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Role of endogenous lipids in the chromophore regeneration of bacteriorhodopsin

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Dedicated to Prof. Mario Della Monica (January 9th 1932, September 9th 2003)

Abstract

The regeneration method of Khorana [J. Biol. Chem. 262 (1987) 9271] has been modified in order to study the effect of endogenous archaeabacterial lipids and, in particular, of glycocardiolipin (GlyC) in the refolding and chromophore regeneration of bacteriorhodopsin (BR).

BR refolding and chromophore regeneration could be obtained in the presence of endogenous lipid mixtures containing or not containing glycocardiolipin; however, the kinetics of bacteriorhodopsin regeneration in the presence of glycocardiolipin was faster than in its absence. These results show for the first time that the interaction of glycocardiolipin with bacteriorhodopsin favours its refolding from the denatured state and the chromophore regeneration.
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1. Introduction

Bacteriorhodopsin (BR), a seven-transmembrane domain protein, is a light-driven proton pump which has the retinal as prosthetic group covalently linked to K216 as a protonated Schiff base [1,2]. Sole protein in the purple membrane (PM) of *Halobacterium salinarum*, bacteriorhodopsin is organized, with a small number of lipid molecules, in a 2D crystalline lattice, being one of the few membrane proteins whose native structure is known at atomic resolution. However, so far, the knowledge of the interactions of α-helices with surrounding lipids and the identification of annular lipids is still incomplete, as is the localization of the lipid head groups [3–5].

Recently, there has been much progress within our knowledge about the lipids of the PM, including the discovery of two novel cardiolipin analogs: glycocardiolipin (GlyC) and archaeal bishosphatidylglycerol (BPG) [6].

The cardiolipin–BR stoichiometries in PM of *H. salinarum* have been estimated by a combination of ^{31}P and ^{1}H-NMR analyses of the lipid extract. While the glycocardiolipin/BR molar ratio is one, bisphosphatidylglycerol is only a minor lipid component of PM [7].

As glycocardiolipin is essential for the trimer stability, it has been suggested that it is located into trimers and directly interacts with BR [7,8].

Bacteriorhodopsin is considered one of the best models for studying the mode of assembly and the folding dynamics of the integral membrane proteins. Studies of bacteriorhodopsin regeneration have indicated that the denatured apoprotein, bacterio-opsin (BO), can be refolded and regenerated in mixed detergent/lipid micelles by the addition of retinal; regeneration, observed as the appearance of the purple absorption band, occurs on a time scale of minutes [9]. The role of individual endogenous lipids in the regeneration process has not yet been investigated. Data of the present regeneration studies in the presence of archaeabacterial lipid extracts containing or not containing glycocardiolipin suggest that glycocardiolipin has an important role in the chromophore regeneration of BR.

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2. Materials and methods

2.1. Materials

DNase I was obtained from Sigma. All organic solvents used were commercially distilled and of the highest available purity (Sigma–Aldrich). β-γ-Dimyristoyl-L-α-phosphocholine (DMPC) was obtained from Sigma. Sodium dodecyl sulfate (SDS) and 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS) were obtained from Sigma, and urea was from Fluka. TLC plates (60 A), obtained from Merck, were washed twice with chloroform–methanol (1:1, v/v) and activated at 120 °C before use.

2.2. Purple membrane isolation

Purple membrane was isolated as reported by Oesterhelt and Stoeckenius [10] from a genetically engineered high-BR-producing strain of *H. salinarum* [11] kindly provided by Richard Needleman. The cells were grown in light at 37 °C in a liquid growth medium containing neutralized peptone (L34, Oxoid), prepared as previously described [10].

2.3. Lipid extraction from PM and whole cells

Total lipids were extracted from PM and whole cells of *H. salinarum*, using the Bligh and Dyer method, as modified for extreme halophiles [12].

To obtain lipid extracts free of retinal, the polar lipids were isolated by precipitation in ice-cold acetone as previously described [12]. The isolated polar lipids were dissolved in chloroform and stored at −20 °C.

2.4. Preparation of denatured delipidated BR and its solubilization in SDS

Denatured BR (BO) has been prepared both as previously described by Braiman et al. [13] and collected during the PM lipid extraction by Bligh and Dyer method. The denatured protein (BO) was solubilized in 8 M urea, 0.5% SDS (3 mg BO/2 ml). Solubilization required 5 h at room temperature. Then, urea was removed by dialysis overnight against 0.5% SDS. After dialysis, the BO/SDS solution was centrifuged at $100,000 \times g$ for 1 h, allowing the precipitation of nonsolubilized BO. The amount of solubilized BO in the supernatant was then determined spectrophotometrically ($\varepsilon_{280} = 6.6 \times 10^4$ cm^{-1} M^{-1}) [14].

2.5. Extraction of residual lipids from BO

Residual lipids remaining associated with BO, after delipidation by the Bligh and Dyer method, were extracted twice with CHCl$_3$/MeOH (1:3, v/v) at 80 °C for 1 h. The combined extracts were brought to dryness under nitrogen; dried lipids were resuspended in a small chloroform volume and saved at −20 °C.

2.6. Lipid analysis

Lipid analysis was performed by thin layer chromatography with the following solvent system: chloroform/acetic acid 90%/methanol (65:35:4, by vol.). The lipids were detected by spraying the plate with 5% sulphuric acid in water and incubating at 120 °C for 45 min.

2.7. Regeneration of bacteriorhodopsin

The regeneration experiments with DMPC/detergent mixtures were performed following the regeneration procedure of Braiman et al. [13]. Where endogenous lipids were used, aliquots of endogenous lipid extracts were dried and dissolved in 2% (w/v) CHAPS at pH 6 (100 mM NaP$_i$). Solutions containing 0.5% (w/v) polar lipids were prepared. Regeneration of BR was carried out by adding 190 μl of clear lipid/detergent solution to 150 μl of an aqueous SDS solution of BO (0.5% SDS, protein concentration 0.5 mg/ml); the volume was brought to 375 μl by the addition of water, and then, 4.5 μl of retinal in ethanol (0.1 mg/ml) were added.

2.8. Absorbance measurements

UV-visible absorption spectra were obtained with a Cary 50 Bio UV-visible spectrophotometer.

In each experiment, the kinetics of chromophore regeneration was studied by following the recovery of the characteristic purple absorption band of the bound retinal. The concentration of renatured BR was determined spectrophotometrically ($\varepsilon_{560} = 5.2 \times 10^{-4}$ cm^{-1}M^{-1}) [14].

3. Results

3.1. Analysis of residual lipids associated with delipidated denatured BR

In our initial approach to the studies of chromophore regeneration, we have reexamined the method of Khorana and carefully analysed residual lipids associated with delipidated denatured BR (named in the following k-BO) obtained by following the procedure described by Braiman et al. [13]. Only 30% of PM lipids were recovered in the lipid extract; although the lipid extraction is clearly incomplete, the procedure of Braiman et al. resulted in a complete retinal removal. Lipids associated with k-BO could be re-extracted with the Bligh and Dyer method; 50% of PM lipids were recovered in the re-extract of k-BO.

Fig. 1 shows the TLC profile of the lipid re-extract of k-BO. It can be seen that all the lipid components of PM are present among residual lipids associated with k-BO; the BO/lipid molar ratio was about 1:5 or higher.

BPG
PG

PGP-Me

PGS
GlyC

S-TGD-1

Residual lipids
(Khorana's method)
(BO/lipids 1:5)

(A)

Residual lipids
(present method)
(BO/lipids 1:2)
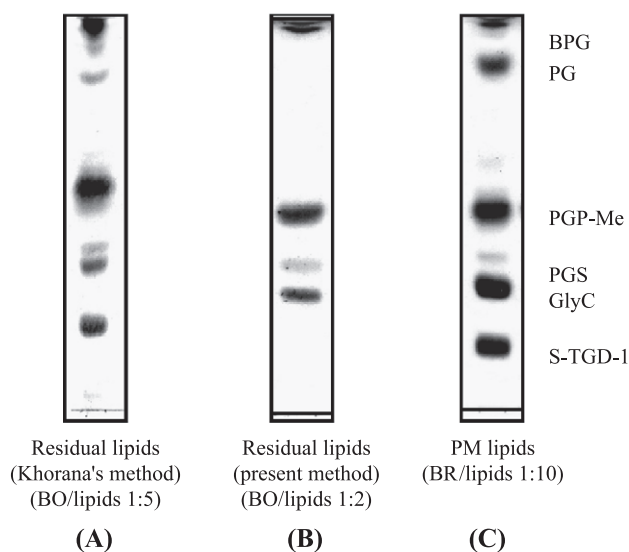
(B)

PM lipids
(BR/lipids 1:10)

(C)

Fig. 1. Lipid profiles of: residual lipids associated with bacterio-opsin (BO) after delipidation by Khorana's method (A); residual lipids associated with BO after delipidation by the Bligh and Dyer method (B); lipid profile of purple membrane (C). TLC plate has been developed in acid solvent system ($CHCl_3$/MeOH/90% acetic acid 65:4:35, v/v), and lipid were stained by spraying with 0.5% H_2SO_4 followed by incubation at 120 °C for 40 min.

It is concluded that, by extracting lipids as described by Braiman et al., only 1/3 of lipids present in PM could be removed, and that BO used in the previous reconstitution studies still contained most of lipids originally present in PM.

Here, in order to prepare a better delipidated protein, we have used the Bligh and Dyer procedure to extract PM lipids instead of using the delipidation method of Braiman et al.

Residual lipids remaining associated with denatured BR after the lipid extraction by the Bligh and Dyer method were also analysed; in this case, only about 10% of PM lipids were not recovered in the lipid extract and therefore remained tightly bound to denatured BR. The composition of residual lipids tightly bound to BO was found to be quite different from PM (see Fig. 1); in the re-extract, there is no S-TGD-1 (3-HSO_3-Gal$p$-β1,6-Man$p$-α1,2-Glc$p$-α1,1-sn-2,3-diphytanylglycerol) and phosphatidylglycerol (PG), indicating that the lipids having an higher affinity to BR, even in the denatured state, are glycocardiolipin, phosphatidylglycerosulfate (PGS), phosphatidylglycerophosphate methyl ester (PGP-Me) and archaeal BPG.

However, BO obtained after lipid extraction with the Bligh and Dyer method ($BO_{B/D}$) is very difficult to solubilize. Only 50% of $BO_{B/D}$ could be solubilized in 0.5% SDS; no residual lipids were associated with the nonsolubilized BO. Therefore, we conclude that residual lipids after the PM lipid extraction are not uniformly distributed in BO, and that it is possible to refold the protein only when some endogenous lipids are still associated with BO. We have estimated that the BR/lipids

molar ratio in the delipidated denatured BO used in the present study is about 1:2.

3.2. Refolding and regeneration of bacteriorhodopsin in DMPC/CHAPS/SDS

Refolding and chromophore regeneration was obtained by addition of DMPC/CHAPS mixed micelles and exogenous retinal to $BO_{B/D}$ in SDS. Renatured BR exhibits the same absorption spectrum of the native PM although a complete regeneration of bacteriorhodopsin could not be obtained. The extent of BR regeneration was 58%, and the ratio A_{280}/A_{560} was 2.6 (not shown).

3.3. Kinetics of bacteriorhodopsin regeneration in the presence of endogenous lipids: the role of glycocardiolipin

We have previously shown that GlyC is very low or absent in the *H. salinarum* cells, while it represents one of the major phospholipids of isolated PM [14]. Therefore, here, in order to study the role of endogenous lipids and in particular of the glycocardiolipin in the folding and regeneration of BR, we have performed refolding and regeneration of bacteriorhodopsin by adding PM and cell lipid extracts, i.e., containing or not containing glycocardiolipin respectively.

Regeneration of bacteriorhodopsin from a partially folded BO was studied by following the increase in absorbance at 560 nm.

Fig. 2 shows the time course of the chromophore regeneration upon addition of retinal to BO in the presence of mixtures of endogenous lipids containing or not containing GlyC. It is possible to observe that the regeneration
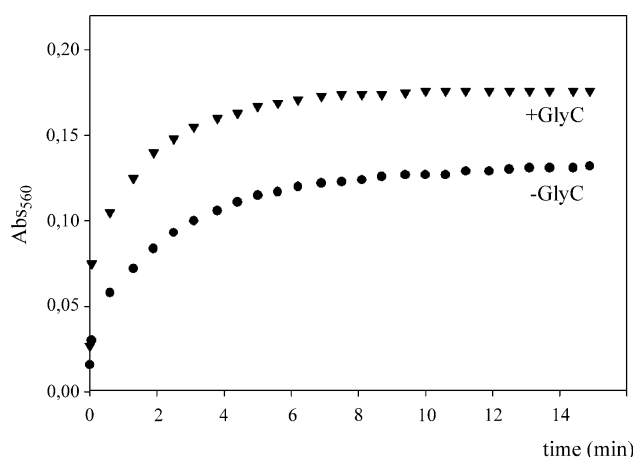

Fig. 2. Time course of the chromophore regeneration upon addition of retinal to BO in presence of mixtures of endogenous lipids containing or not containing GlyC. The chromophore regeneration was obtained by adding the endogenous lipids in CHAPS containing solution (2% CHAPS at pH 6, lipid concentration 0.5%) to BO in SDS (0.5% SDS, protein concentration 0.5 mg/ml). The kinetics of the regeneration process was studied by following the increase of the UV absorbance at 560 nm.

Table 1
Experimentally determined rate constants for bacteriorhodopsin regeneration (BO–BR)

Lipids	k_1 (s^{-1})	k_2 (s^{-1})
(a) +GlyC	0.59	0.008
(b) −GlyC	0.22	0.005
(c) DMPC	0.22	0.006

Regeneration was obtained by adding 0.5% lipids in 2% CHAPS: endogenous lipids (a) containing or (b) not containing glycocardiolipin; (c) DMPC.

yields depend on the nature of endogenous lipids used; in particular, the yield of regeneration in experiments carried out using the extract at high GlyC content was 25% higher than that recorded in the presence of the extract not containing GlyC. The rate of the regeneration process was also influenced by the nature of the lipids. Table 1 shows a report on the experimental rate constants relative to bacteriorhodopsin regeneration in the presence of the two different lipid mixtures; in both cases, the regeneration process was well described by two first-order kinetic phases.

It has been previously shown that addition of retinal to partially folded BO regenerates bacteriorhodopsin, in a reaction that consists of at least two stages: first was noncovalent binding of retinal, and second was Schiff base formation [9,15].

The kinetics of bacteriorhodopsin regeneration in the presence or in the absence of glycocardiolipin can also be adequately described by the sum of two single exponentials, of which, the fast one corresponds to the noncovalent retinal binding while the slow one to the Schiff base formation. The rate constants of the two phases are close to those previously described in the literature [9,16].

Interestingly, after the addition of a mixture of endogenous lipids not containing glycocardiolipin, slower kinetics for both processes are observed. The kinetics of the regeneration of BR in the absence of glycocardiolipin is similar to that observed when exogenous lipids (DMPC) are used in the refolding/regeneration steps.

4. Discussion

There are many experimental restrictions to the study of the folding of membrane proteins in vitro, essentially because it is difficult to solubilize these proteins without causing irreversible denaturation.

On the contrary, BR refolding has been extensively studied in vitro because, unlike most of membrane proteins, its renaturation is reversible, and it can be easily prepared on a large scale.

In this preliminary work, we have modified the refolding/regeneration method of Khorana with the aim of studying the effect of endogenous lipids. In particular, we have examined the role of the recently discovered glycocardiolipin, which has important roles in the function and stability of BR [8].

The denatured BR used in the present study as starting material to study refolding and chromophore regeneration contains much less endogenous lipids than that one used in previous refolding studies.

We could refold BR and regenerate the chromophore by adding DMPC/CHAPS or a mixture of endogenous lipids in CHAPS. The presence of glycocardiolipin in the lipid mixture used to reconstitute BR allowed a faster regeneration process.

In conclusion, the results of lipid analyses and of chromophore regeneration studies illustrated in the present work suggest that it is not possible to dissociate glycocardiolipin from BR without causing irreversible denaturation, and that its presence accelerates the protein refolding and retinal binding.

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