BIOGENESIS OF PURPLE MEMBRANE: REGULATION OF BACTERIO-OPSIN SYNTHESIS

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1. Introduction

In the cell membrane of Halobacteria a photoreceptor protein, bacteriorhodopsin, forms a two-dimensional hexagonal crystalline network within the lipid matrix [1–4]. This specialized domain of the membrane has been termed purple membrane [5,6].

Besides its attractiveness for bioenergetic studies, the purple membrane appears highly suited for studying the biogenesis of a membrane: Purple membrane contains only one protein species (bacterio-opsin), one pigment (retinal) bound to bacterio-opsin and two main lipid species. Its biogenesis is inducible: A situation of low oxygen supply [7] and/or of nutrient exhaustion triggers the membrane synthesis.

In a recent paper [8] it was shown that (1) the synthesis of both the membrane components bacterio-opsin and retinal are strictly coordinated. Only inducing conditions turn on both biosynthetic pathways. (2) Another differentiated domain of the plasma membrane termed the 'brown membrane' is involved in the biogenesis of the purple membrane. This domain has a lower buouyant density than purple membrane, contains bacteriorhodopsin and a cytochrome *b*-type protein besides lipid and some minor protein species. (3) Newly formed bacterio-opsin is initially incorporated in the brown membrane and can only 'crystallize' to form the purple membrane patches after reaction with retinal and a subsequent modification step.

2. Materials and methods

2.1. *Halobacteria strain and media*

Growth of the mutant R_1M_1 of H. halobium which does not synthesize bacterioruberin was performed as described in ref. [9]. Basal salt solution contained per liter 250 g NaCl, 20 g $MgSO_4 \cdot 7\ H_2O$ and 2 g KCl.

2.2. *Growth conditions inducing purple membrane production*

Large scale: 14 liter Magnaferm fermenter (New Brunswick); aeration 120 liter/min, 300 rev/min, 39°C. Small scale: 230 ml medium in a 500 ml Erlenmeyer flask or 10 ml in a 25 ml Erlenmeyer flask, shaken with 100 reciprocating movements per min. These conditions of limited oxygen supply will be denoted in the following as 'standard conditions'.

Sufficient aeration (nearly no purple membrane production) occurs if the fermenter is run at 180 liter air/min and 500 rev/min or a 500 ml Erlenmeyer fask is filled with only 90 ml medium under otherwise identical conditions.

2.3. *Isolation of the membrane fractions*

Cells were lyzed in 0.2 M NaCl solution. A small amount of DNase was added to reduce viscosity. The lyzate was centrifuged in a Beckman 50 Ti rotor at 35 000 rev/min for 2 h. The sedimented membrane fractions were resuspended and analyzed either directly by dodecylsulphate gel electrophoresis or further separated by sucrose density gradient centrifugation (20–45% sucrose) in a Beckman SW 41 rotor (36 000 rev/min, 6–8 h). This procedure yields the membrane fractions RM 340, brown membrane and purple membrane. Spectra were taken with an Aminco DW 2 spectrophotometer.

2.4. *Gel electrophoresis*

Dodecylsulphate gradient gel electrophoresis was performed as described in ref. [10]. A linear gradient (10–25% acrylamide) was used.

3. Results and discussion

The alkaloid nicotine selectively inhibits synthesis of the purple membrane component retinal [11] by blocking the cyclization of lycopene to β-carotene [12] In cells grown in the presence of 1 mM nicotine (in the following denoted as 'nicotine cells') production of the purple membrane is completely blocked, synthesis of the membrane protein bacterio-opsin yet remains inducible: Nicotine cells grown under conditions of limited oxygen supply contain 4–5 fold higher levels of bacterio-opsin than aerobically grown nicotine cells. This observation indicates that the signal inducing the cell to produce purple membrane directly controls the protein synthesis of bacterio-opsin.

Bacterio-opsin accumulating in nicotine cells does not form the crystalline lattice of the purple membrane, but is found in another differentiated membrane fraction that was called the brown membrane. Figure 1 shows the membrane protein pattern (fig.1A) and some spectroscopic properties (fig.1B and C) of the brown membrane. In addition to bacterio-opsin, one further protein component together with some minor protein species are detectable by dodecylsulphate gel electrophoresis. The absorption spectrum of the brown membrane purified from nicotine cells (fig.1B) displays maxima at 418 nm and 276 nm (not shown) in the oxydized form and at 560 nm, 527 nm, 427 nm and 276 nm in the dithionite-reduced form, indicating the presence of a heme protein. Addition of retinal converts the bacterio-opsin of the brown membrane to bacteriorhodopsin, thereby forming the 570 nm chromophor. Brown membrane preparations isolated from cells grown in the absence of nicotine contain most of their bacterio-opsin already complexed with retinal, only about 10–20% free bacterio-opsin are detectable (fig.1C).

In order to follow the kinetics of purple membrane formation, nicotine cells containing bacterio-opsin were resuspended in a nicotine-free medium and further incubated at 37°C. As shown in fig.2A, the bacterio-opsin of the brown membrane becomes gradually converted to bacteriorhodopsin. Obviously, endogenous retinal synthesis immediately resumes under these conditions. After a lag period of about 2 h the bacteriorhodopsin of the brown membrane begins to 'crystallize', thereby forming purple membrane patches. If this experiment is repeated in the presence of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) purple membrane formation is completely

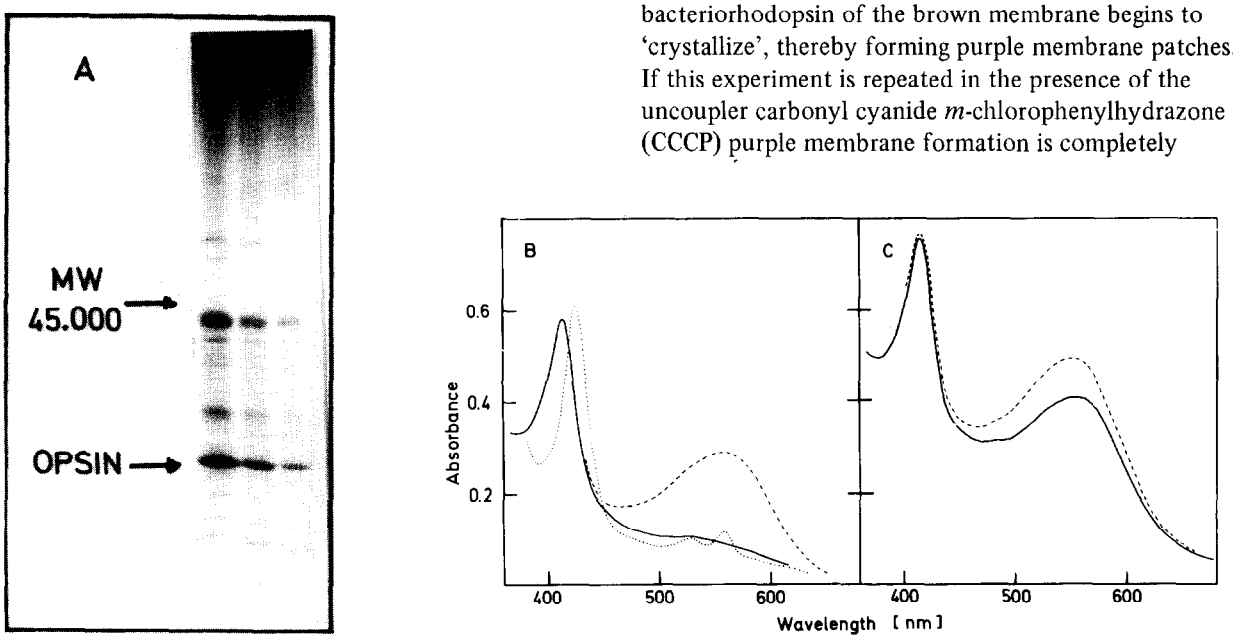

Fig.1 Protein composition and spectral properties of the brown membrane. (A) Dodecylsulphate gradient gel electrophoresis of the brown membrane. (B) Absorption spectrum of the brown membrane isolated from nicotine cells: (———) air oxidized form, (. . . .) dithionite-reduced form and (- - -) air oxidized form after the addition of 15 nmol/ml all-trans retinal. (C) Absorption spectrum of the brown membrane isolated from cells grown in the absence of nicotine: (———) air oxidized form, (- - -) air oxidized form after the addition of 15 nmol/ml retinal.

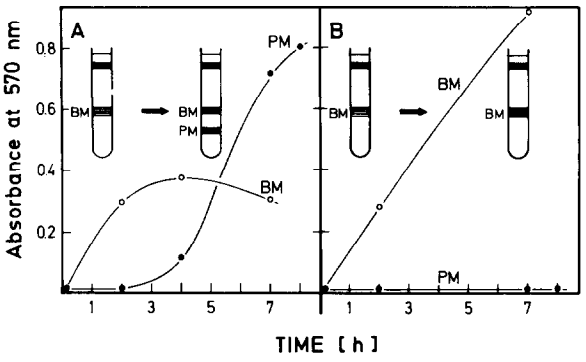

Fig.2. Kinetics of purple membrane formation. Halobacteria were grown in the magnaferm fermenter under stanard conditions. After 90 h, the cells were harvested by centrifugation, washed in basal salt solution and resuspended in basal salt solution containing 0,5% L-alanine (cell concentration: A_{578} = 1.5). 200 ml aliquots were shaken in 500 ml Erlenmeyer flasks at 39°C for the times indicated. After incubation, the cells were harvested and lyzed. The membrane fractions were separated by sucrose density gradient centrifugation as described in Materials and methods. Brown membrane fractions (BM) and purple membrane fractions (PM) were diluted to 2.0 ml and analyzed spectrophotometrically at 570 nm. (A) incubation in basal salt containing L-alanine. (B) as (A), except for addition of 2 × 10^{-5} M carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). The inserts show schematically the changes of the membrane patterns (sucrose gradients).

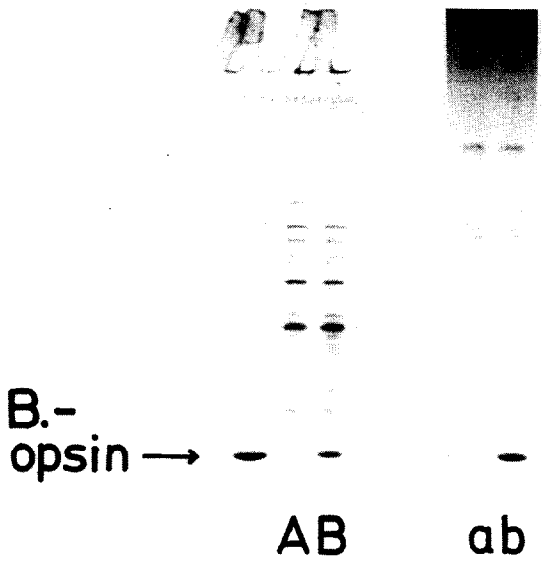

Fig.3. Induction of bacterio-opsin synthesis by retinal. Two 10 ml aliquots of a culture of nicotine cells grown under standard conditions for 4 days were transferred into 25 ml Erlenmeyer flasks. To each flask 5 μCi [^{14}C]valine were added, to flask B 0,3 μmol retinal was added in addition. The flasks were shaken at 37°C. After 30 h, the cells were harvested and lyzed. Membrane proteins were analyzed by dodecylsulphate gel electrophoresis as described in Materials and methods. (A) Membrane protein pattern of reference cells. (B) Membrane protein pattern of the retinal-treated cells. (a) And (b) autoradiography of the patterns (A) and (B), respectively.

blocked, though the endogenous retinal synthesis again resumes: All the bacterio-opsin present is converted to bacteriorhodopsin, but remains localized in the brown membrane (fig.2B). The same type of inhibition is observed in the presence of 0.1 mM cyanide. Formation of purple membrane is also blocked by the presence of the protein biosynthesis inhibitor puromycin, though retinal synthesis again remains unaffected. Apparently, an energy-dependent (protein synthesis-dependent) step is involved in the process of sorting out and assembling bacteriorhodopsin in the brown membrane to form the purple membrane patches.

As mentioned above, synthesis of bacterio-opsin remains inducible even under conditions of complete inhibition of retinal synthesis. However, these nicotine cells contain only 20–30% of the bacterio-opsin present in cells grown in the absence of nicotine but under otherwise identical conditions. This finding suggests that free bacterio-opsin (but not bacteriorhodopsin) is able to inhibit its own synthesis. If this were the case, one would expect that addition of retinal to a culture of nicotine cells would stimulate synthesis of bacterio-opsin. Figure 3 shows the result of such an experiment: One half of a nicotine cell culture of the stationary growth phase was treated with retinal, the other half used as reference. The analysis reveals a nearly 5-fold stimulation of bacterio-opsin production in the retinal-treated cells. If a labeled amino acid is added together with retinal, bacterio-opsin becomes highly labeled (fig.3b). Thus retinal stimulates indeed de novo protein synthesis of bacterio-opsin. This conclusion was also reached on the basis of similar experiments using the protein synthesis inhibitor puromycin. Simultaneous addition

Table 1
Bacteriorhodopsin content of cells grown under different conditions

Culture	Growth conditions			Bacteriorhodopsin production (nmol/100 ml culture)
	High O_2 supply	Low O_2 supply	Addition of retinal	
1	+	–	–	12
2	+	–	+	14
3	–	+	–	61
4	–	+	+	69

Cells were grown under conditions of limited or high oxygen supply, respective (see Materials and methods). To culture 2 and 4, 0.5 nmol retinal per ml culture were added every 10 h. After 4 days, the cells were harvested and lyzed. The total membrane fraction was analyzed spectroscopically for bacteriorhodopsin content.

of retinal and puromycin (20 μg/ml) to nicotine cells completely abolish the increase of bacterio-opsin production.

Our results so far demonstrate that bacterio-opsin controls its own synthesis. These results imply that this control is exerted either at the level of transcription (a repressor function of bacterio-opsin) or at the level of translation. The data available at present allow no distinction among these possibilities.

The experimental results obtained so far show the existence of at least two different control mechanisms regulating bacterio-opsin synthesis: (1) Low oxygen concentration induces bacterio-opsin synthesis and (2) free bacterio-opsin inhibits its own synthesis. Retinal cancels this inhibition, thereby inducing bacterio-opsin synthesis. In order to find out the hierarchy of these control functions, the following experiment was made: Halobacteria were grown under conditions of efficient aeration and every 10 h retinal was added to the culture. Under these conditions, control mechanisms 1 and 2 should regulate in conflicting ways. Mechanism 1 does not allow bacterio-opsin synthesis, whereas mechanism 2 allows synthesis. The experimental results summarized in table 1 indicate that the control pathway exerted by oxygen concentration is dominant and determines the maximum rate of opsin synthesis for a given growth condition. It appears plausible that the main job of control mechanism 2 is to coordinate the bacterio-opsin and retinal synthetic pathways.

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CONFORMATIONAL DYNAMICS OF PORCINE PANCREATIC COLIPASE: A 360 MHz PROTON NUCLEAR MAGNETIC RESONANCE STUDY

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1. Introduction

The present study is devoted to colipase II, one of the two main molecular forms of porcine pancreatic colipase [1,2]. This small protein has a molecular weight of 8700 and consists of a single polypeptide chain of 84 amino acid residues [3,4]. Its amino acid sequence has been determined [3] and corresponds to a rather compact structure maintained by 5 disulfide bridges [4]. The biological role of colipase is of great importance since it acts in vivo as a cofactor preventing the inhibitory effect of physiological concentrations of bile salts on the intraduodenal lipolysis of dietary triglycerides [1,5,6]. In an attempt to explain the physiological effect of colipase, binary and ternary associations of colipase, lipase and bile salts have been recently investigated under a variety of experimental conditions [7–13]. Typically, colipase has been shown to bind stoichiometrical amounts of bile salt micelles and to form a binary complex that pancreatic lipase can in turn recognize. It is likely that these binding and recognition processes involve specific conformational changes creating and/or unveiling appropriate sites on the colipase molecule [11].

The potential of high resolution n.m.r. in describing the conformational rearrangements of proteins under various perturbing factors (pH, temperature, ionic strength, ligand binding etc...) is now widely recognized. In this paper, I report and describe the first n.m.r. spectra of porcine colipase, obtained with the help of the most recent advances in the instrumentation for high field proton n.m.r. spectroscopy. Several resonances can be ascribed to specific residues or classes of residues and provide useful natural probes to study the conformational dynamics of colipase II in solution.

Abbreviations: n.m.r., Nuclear Magnetic Resonance. DSS, Sodium 2,2-dimethyl-2-silapentane-5-sulfonate. HMDS, Hexamethyldisiloxane. ppm, parts per million.

2. Material and methods

Porcine pancreatic colipase II was a generous gift of Mrs. M. Astier and Dr. M. Charles [1]. Fourier Transform n.m.r. spectra were obtained on the Bruker HXS-360 MHz spectrometer located at the Stanford Magnetic Resonance Laboratory (Stanford University California, USA). This spectrometer is interfaced with a Nicolet NIC-80 16K computer equipped with a Nicolet NIC-294 disc system. Protein samples were dissolved in pure D_2O (Diaprep) at a concentration of 1.5 mM. and pH was adjusted with dilute NaOD and DCl. The pH values were measured on a Radiometer pH Meter Model 26 employing a thin combination glass electrode and are given without correction for deuterium isotopic effect. Chemical shifts were measured with respect to HDO and subsequently converted to HMDS by measuring the position of HDO resonance with respect to an external capillary of HMDS. No correction for bulk magnetic susceptibility was applied. For the sake of a better comparison with other n.m.r. studies of proteins, chemical shifts were finally expressed in ppm from DSS. All spectra were recorded at 30°C using 5 mm precision n.m.r. cells (Wilmad). Sensitivity enhancement was achieved using negative exponential multiplication of the free