SELECTIVE FORMATION OF BACTERIO-OPSIN TRIMERS BY CHEMICAL CROSSLINKING OF PURPLE MEMBRANE

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1. Introduction

The purple membrane of *Halobacterium halobium* contains a single protein known as bacteriorhodopsin, which is composed of 1 mol retinal/mol bacterio-opsin [1]. The organization of the protein within each purple membrane sheet is that of an exact 2-dimensional crystal. X-ray and electron diffraction data have revealed that the bacteriorhodopsin in purple membrane is arranged as trimeric units in a hexagonal lattice [2]. The average distance between bacteriorhodopsin molecules within a trimeric unit is significantly shorter than the spacing between bacteriorhodopsin molecules from adjacent trimers.

One could predict that crosslinking of purple membrane with bifunctional reagents would lead to the selective formation of crosslinked trimers. We demonstrate here that this is indeed the case.

The biosynthetic precursor of purple membrane is the brown membrane, which also contains bacterio-opsin [3]. When this membrane is treated with crosslinking reagents, polymeric protein molecules are formed exclusively.

Crosslinked purple membrane exhibits the same photointermediates as the native membrane, although the cycle of events is slowed down.

2. Materials and methods

2.1. *Chemicals*

Dimethyl-3,3′-dithiobispropionimidate·2 HCl (DTBP) and dimethylsuberimidate·2 HCl (DMS) were obtained from Pierce, Rotterdam. Tartryldihydrazide for the preparation of tartryldiazide (TDA) was obtained from Fluka, Neu-Ulm, and L-[^{35}S]methionine (500 Ci/mmol) from Amersham Buchler, Braunschweig.

2.2. *Crosslinking of bacteriorhodopsin*

2.2.1. DTBP

Purple membrane containing of 6 nmol bacteriorhodopsin was suspended in 200 μl 0.2 M *N*-methylmorpholine acetate pH 8.0, 2.5 mg DTBP·2 HCl (dissolved in 100 μl 0.1 M *N*-methylmorpholine) were added and the mixture incubated at 37°C for 2 h.

2.2.2. DMS

Purple membrane containing 6 nmol bacteriorhodopsin was suspended in 500 μl 0.1 M $NaHCO_3$ and 1 mg DMS·2 HCl (dissolved in 25 μl water) added and the mixture incubated at 37°C for 2 h.

2.2.3. TDA

TDA was prepared from tartryldihydrazide as in [4]. Purple membrane containing 6 nmol bacteriorhodopsin was suspended in 100 μl 1 M *N*-methylmorpholine acetate, pH 8.0 and 100 μl TDA solution (15 μmol) added and the mixture neutralized with 2 M KOH then incubated at 56°C for 1 h.

After the crosslinking reaction the purple membrane was collected by centrifugation and the precipitate washed 3 times with water.

2.3. *Sodium dodecyl sulphate–polyacrylamide gel electrophoresis and fluorography*

Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis was performed using slab gels con-

taining 12% acrylamide as in [5]. Proteins were visualized by staining with Coomassie blue. Fluorography was carried out as in [6].

2.4. *General procedures*

Purple membrane and brown membrane were isolated as in [3,7,8]. The kinetics of the light induced photochemical changes were measured as in [9]. Experimental conditions of pulse labelling using L-[^{35}S]methionine will be published [10].

3. Results and discussion

In this study bacteriorhodopsin in purple membrane was crosslinked using several bifunctional reagents. Analysis by SDS–polyacrylamide gel electrophoresis revealed the presence of mainly trimeric bacteriorhodopsin molecules (fig.1). Dimers, tetramers and higher oligomers were detected in much smaller amounts. TDA and DTBP produced trimers more selectively than DMS. An average distribution of crosslinking products is given in table 1.

It was possible to crosslink bacteriorhodopsin almost quantitatively with DTBP, but TDA and DMS left up to 50% protein as monomer. Purple membrane sheets tend to aggregate when in suspension, therefore some sheets may have been protected from the crosslinking reagent. This may explain why only trimeric and monomeric bacterio-opsin molecules were found without extensive formation of dimers.

Newly synthesized bacterio-opsin is initially found in a non-crystalline state within the brown membrane [3]. Crystallization of bacterio-opsin to form purple membrane patches occurs after reaction with retinal in an energy-dependent step. When bacterio-opsin was pulse-labelled in vivo for 15 min with a radioactive amino acid the subsequent membrane fractionation

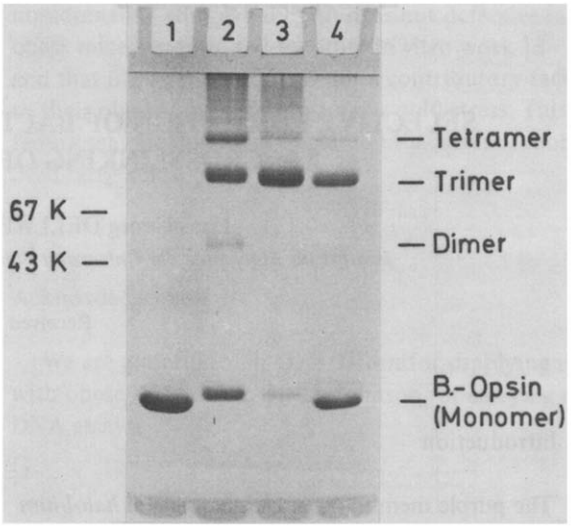

Fig.1. SDS–polyacrylamide gel electrophoresis of crosslinked purple membrane. The figure shows the control (1) and the effect of treatment with the bifunctional crosslinkers DMS (2), DTBP (3), and TDA (4). The photograph was taken after Coomassie blue staining. Reference proteins were hen egg albumin (43K) and bovine serum albumin (67 K). For further details see section 2.

revealed an almost equal distribution of labelled bacterio-opsin between the brown membrane and purple membrane fractions (fig.2A). The radioactively-labelled membrane fractions were used to compare the crosslinking behaviour in the brown membrane and purple membrane, respectively.

The result of crosslinking with DTBP is shown in fig.2B. Crosslinking of purple membrane leads mainly to the formation of trimeric bacterio-opsin, whereas brown membrane is crosslinked to polymeric products.

When purple membrane crosslinked with DTBP was treated with the SH-reagents β-mercaptoethanol,

Table 1
Typical distribution of bacterio-opsin oligomers after crosslinking

	Monomer	Dimer	Trimer	Tetramer	Polymers
DMS	33%	9%	26%	16%	16%
DTBP	10%	0%	69%	12%	9%
TDA	50%	0%	43%	3%	3%

The polyacrylamide gel was stained with Coomassie blue and evaluated by densitometry

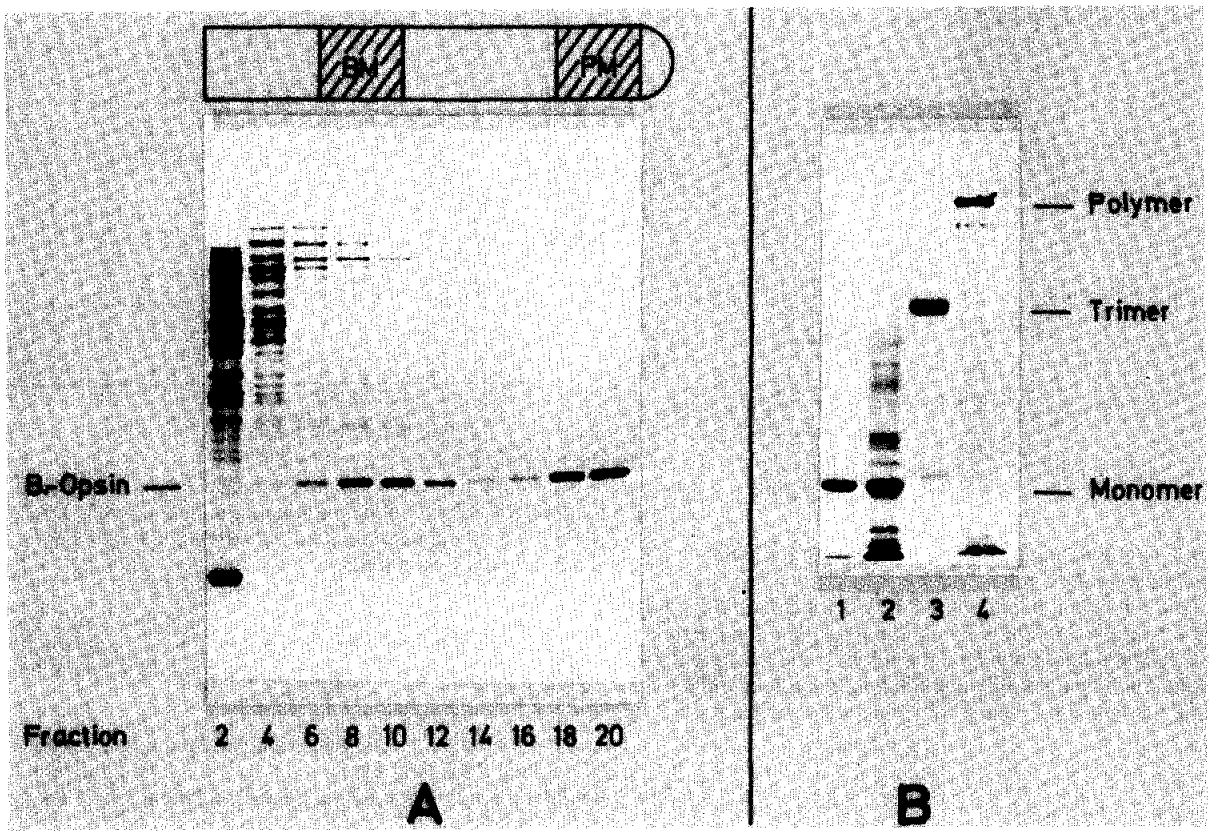

Fig.2. SDS–polyacrylamide gel fluorograms of pulsed bacterio-opsin in different membrane fractions of halobacteria before (A) and after (B) chemical crosslinking. Details: A. Cells were pulsed with L-[^{35}S]methionine (500 Ci/mmol) for 15 min and then immediately disintegrated. The membranes were separated by sucrose density gradient centrifugation (25–45% sucrose), the gradients fractionated and the fractions analysed by polyacrylamide gel electrophoresis; B. Fraction 18 (purple membrane, PM) and 10 (brown membrane, BM) of the gradient shown in A were crosslinked with DTBP. Samples 1–4 show purple membrane, brown membrane, crosslinked purple membrane, and crosslinked brown membrane, respectively.

1,4-dithioerythritol, or thioglycolic acid, a poor recovery of monomers was found. A known side reaction of imidate-crosslinkers is the reaction of the protein *N*-alkyl imidate with another appropriately positioned protein amino group to give a disubstituted amidine [11–13]. Thus an intermolecular crosslink can be produced with what is nominally a monofunctional reagent. If this happened in the case of bacterio-opsin crosslinked with DTBP, then the low recovery of monomers after disulfide cleavage could be explained. In order to substantiate this possibility purple membrane was treated with propionimidate and subjected to electrophoresis. Figure 3 shows that trimer formation occurred.

Formation of trimeric bacteriorhodopsin does not affect the A_{570} of the protein. Typically the recovery of A_{570} was more than 90% after the crosslinking reaction. However, during the crosslinking reaction, the A_{max} was shifted to 500 nm. After washing the crosslinked membranes the 570 nm chromophore was restored.

In the experiment of fig.4 native purple membrane and crosslinked purple membrane (70% trimers) were illuminated with light ($\lambda > 515$ nm) and the forma-

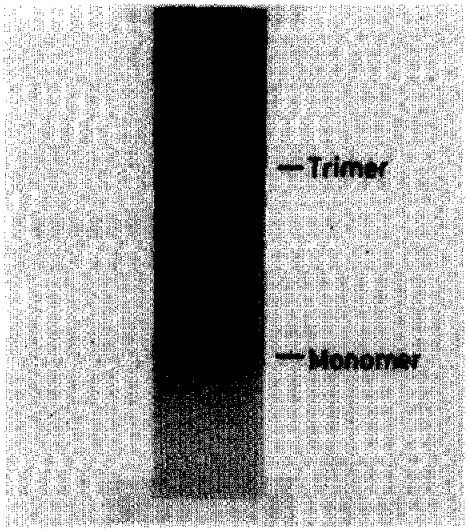

Fig.3. Crosslinking of purple membrane with the monofunctional imidate propionimidate. The crosslinking was carried out under the same conditions as for crosslinking with DTBP (section 2). SDS–polyacrylamide gel electrophoresis was performed as in section 2. The gel shows (1) untreated membrane, (2) membrane treated with 2.5 mg propionimidate, (3) membrane treated with 5 mg propionimidate.

tion of the 412 nm chromophore followed at low temperature. The crosslinked purple membrane could still be reversibly bleached by light. This result was not surprising since purple membrane after treatment with crosslinking reagent still exhibited proton pump activity when incorporated into vesicles [14]. However, the kinetics of the photochemical cycle were influenced by the crosslinking; the decay of the 412 nm intermediate was much slower than in the untreated control.

Acknowledgements

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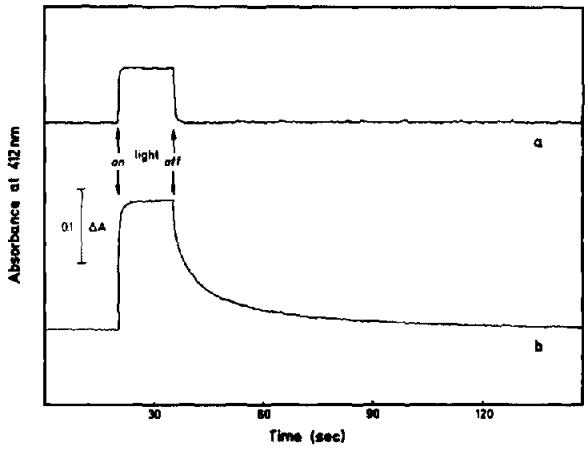

Fig.4. Kinetics of the 412 nm chromophore formation. Purple membrane (a) and purple membrane crosslinked with DTBP (b) of equal A_{570} were irradiated with light of $\lambda > 515$ nm and the ΔA_{412} monitored.

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