LOCALIZATION OF THE METHYLATION SYSTEM INVOLVED IN SENSORY BEHAVIOR OF *HALOBACTERIUM HALOBIUM* AND ITS DEPENDENCE ON CALCIUM

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1. Introduction

Halobacterium halobium has two sensory photosystems, PS 370 and PS 565, by which it can detect UV- and visible light [1]. It can also detect chemical gradients [2]. Both light and chemical stimuli modulate the frequency of reversals of the swimming direction: chemoattractants and attractant visible light suppress the reversals for a certain time while chemorepellents and repellent UV-light increase the frequency of reversal responses [1,3]. Membrane proteins are methylated in the presence of attractants and demethylated in the presence of repellents [4]. Ca^{2+} is supposed to play a role in controlling the direction of flagellar rotation [5]. This paper describes the influence of Ca^{2+} on the activitities of methyltransferase and methylesterase and the localization of the 2 enzymes and of methylated membrane proteins in the cell.

2. Materials and methods

Halobacterium halobium, strain R_1, was grown in peptone medium [2] to the stationary phase. Bacteria were centrifuged at 13 000 × *g*, washed, and resuspended in peptone-free medium.

2.1. *Methylation of membrane proteins in vivo*

Bacterial suspension (40 ml) were shaken in the presence of 30 μg puromycin/ml (Boehringer, Mannheim) for 60 min at 37°C to block protein biosynthesis [4,6]. 400 μCi of L-[*methyl*-^{3}H]methionine, 75 Ci/mmol, (Amersham, Buchler, Braunschweig) were then added. After 60 min, bacteria were centrifuged at 13 000 × *g* for 15 min. Soluble proteins, red membrane and purple membrane were isolated as in [7].

2.2. *Methylation and demethylation in vitro*

A culture of 50 ml was centrifuged and resuspended in 5 ml peptone-free medium. The suspension was sonicated for 10 s at 70 W (Branson sonifier B12, microtip, Danbury CT). Cell envelopes and soluble fraction were separated by centrifugation at 10 000 × *g* for 5 min, the pellet containing the envelopes was washed and resuspended in a 4.3 M NaCl-solution at pH 7.0.

Methylation: 200 μl envelope suspension (400 μg protein/ml) were incubated with 20 μCi *S*-adenosyl-L-[*methyl*-^{3}H]methionine, 64 Ci/mmol, (Amersham, Buchler, Braunschweig) for different times and at different free [Ca^{2+}] at 37°C. Stimulus-dependent methylation was measured after adding glucose (6 mM) or after illumination with light of 565 nm wavelength for 60 min with an intensity of $4.8 \cdot 10^{14}$ $h\nu \cdot cm^{-2} \cdot s^{-1}$ (100 W tungsten lamp, 565 nm interference filter, half-width 5.9 nm, Schott, Mainz). Envelopes were then centrifuged at 10 000 × *g* for 5 min.

Demethylation: 100 μl radioactively labeled, methylated envelopes were incubated with 1 ml soluble fraction (6 mg protein/ml) at 37°C for different times and at different concentrations of free Ca^{2+}. Stimulus-dependent demethylation was measured after exposing the samples to 5 mM phenol or illumination with UV-light with an intensity of $2.9 \cdot 10^{13}$ $h\nu \cdot cm^{-2} \cdot s^{-1}$ for 30 min (100 W tungsten lamp, 370 nm interference filter, half-width 12.2 nm, Schott, Mainz).

Free [Ca^{2+}] was adjusted by 1 mM EGTA and different amounts of $CaCl_2$ at pH 7.2 according to [8]. The Ca^{2+} activities were not corrected for the high ionic strength of the NaCl-solution and therefore cannot be regarded as absolute values.

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2.3. *SDS-gel electrophoresis*

Membrane fragments or envelopes (40 μg protein each) were resuspended in 100 μl 20 mM phosphate buffer at pH 6.8, solubilized in SDS-cocktail at 57°C for 10 min and run on SDS–polyacrylamide gels [9]. Dansylated bovine serum albumin [10] was added as a marker. Gels were cut into 1.5 mm slices (15 above and 15 below serum albumin) and solubilized in 4 ml toluene-based scintillation cocktail containing 10% NCS solubilizer (Amersham/Searle, USA). Radioactivity was measured in a liquid scintillation spectrometer. Protein content was determined as in [11].

3. Results and discussion

3.1. *Localization of methylated membrane proteins*

In *H. halobium*, the cell membrane can be further fractionated into the purple membrane and the red membrane [7]. Fig.1 shows that radioactive methyl groups from methionine were transferred only to proteins located in the red membrane. The methyltransferase seems to be rather specific, soluble proteins were not found to be methylated and no radioactivity could be detected in the purple membrane. In the red membrane fraction, 5 radioactive protein bands were observed. These do not necessarily correspond to 5 different proteins, they may be due to multiple methylation of fewer proteins which alters their electrophoretic mobility [12,13].

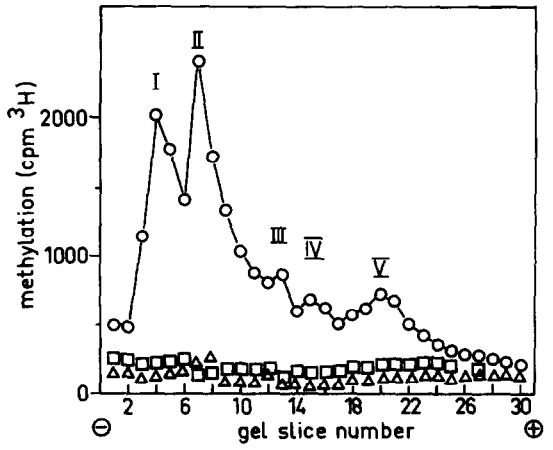

Fig.1. Protein methylation in vivo. *H. halobium* was incubated with L-[*methyl*-^{3}H]methionine after inhibition of protein biosynthesis by puromycin: (△) soluble proteins; (□) purple membrane; (○) red membrane were isolated and run on SDS-gels. Radioactivity was determined in 1.5 mm gel slices.

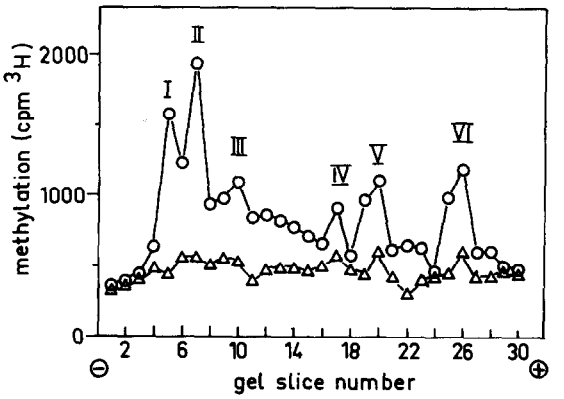

Fig.2. Methylation and demethylation of membrane proteins in vitro. Methylation was carried out by incubating envelopes with *S*-adenosyl-L-[*methyl*-^{3}H]methionine. Methylated envelopes were divided into 2 portions. One was analyzed for incorporated methyl groups directly. Demethylation was measured after addition of soluble proteins to the second portion. Incubation was 60 min at 37°C. Envelopes were run on SDS-gels and radioactivity was determined in 1.5 mm gel slices: (○) methylation; (△) demethylation.

3.2. *Methylation and demethylation in vitro*

Methylation of membrane proteins can be measured in vitro with cell envelopes by using radioactively labeled *S*-adenosyl-methionine as methyl donor. Methylation in vitro yields 6 radioactive protein bands on SDS gels (fig.2).

The methyltransferase of *S. typhimurium* is a soluble protein [14]. In *H. halobium*, the enzyme seems to be attached to the envelope fraction, since methylation occurred in the absence of the soluble fraction. In the presence of the soluble fraction, much less radioactivity was incorporated (not shown). This may be due to the competitive activity of a methylesterase in the soluble fraction. Addition of soluble proteins to methylated envelopes led to a loss of radioactivity from membrane proteins (fig.2) indicating demethylation. Thus, the methylesterase is obviously located in the soluble fraction like in *S. typhimurium* [15]. When envelopes in the presence of *S*-adenosyl methionine were stimulated by attractants, the level of methylation was found to be higher than in unstimulated samples (table 1a). Bands I–III (see fig.2) had increased upon treatment with glucose as well as upon illumination with light of 565 nm wavelength. With glucose, the highest increase was found in bands I and II, illumination caused the highest increase in band III. Bands IV–VI were not changed significantly.

Stimulation of methylated envelopes by repellents

Table 1
Stimulus-dependent methylation and demethylation in vitro

(a) Methylation: Attractant stimulus	cpm ^3H (t = 0 min)	cpm ^3H (t = 60 min)
None	190	6100
Glucose	187	9500
Illumination at 565 nm	200	10 900
(b) Demethylation: Repellent stimulus	**cpm ^3H (t = 0 min)**	**cpm ^3H (t = 30 min)**
None	6200	3800
Phenol	6180	2050
Illumination at 370 nm	6230	1770

Experiments were carried out as in fig.2.

(a) Methylation: Together with radioactive *S*-adenosyl methionine, 6 mM glucose were added or the sample was illuminated at 565 nm wavelength ($4.8 \cdot 10^{14}$ $h\nu \cdot cm^{-2} \cdot s^{-1}$). The level of methylation is given as the total amount of radioactivity found in bands I–III (see fig.2).

(b) Demethylation: Methylated envelopes in the presence of methylesterase were stimulated by 5 mM phenol or illumination at 370 nm wavelength ($2.9 \cdot 10^{13}$ $h\nu \cdot cm^{-2} \cdot s^{-1}$). Methylation level is given as the total amount of radioactivity found in bands II–IV

in the presence of soluble proteins activated demethylation (table 1b). Upon addition of phenol or illumination with light of 370 nm wavelength, more methyl groups were removed from bands II–IV than in the control sample. Obviously, the receptors were not lost during the sonication procedure which indicates that they are located in the membrane rather than in the periplasmic space.

Fig.3 shows that methylation occurs much slower than demethylation. A similar time course has been reported for *E. coli* [16].

3.3. *Calcium-dependence of methylation and demethylation*

Attractant stimuli raise the methylation of membrane proteins and suppress the frequency of reversals of the swimming direction; repellent stimuli cause demethylation and an enhanced frequency of reversals. In *B. subtilis* [17] and in *H. halobium* [18,19] low external [Ca^{2+}] suppress the reversals while high [Ca^{2+}] in the presence of a Ca^{2+} ionophore increase the reversal frequency. One might therefore expect some correlation between the [Ca^{2+}] and the activities of methyltransferase and methylesterase. Fig.3 shows that methylation in vitro was inhibited by Ca^{2+} while demethylation was activated. Inhibition of the methyltransferase by Ca^{2+} has also been reported for *B. subtilis* [20]. These findings correspond to the influence of Ca^{2+} on the behavioral reactions. The Ca^{2+}-dependence of methylation and demethylation of membrane proteins reported here may be the key to understand the effect of Ca^{2+} on the behavioral responses of *H. halobium* and other bacteria.

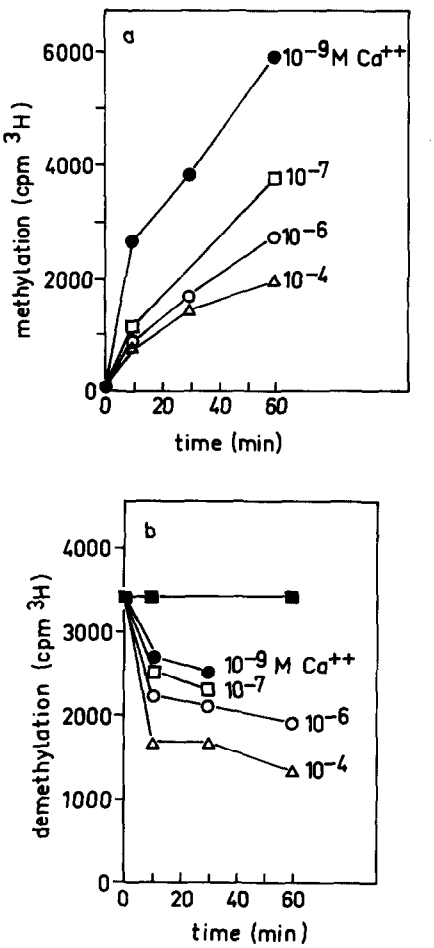

Fig.3. Calcium dependence of methylation (a) and demethylation (b). Experiments were carried out as in fig.2. The closed squares in (b) indicate the methylation level in the absence of methylesterase-containing soluble proteins. All samples contain 1 mM EGTA at pH 7.2. 10^{-9} M Ca^{2+}: no addition of $CaCl_2$; 10^{-7} M: addition of 0.55 mM $CaCl_2$; 10^{-6} M: 0.93 mM $CaCl_2$; 10^{-4} M: 1.1 mM $CaCl_2$. On the ordinate the total amount of radioactivity found in bands I–VI (see fig.2) is given.

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