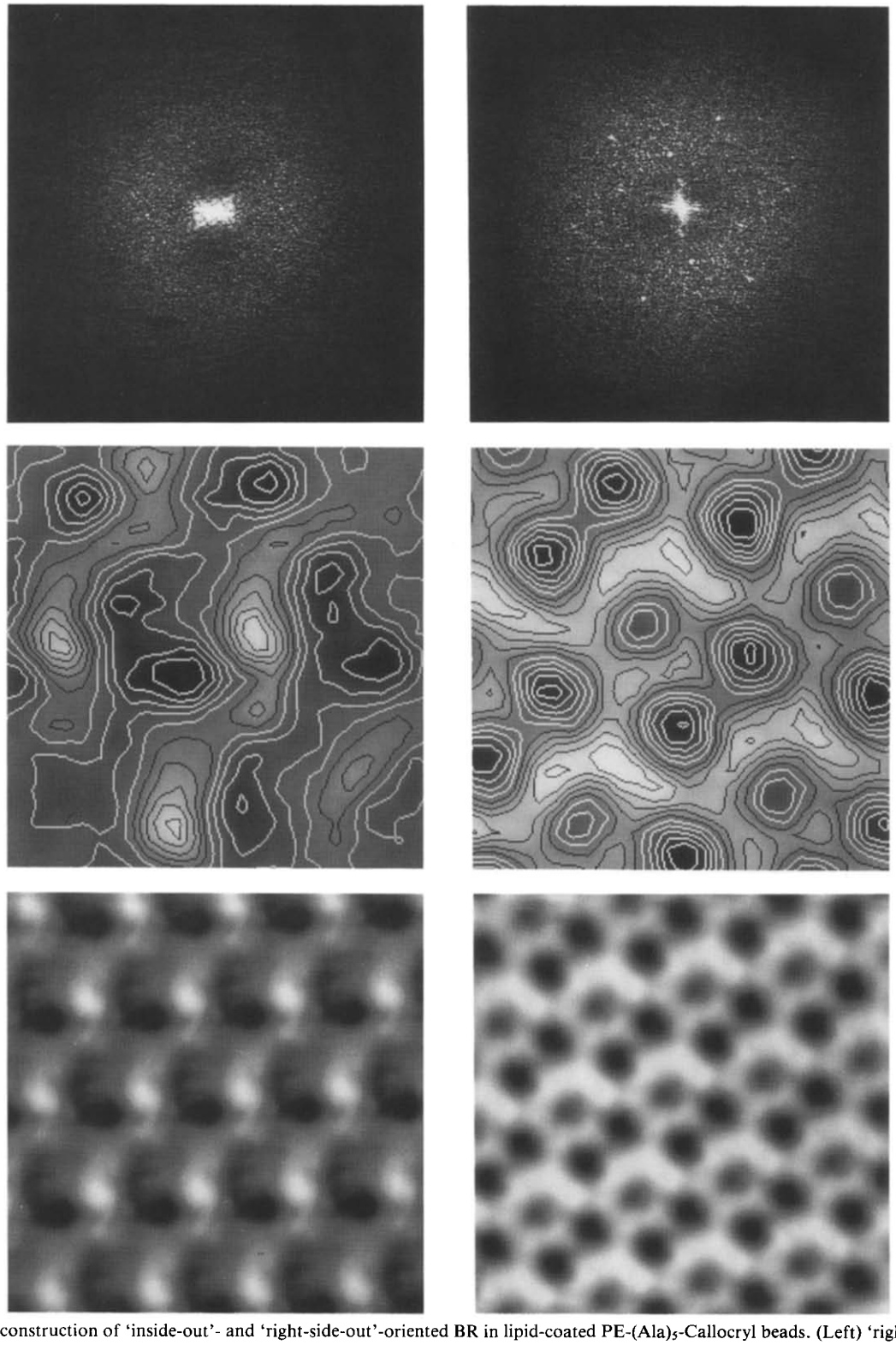

Fig. 4. Image reconstruction of 'inside-out'- and 'right-side-out'-oriented BR in lipid-coated PE-$(Ala)_5$-Callocryl beads. (Left) 'right-side-out' BR layer. (Right) 'inside-out' BR layer. (Upper panel) Optical diffraction patterns (power spectra). (Middle panel) Reconstructed BR layer after correlation averaging, contour levels superimposed; metal deposited on the surface is black. (Lower panel) Crystalline area containing about 16 unit cells (reconstituted from the averaged unit cell in the computer).

allowed us to find regions with high structural order and to determine the unit cell constants. All micrographs of the 'inside-out' preparations inspected showed the same structural order and no random distribution was observed. Image processing of the selected regions showed a regular hexagonal pattern of

BR trimers. Reconstruction of the 'right-side-out' pumping BR population was far more complicated. The reflection spots in power spectra were much weaker or even undetectable in some micrographs, i.e. decoration of the regular unit cells was obscured by irregularly deposited metal grains. The results are in accordance with a report of Neugebauer and Zingsheim [22] who revealed structural differences of the two faces of the PM by silver decoration. The cytoplasmic side showed a hexagonal decoration pattern whereas decoration of the extracellular side was faint and more irregular. The values for the lattice constant of BR in the PM varied between 6.0 and 6.2 nm [23] and that of a lipid-depleted PM was found to be only 5.7 nm [24]. The values from our experiment were 5.7 nm for the 'inside-out'-oriented BR layer and 6.0 nm for the 'right-side-out' orientation and are thus within the limits of a BR arrangement as in the PM.

The lipid-coated beads are comparable with liposomes regarding their capability of protein reconstitution but are much more stable against mechanical and osmotical stress. The particles are 'reutilizable' because after washing out the outer lipid layer (together with the incorporated protein) by detergents or organic solvents, the particles could be coated with new lipid and BR was incorporated to the same extent as before (results not shown). The lipid-carrier system described here allows the formation of asymmetric lipid bilayers and by introduction of hydrophilic spacers and variation of surface charge, an optimal environment for different membrane proteins could be created. This might be of importance for the functional analysis of membrane proteins but could also be of biotechnological interest for the construction of biosensors.

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