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Our studies concern the molecular basis of sensory and energy transduction by archaeobacterial rhodopsins, phototransducers in *Halobacterium halobium*. Two of these rhodopsin-like proteins, bacteriorhodopsin (BR) and halorhodopsin (HR), are light-driven ion pumps. The other two, sensory rhodopsins I and II (SR-I, λ_{max} 587 nm and SR-II, λ_{max} 487 nm), regulate the cells' swimming behavior, enabling them to migrate into preferred regions of light intensity and color (phototaxis). Photoexcitation of SR-I generates a long-lived intermediate absorbing in the near-UV (373 nm). This species, S₃₇₃, is also photochemically reactive and functions as a third phototaxis receptor. Retinal analog studies show that all-*trans*/*13-cis* isomerization of the chromophore is required for SR-I and SR-II photochemical reactions and receptor signaling *in vivo*. We have identified the chromoproteins of SR-I and SR-II, both hydrophobic proteins of ~25 kD, and a second SR-I component, a 94 kd integral membrane protein which undergoes reversible covalent modification by carboxymethylation. We are isolating fragments of this protein, whose sequence

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18. bacterial motility, photosensory receptors, retinal, color-sensing, photodetection, spectroscopy
19. will be used for preparation of an oligonucleotide probe for cloning. In recent progress, we have demonstrated that photostimuli control the *in vivo* demethylation rate through photoactivation of SR-I in either its attractant or repellent signaling form as well as through the repellent receptor SR-II. Simultaneous photoactivation of the SR-I attractant and SR-II repellent receptors cancel in their effects on demethylation, demonstrating the methylation system is regulated by an integrated signal. Our working model is that the three known phototaxis signaling receptor states are coupled to two distinct transducers whose demethylation is controlled by one integrated signal. We are studying the role of methylation in photosignal transduction by biochemical and spectroscopic analysis of the chromoproteins and methylation system components in photosignaling mutants.



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ANNUAL REPORT ON GRANT N00014-89-J-1629

PRINCIPAL INVESTIGATOR: Dr. John L. Spudich

CONTRACTOR: Albert Einstein College of Medicine
Bronx, NY 10461

TITLE: Role of Protein Methylation in *Halobacterium halobium* Phototaxis

RESEARCH OBJECTIVES: This project is part of an effort to understand the physical chemical basis of sensory and energy transduction by biological membranes using a model system: the bacterial rhodopsins of the archaebacterium *H. halobium*. Our objective is to gain insight into the mechanism of signaling and adaptation to phototaxis stimuli by studying the role of protein methylation in signaling by the phototaxis receptors sensory rhodopsin I and II (SR-I and SR-II). Methylation of a set of integral membrane proteins is required for taxis adaptation in *H. halobium* and a 94 kd protein has been linked specifically to SR-I. We aim to determine the relationship between this protein and the 25 kd chromophoric polypeptide of SR-I, and the role of methylation in modulation of phototaxis signals.

PROGRESS (Year 1): The support from this grant has enabled us to: (1) implement a new analysis method for the methylation system to test its relationship to signaling by the receptors photoreactions; (2) determine by using a retinal analog "locked" in a 13-*trans* configuration that all-*trans* to 13-*cis* isomerization of retinal is the trigger for phototaxis signaling by sensory rhodopsins I and II (SR-I and SR-II); and (3) characterize the spectral properties of SR-II, for which previously only rough spectra were available. This primitive retinylidene protein has unique properties which became evident in this study (see (3) below).

(1) We have been able to demonstrate directly that phototaxis stimuli control the methylation system *in vivo* through photoactivation of SR-I in either its attractant or repellent signaling form as well as through SR-II. The effects of positive stimuli which suppress swimming reversals (i.e. an increase in attractant or decrease in repellent light) and negative stimuli which induce swimming reversals (i.e. a decrease in attractant or increase in repellent light) through each photoreceptor were monitored by assaying release of volatile [³H]methyl groups (methanol from methylester hydrolysis). In *H. halobium* positive photostimuli produce a transient increase in the rate of demethylation followed by a decrease below the unstimulated value, whereas negative photostimuli cause an increase followed by a rate similar to that of the unstimulated value. Simultaneous photoactivation of the SR-I attractant and SR-II repellent receptors cancel in their effects on demethylation, demonstrating the methylation system is regulated by an integrated signal.

Mutant analysis indicates the source for the volatile methyl groups are intrinsic membrane proteins distinct from the chromoproteins which share the membrane. We found the methyl-accepting protein (94 kD M_r) previously correlated in amount with the SR-I chromoprotein (25 kD M_r) is missing in a recently isolated SR-I⁻SR-II⁺ mutant (Flx3b), thus confirming the association of this protein with SR-I. Photoactivated SR-II in Flx3b controls demethylation, predicting the existence of a photomodulated methyl-accepting component distinct from the 94 kD M_r protein of SR-I. Based on these results we have developed a model in which the three known phototaxis signaling receptor states (the attractant receptor SR-I₅₈₇, its repellent form S₃₇₃, and the repellent receptor SR-II₄₉₀) are coupled to two distinct transducers whose demethylation is controlled by one integrated signal. This work resulted in the publication of a paper in PNAS (reprint enclosed), and contributed to one submitted to J. Bacteriology (manuscript enclosed).

(2) An analog of all-*trans* retinal in which all-*trans*/13-*cis* isomerization is blocked by a carbon bridge from C12 to C14 was incorporated into the apoproteins of SR-I and SR-II in retinal-deficient *H. halobium* membranes. The "all-*trans* locked" retinal analog forms SR-I and SR-II analog pigments with similar absorption spectra as the native pigments. Blocking isomerization prevents the formation of the long-lived intermediate of the SR-I photocycle (S₃₇₃) and those of the SR-II photocycle (S-II₃₆₀ and S-II₅₃₀). A computerized cell tracking and motion analysis system capable of detecting 2% of native pigment activity was used for assessing motility behavior. Introduction of the locked analog into SR-I or SR-II apoprotein *in vivo* did not restore phototactic responses through any of the three known photosensory systems (SR-I attractant, SR-I repellent, or SR-II repellent). The results demonstrate that unlike the phototaxis receptor of *Chlamydomonas reinhardtii*, which has been reported by Foster and coworkers to mediate physiological responses without specific double bond isomerization of its retinal chromophore, all-*trans*/13-*cis* isomerization is essential for SR-I and SR-II phototaxis signaling. In this regard, photoactivation of these primitive archaeobacterial photoreceptors resembles that of evolutionarily distant visual pigments in higher organisms. This work is in press in the Biophysical Journal (manuscript enclosed).

(3) Using the SLM Aminco DW2000 spectrophotometer provided by ONR funds, we have conducted band shape analysis of SR-II and probed its retinal-binding cleft with chromophore analogs designed to elucidate retinal/apoprotein interactions. We found the SR-II absorption spectrum is defined by vibrational fine structure, which no other retinylidene pigment exhibits (e.g. mammalian rhodopsins with similar absorption maximum and bandwidth as SR-II nevertheless shows unstructured absorption). Analysis with analog chromophores indicates that this unique property is due to two factors: (i) the protein forces the retinal ring to be coplanar with the retinal polyene chain, and (ii) retinal/protein electrostatic interactions which diffuse vibrational modes in visual pigments and other bacterial rhodopsins, are lacking in SR-II. This finding has significance to understanding wavelength tuning as well as the range of chromophore structures possible in retinylidene proteins. We will shortly be submitting a paper describing this work to Biochemistry.

WORK PLAN (Year 2): A major focus will be to characterize the chemical properties of the methylated 94 kd protein, which appears to play a key role in SR-I signaling. Methyl-accepting chemotaxis proteins in *E. coli* are methylated by formation of a carboxymethylester on glutamate residues at multiple sites on the protein. We will apply high performance liquid chromatography (HPLC), using the system we purchased with the ONR equipment allocation, to isolate fragments of the 94 kd protein to establish the nature and number of methylation sites, and the sequence of the methylated region of the 94 kd to compare with known eubacterial methyl-accepting transducers. The 94 kd protein is the first methyl-accepting taxis protein in an archaeobacterium. The comparison of sequence is therefore interesting from an evolutionary perspective. In terms of mechanism an important question is whether there is homology in the domain near the eubacterial methylation sites implicated in the excitation process.

The isolated 94 kd protein will be fragmented and sequenced and oligonucleotide probes used to clone from an existing *H. halobium* genomic library in pUC12. We have begun the fragment purifications and within 2 months should have material suitable for sequencing after which we can initiate the cloning work.

Also in this period we will begin the characterization of absorption properties of SR-I and SR-II *in vivo* and in vesicle membranes. Our objective is to characterize the pigments in wild type cells for comparison with cells and membranes in which methylation is altered genetically and by photostimulation. SR-II in particular is difficult to monitor because of its low concentration in the membrane, however as noted above, the SLM DW2000 spectrophotometer is now installed in our laboratory and has greatly extended our capability to analyze the absorption spectra.

INVENTIONS: None.

PUBLICATIONS AND REPORTS (Year 1):

1. Spudich, E. N., Takahashi, T. and Spudich, J. L. (1989) Sensory rhodopsins I and II modulate a methylation/demethylation system in *Halobacterium halobium* phototaxis. Proc. Natl. Acad. Sci. USA 20:7746-7750.
2. Yan, B., Takahashi, T., Johnson, R., Derguini, F., Nakanishi, K. and Spudich, J. L. All-*trans*/13-*cis* isomerization of retinal is required for phototaxis signaling by sensory rhodopsins in *Halobacterium halobium*, Biophysical J., *in press*.
3. Sundberg, S. A., Alam, M., Lebert, M., Spudich, J. L., Oesterhelt, D., and Hazelbauer, G. L. Characterization of mutants of *Halobacterium halobium* defective in taxis, *J. Bact.* *in press*.
4. Takahashi, T., Yan, B., Mazur, P., Derguini, F., Nakanishi, K., and Spudich, J. L. Color-regulation in the archaeobacterial phototaxis receptor phoborhodopsin (sensory rhodopsin II), *in preparation for Biochemistry*.

TRAINING ACTIVITIES: Ms. Virginia Yao (graduate student) has been trained on the high performance liquid chromatography (HPLC) system purchased on this grant. Dr. Tetsuo Takahashi (post-doctoral fellow) and Mr. Bing Yan (graduate student who is doing his thesis on a collaborative project between my laboratory and that of Prof. Koji Nakanishi, Department of Chemistry, Columbia University, New York) have received intensive training in spectroscopy and photochemistry in this period. Dr. Padmaja Deval joined the laboratory in December, 1989, and has begun work on the HPLC fragment analysis.

AWARDS AND FELLOWSHIPS: None.