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Large scale preparation of homogeneous bacteriorhodopsin

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Homogeneous bacteriorhodopsin was obtained preparatively (100 mg batches) from purple membrane of *Halobacterium halobium* cells. The homogeneity of the protein was considerably affected by variations in the growth conditions of the bacteria. Fully maturated bacteriorhodopsin having a blocked N-terminus and a homogeneous C-terminus, was reproducibly obtained when cells were grown in a sufficiently aerated medium.

Bacteriorhodopsin; Halobacterium; Heterogeneity; Purification

1. INTRODUCTION

Bacteriorhodopsin, the only protein in the purple membrane of the archebacteria, *Halobacterium halobium*, functions as a light-driven proton pump [1]. The structure of this integral membrane protein (M_r 26000), which binds the chromophore retinal, is of great interest because of its analogy in structure and mechanism with the visual pigment, rhodopsin, active in the vertebrate retina. The primary structure of bacterio-opsin and that of its gene are known [2,3], and a model of its three-dimensional structure has been proposed based on electron microscopy studies [4,5]. The protein isolated from the membrane exhibits charge and/or size heterogeneity due to incomplete maturation and/or uncontrolled differential proteolysis (for reviews see [6,7]).

This paper reports the effects of various parameters of the culture, including light, aeration, and stirring, on the structure of bacteriorhodopsin. The protein obtained under various conditions was analyzed by electrophoresis, isoelectric focusing, and microsequencing. The conditions required for the reproducible large-scale preparation of mature and homogeneous bacteriorhodopsin are described.

2. MATERIALS AND METHODS

2.1. *Materials*

Bacteriological pepton (L-37) was purchased from Oxoid (Basingstoke, Hampshire, England). Antifoam (cat. no. A-5757), leupeptin, pepstatin, diisopropylfluorophosphate, and chromatographically purified deoxyribonuclease 1 from bovine pancreas (containing only a low level of protease activity; cat no. D-4527) were obtained from Sigma (St. Louis, MO). Octyl-β-D-glucopyranoside, benzamidin, and inhibitor E-64 were purchased from Calbiochem-Behring Corp. (La Jolla, CA). Octyl glucoside was purified as previously described [8]. α_2-Macroglobulin, aprotinin, leupeptin, pepstatin, phenyl-methyl sulfonylfluoride, and L-pyroglutamate aminopeptidase were purchased from Boehringer (Mannheim, FRG). Pharmalytes and Immobiline dry plates were purchased from Pharmacia-LKB Biotechnology (Piscataway, NJ).

2.2. *Growth of* H. halobium

H. halobium cells (strain S-9) were grown in the medium (4 M NaCl, 0.2 M KCl, 0.08 M $MgSO_4$, 15 g/l pepton, and 0.01 M sodium citrate, pH 7.0) described by Oesterhelt and Stoeckenius [1]. Prior to the addition of pepton, the basal medium was filtered on 0.22 μm nylon filters (Rainin, Emeryville, CA). Antifoam was added in large scale cultures (5 ml/10 liters of medium). Small cultures (50 ml medium inoculated with cells from single or mixtures of colonies) were done at 37°C, either in a shaking waterbath or on a thermostated rotatory shaker. Aliquots, taken in pre-stationary growth phase (8–10 days), were inoculated in fresh medium (1 ml/50 ml). These cultures were used to inoculate the fermentor (100 ml/10 liters of medium) when they arrived at the end of the exponential growth phase (4 days). Bacterial growth (monitored at 660 nm) was compared in 3 types of vessels: (i) 1 liter medium in 2-liter Erlenmeyer flasks placed on a rotatory shaker (200 rpm, no illumination); (ii) 10 liters of medium in a glass fermentor surrounded by 12 fluorescent tubes, stirred at 50–100 rpm, with or without low aeration (0.2 liters/min) (37°C); (iii) 10 liters of medium in a 16-liter steel fermentor (New Brunswick Scientific, Edison, NJ), stirred at 300 rpm, without illumination and with high aeration (3 liters air/min, vessel pressure = 5 psi).

2.3. *Preparation of purple membrane*

All steps were performed under dim light. Cells were harvested by centrifugation (8000 rpm, 30 min), washed twice with basal medium, and the pellet resuspended in water (250 ml for the cells from a 10 liter culture) with purified deoxyribonuclease (1 mg/50 ml), and stirred for 15 h at 4°C in the dark. Purple membrane was recovered by centrifugation (45000 rpm, 45 min), resuspended in water (in the centrifuge tubes), and transferred to clean tubes repetitively until supernatant was free of any UV-absorbing material (range 250–700 nm). The purple membrane was analyzed by centrifugation on a sucrose gradient by loading a 2 ml membrane suspension on gradients made of 10 ml of a 10% w/v sucrose solution on top of a 40 ml sucrose gradient (10% to 30% w/v) with a 15 ml cushion of 60% w/v sucrose. Centrifugation was done for 15 h at 45000 rpm in a 45Ti rotor (Beckman Instruments, Palo Alto, CA). Sodium azide (0.05% w/v) and the following protease inhibitors (used in various combinations)

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Abbreviations: BR, bacteriorhodopsin; SDS, sodium dodecyl sulfate

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were added during the preparation of the membrane: 10^{-6} M leupeptin, 10^{-6} M pepstatin, 10^{-4} M diisopropylfluorophosphate, 10^{-4} M phenyl-methyl sulfonylfluoride, 10^{-3} M benzamidin, 10^{-5} M E-64, 10^{-6} M aprotinin, 3×10^{-8} M α_2-macroglobulin, or 10^{-4} M EDTA.

2.4. *Solubilization of bacteriorhodopsin*

Purple membrane (2 mg) was suspended in 1.5 ml solution containing 10 mM sodium phosphate buffer, pH 6.0, 5% w/v octyl-β-D-glucoside and 0.05% w/v sodium azide. The mixture was stirred for 15 h at 4°C and insoluble material removed by centrifugation (100 000 rpm, 350 000 × g, 30 min). The solubilized protein was concentrated on a YM 30 membrane (Amicon, Danvers, MA) and dialysed versus a 1% w/v octyl glucoside aqueous solution.

2.5. *Analytical methods*

Electrophoresis was performed in polyacrylamide gels (10 cm × 6 cm, 0.5 mm thickness) containing 0.1% w/v sodium dodecyl sulfate according to a previously described procedure [9]. Isoelectric focusing was performed in polyacrylamide gels (10 × 10 cm; 0.2 mm thickness) containing 2% w/v Pharmalytes and 1.5% w/v octyl glucoside and in Immobiline gels (pH range 4–7) containing 2% w/v octyl glucoside and 1% w/v ampholytes (pH range 3–10). Proteins were stained either with Coomassie blue R-250 or with silver [10].

N-terminal sequence analyses were done using the automated Edman-Chang technique [11] in an Applied BioSystems sequencer. C-terminal residues were identified by N-terminal microsequencing of the 14–17 amino acid long C-terminal peptide released by proteolytic treatment of purple membrane by papain. Proteolysis was done under conditions as previously described [12]. Amino acid sequences could be identified in polypeptide mixtures because the sequence of the protein precursor was known [3] and because the sequences of the N- and C-termini of bacteriorhodopsin differ significantly.

3. RESULTS AND DISCUSSION

Bacteriorhodopsin isolated from purple membrane preparations has been reported to be a heterogeneous protein albeit only a single gene corresponding to a polypeptide chain of 248 amino acids is known to code for the protein [3]. This heterogeneity was visualized using techniques based on separation by charge or size. The bacterio-opsin polypeptide is synthesized as a precursor containing a 13 amino acid signal sequence which is then maturated in several steps leading to a blocked N-terminus after cyclization of the Gln-1 residue [12,13]. Incomplete maturation at the N-terminus as well as partial proteolysis (and possibly partial removal of the aspartic residue in position 249 [3,13]) at the C-terminus, have been reported to be at the origin of the multiband patterns observed in electrophoresis or isoelectric focusing [7,12].

Bacteriorhodopsin prepared in this laboratory exhibited a more discrete heterogeneity than that reported in literature. Nevertheless the quality of the protein was considered to be inadequate for protein crystallization experiments. The small differences between the individual forms, together with variation from batch to batch, and the poor resolution of preparative separation techniques, made the purification of a single species difficult and irreproducible. These difficulties were circumvented by altering the bacterial culture condions (indeed it is known that some proteins can be isolated in the native form only when the cells are in a given physiological state; see e.g. [14–16]). Preliminary observations indicated that variability of the quality of bacteriorhodopsin could be related to the growth conditions with several factors affecting the maturation of the protein including light, stirring, temperature, pH, aeration, and composition of the medium.

Previous studies had indicated that aeration (i.e. the dissolved oxygen concentration) can be an important parameter for the growth of these aerobic halobacteria. Indeed cells synthesize bacterio-opsin and retinal when the oxygen concentration is kept low [17–19] but little purple membrane is formed when the medium is saturated with air [19]. Recently published reports, dealing with the heterogeneity of bacteriorhodopsin, had at least one common point: aeration of the medium was either limited during the whole growth [12] or was stopped when cells reached the end of the logarithmic growth phase [7] (for the above-mentioned reasons and because bacteriorhodopsin functions when cells are in anaerobiosis). Illumination of the culture medium during bacterial growth has been reported to stimulate the synthesis of purple membrane at low oxygen concentration [19,20]. Nevertheless the effect of light was not well understood since some investigators illuminated their cultures whereas others did not.

The growth of *H. halobium* was compared in two different vessels: a 2-liter Erlenmeyer flask placed on a rotatory shaker and a 16-liter steel fermentor were chosen. Both cultures were started from the same medium inoculated at $t = 0$ and were performed simultaneously at 37°C without illumination. The flask was well shaken and the medium in the fermentor well stirred. In addition, in the absence of illumination, copious aeration was provided in the fermentor whereas the flask was closed (to reduce evaporation). It can be seen in fig.1 that, although both cultures reached the same maximal absorbance, the shaken-flask culture needed two additional days to reach the pre-stationary growth phase. After 4, 6, 8 and 10 days aliquots of the medium (400 ml) were withdrawn from both cultures, purple membrane was prepared and bacteriorhodopsin isolated. Analysis of the protein obtained from the flask-culture by SDS-polyacrylamide gel electrophoresis showed that it was composed of a mixture of 3 polypeptide chains: the minor ones had a slightly higher apparent M_r than the major polypeptide (fig.2A). With isoelectric focusing, the same samples displayed at least 4 solubilized protein species differing in charge (fig.2B). The distribution of the protein bands in these patterns changed from the fourth to the sixth day but remained stable through the tenth day of the culture (fig.2). Similar electrophoresis and isoelectric focusing patterns composed of multiple bands have been reported by several authors (see e.g. [7,12]).

Bacteriorhodopsin contained in purple membrane of cells grown in the fermentor behaved as a single polypeptide migrating as one band in both elec-

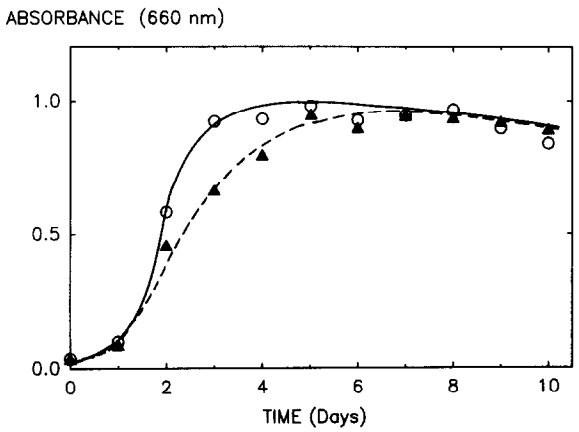

Fig.1. Comparison of the growth of *H. halobium* in a shaken flask or in a stirred steel fermentor. Both cultures were started at $t = 0$ from the same inoculated medium in the absence of light. (▲) The flask was shaken at 200 rpm and (○) the fermentor stirred at 300 rpm and aerated at 3 liters/min. For technical details see section 2.

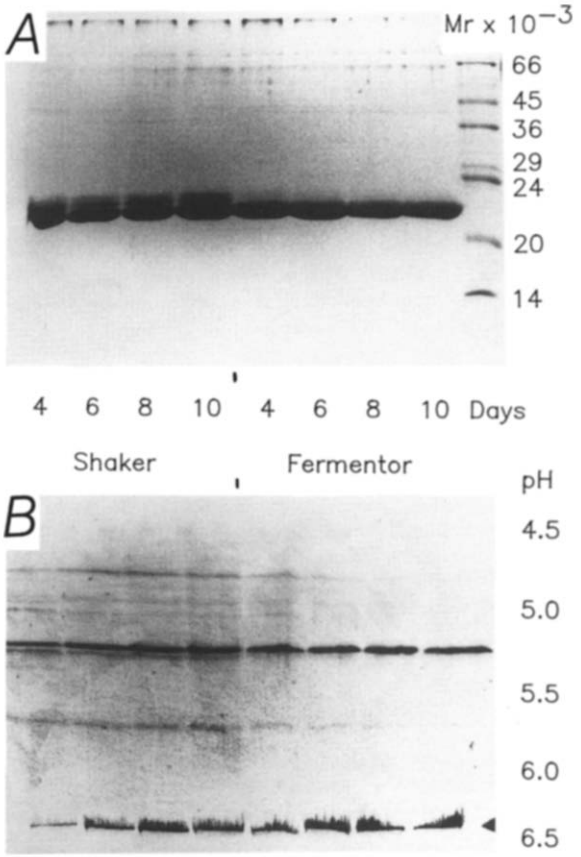

Fig.2. Analysis of bacteriorhodopsin by SDS-polyacrylamide electrophoresis and isoelectric focusing after various growth times in a shaken-flask or in a stirred fermentor. Cells were harvested after 4, 6, 8 or 10 days. Bacteriorhodopsin was isolated from purple membrane as described under section 2. (A) Purple membrane samples (containing 10 μg bacteriorhodopsin) analyzed by SDS-gel electrophoresis and stained with Coomassie blue. The scale on the right gives the apparent M_r. (B) Solubilized bacteriorhodopsin (10 μg) analyzed by isoelectric focusing in an immobilized pH gradient and stained with silver. Approximate pH is indicated. Traces of insoluble protein remaining on the surface of the gel where samples were deposited (arrow) are overstained.

trophoresis and in isoelectric focusing (fig.2). Protein was detected as a single band on the fourth day in electrophoresis (and in isoelectric focusing gels stained with Coomassie blue), but displayed a few minor bands accompanying the major band in isoelectric focusing gels stained with silver. The protein was actually homogeneous from the fifth or sixth through the tenth day of the culture (i.e. at the beginning of the stationary growth phase). The homogeneous bacteriorhodopsin had an apparent M_r of 22 000 (in electrophoresis in the presence of SDS) and an isoelectric point (pI) of 5.2 (in free or immobilized pH gradients). Gels, loaded with increasing amounts of protein and stained with silver in addition to Coomassie blue, occasionally displayed after extensive staining an extremely faint band (pI = 5.7) comprising less than 5% of the total protein which corresponded to a band found in the samples from flask cultures. Isoelectric focusing, in free ampholyte pH gradient gels, showed artifactual smears (absent in immobilized pH gradient gels) suspected to be due to binding of ampholytes by the protein (result not shown). The spectra of bacteriorhodopsin, prepared under both culture conditions and solubilized in purified octyl glucoside, were identical (result not shown). After gel filtration in the presence of octyl glucoside, as described previously [21], the optical purity of the heterogeneous or homogeneous protein was comparable to the best values reported (A_{280} nm/A_{548} nm = 1.6) [7]. A 10-liter culture in the steel fermentor gave about 100 g wet cells and yielded about 300–400 mg purple membrane (calculation based on ϵ = 63000 at 560 nm). This quantity (obtained in non-illuminated cultures) was comparable to that reported for cultures done under illumination [7].

Table 1 gives the results of sequence analyses of bacterio-opsin obtained under various culture conditions and isolated in the presence or absence of protease inhibitors. Purple membrane, prepared from cells grown in shaken flasks or under low aeration in the glass fermentor, contained bacterio-opsin populations incompletely maturated at their N-terminus. Sequences started at positions −6, −4 or −3 in addition to the protein which had been completely processed and had the blocked N-terminus (table 1). Protease inhibitors, added during the preparation of the membrane and during the solubilization of the protein, did not decrease its heterogeneity when the culture was performed under illumination, with or without low aeration. The protein obtained from the glass fermentor culture exhibited a major band in isoelectric focusing (pI 5.2) and a few minor bands. Only traces of PTH amino acids were found after the Edman degradation indicating that most of the protein had a blocked N-terminus (table 1). This sequence could be unblocked by L-pyroglutamate aminopeptidase confirming that Gln-1 had undergone cyclization.

Table 1

Primary structure of bacteriorhodopsin obtained under various growth and isolation conditions

Growth conditions	isolation conditions + or − inhibitors	Amino acid sequence[a]	
		N-terminus	C-terminus
Shaken flask (single colony, 1 liter of medium)		(b) −5 −1 +1 5	240 248
(1) no illumination, no aeration	+ or −	1/2 A V̇ E G V Ṡ Q̇ A Q I Ṫ (c)	A P E P S Ȧ G D G A A A T Ṡ
		1/4 E G V Ṡ Q̇ A Q I Ṫ (c)	E A P E P S Ȧ G D G A A A T Ṡ
		1/4 G V Ṡ Q A Q I Ṫ (c)	
		Possibly also blocked (d)	+ traces of Asp in position 249
Glass fermentor (mixture of colonies, 10 liters of medium)		−5 −1 +1 5	
		2/3 A V̇ E G V Ṡ Q̇ A Q I Ṫ	n.a.
(2) continuous illumination, aeration during the two first days only	+ or −	1/3 G V Ṡ Q̇ A Q I Ṫ	
		Possibly also blocked (d)	
(3) continuous illumination, continuous low aeration	+ or −	>4/5 (b) Blocked (e)	240 248
		+ traces of	A P E P S Ȧ G D G A A A T Ṡ
		A V̇ E G V Ṡ Q̇ A Q I Ṫ	E A P E P S Ȧ G D G A A A T Ṡ
		and G V Ṡ Q̇ A Q I Ṫ	
Steel fermentor (single colony, 10 liters of medium)			240 248
(4) no illumination, strong continuous aeration	+ or −	Blocked	A P E P S Ȧ G D G A A A T Ṡ
			E A P E P S Ȧ G D G A A A T Ṡ

[a] N-terminal and C-terminal sequences are displayed on the same line for clarification. This does not imply that they correspond to the same polypeptide chain. N-terminal sequences were obtained by microsequencing of bacterio-opsin contained (in purple membrane or after solubilization of the protein with octyl glucoside). C-terminal sequences correspond to the result of N-terminal microsequencing (by the Edman-Chang technique) of the C-terminal peptides, released by mild proteolysis of bacteriorhodopsin, contained in purple membrane, with papain (see under section 2)

[b] The estimated amount of each polypeptide is given as the fraction of the unblocked sequences except for growth conditions 3 where the estimated amount of blocked sequences is indicated

[c] No PTH amino acids were detected in 10 additional cycles when purple membrane was analyzed

[d] The amount of blocked sequence could not be evaluated

[e] N-terminus could be unblocked with L-pyroglutamate aminopeptidase

n.a. = not analyzed

When the culture was well aerated, the protein was homogeneous and no inhibitors were needed. The presence of a blocked N-terminus indicated that the precursor had been fully maturated. Ser-248 was found at the C-terminus indicating that the additional aspartic acid residue in position 249 had been completely removed, contrary to the protein isolated from cells grown in the shaken-flasks containing traces of Asp-249 (table 1). (The sequence corresponding to the minor band, with $pI = 5.7$, infrequently observed in isoelectric focusing gels, could not be determined.) The homogeneous protein, stored in the form of either a purple membrane suspension (in water) or solubilized protein (in a 1% w/v aqueous solution of purified octyl glucoside), was stable in the absence of any protease inhibitor when sodium azide was added as a bactericidal agent.

The results in table 1 demonstrate that illumination was not required for the synthesis of large amounts of purple membrane. On the contrary, aeration of the culture medium was a limiting factor for the maturation process. Fully maturated protein could only be obtained under sufficient aeration; decreased aeration yielded various forms of incompletely maturated protein (some populations detected in gels may correspond to protein molecules lacking the chromophore or to various protein conformations). Consequently, it may be postulated that either the biosynthesis, the function or the regulation of the peptidase(s) responsible for the maturation of the bacterio-opsin precursor, are directly or indirectly oxygen dependent. No information is available concerning the aminopeptidase(s) cleaving the signal sequence or the carboxypeptidase responsible for the cleavage of Asp-249. It is also not known whether the cyclization of Gln-1 is an enzyme-catalyzed reaction or is spontaneous, since both mechanisms are possible [23].

It may be argued that the ability to produce homogeneous bacteriorhodopsin is strain-dependent. The strain S-9 overproduces purple membrane, does not form gas vacuoles, and lacks the C-50 carotenoids (bacterioruberins) [24,25]. As a consequence of these

mutations, the isolation of the purple membrane was simplified. When analyzed by centrifugation on a sucrose gradient, the purple membrane from homogeneous or heterogeneous preparations gave a single band. Previous reports have shown that several strains, including S-9, produce heterogeneous bacteriorhodopsin when grown under limited aeration [7]. It cannot be excluded that strains, lacking the peptidase(s) involved in the maturation process or enzymes responsible for post-translational modifications (e.g. cyclization of glutamine), can produce other bacteriorhodopsin forms. The results presented here demonstrate that the same strain can yield a protein of another quality when grown under different conditions. Cultures, inoculated with a mixture of colonies or with cells from a single clone, produced homogeneous protein when the medium was sufficiently aerated. Although this procedure can eliminate initial contamination by mutants unable to mature the protein, those may reappear because of the high mutation frequency in these bacteria [24]. It was preferable to harvest the cells at the end of the pre-stationary phase (after 5–6 days), rather than later, because changes in the metabolism occur in the stationary phase leading to the formation of products which can interfere with the preparation of the protein (e.g. a white pellet of increasing size with the age of the culture was observed when the purple membrane was prepared from cells in the stationary phase).

The amount of dissolved oxygen available to the cells is suspected to be the important parameter for growth as well as for the synthesis and maturation of bacteriorhodopsin. This results from the amount of oxygen dissolved in the medium (which is dependent upon aeration and mixing) but also from the number of cells present in the medium. The apparent discrepancies between previously reported results and those presented here, regarding the intensity of the aeration, suggest the existence of an optimal oxygen concentration at which both, bacteriorhodopsin biosynthesis and complete maturation processes, can occur simultaneously. Under these conditions illumination is not required to obtain large amounts of protein. This explanation is also in agreement with the facts that insufficient aeration can lead to incomplete maturation whereas air saturation inhibits the synthesis of bacteriorhodopsin [26]. Optimal conditions may be different for individual strains especially in the case of strains overproducing purple membrane when compared to the wild-type.

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