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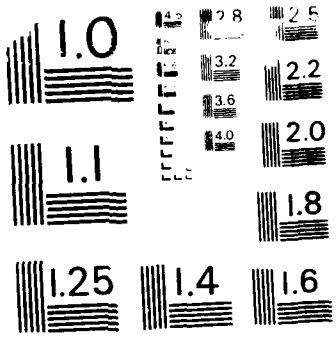

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19 ABSTRACT (Continue on reverse if necessary and identify by block number)

The objective of this project is to use primarily an electrochemical approach to study the fundamental molecular processes that underlie the light-mediated sensory (visual) and energy (photosynthetic) transduction in model retinal protein membranes. We take advantage of the chemical similarity between rhodopsin (a visual pigment) and bacteriorhodopsin (a photosynthetic pigment) to explore possible common designs in the photosynthetic and the visual membranes.

Specifically, we use an electrochemical concept of chemical capacitance and an electrophysiological method of tunable voltage clamp measurement to study the light-induced rapid charge movements in reconstituted bacteriorhodopsin and rhodopsin membranes. We were able to separate photoelectric signals that are originated from the charge movements inside the membrane as well as from the two membrane-solution interfaces, i.e., we are able to

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obtain site-specific kinetic information by electrical means. In conjunction with chemical modifications, we began to probe the site of proton uptake at the membrane surface. We found that N-bromoacetamide modification also affect the turnover time of the photocycle. Along with the experimentation, we establish a new concept of local reaction conditions that may have a significant impact on future interpretation of spectroscopic data as well as photoelectric data. A theoretical analysis of the effect of internal electric field allowed us to resolve an apparent paradox about the ionic strength effect on the bacteriorhodopsin photocycle. Our analysis further leads to the proposal of a mechanistic model of photosensory transduction in the visual membrane.

INTRODUCTION:

The objective of this project is to use primarily an electrochemical approach to study the fundamental molecular processes that underlie the light-mediated sensory and energy transduction in model retinal protein membranes. Most light-mediated energy transducing membranes utilize chlorophyll protein complexes as the "reaction centers" whereas most light-mediated sensory transducing membranes utilize retinal proteins as the light-sensing elements. At a superficial level the only thing in common to both types of membranes is the asymmetrical orientation of the membrane-bound pigment-proteins. The retinal protein, bacteriorhodopsin, from the purple membrane of Halobacterium halobium is a light-driven proton pump (Stoeckenius and Bogomolni, Ann. Rev. Biochem. 51:587, 1982). It is unique in the sense that it is similar to the visual pigment rhodopsin chemically but similar to the chlorophyll protein complexes functionally. The bacteriorhodopsin system thus presents an unusual opportunity to gain insights into possible common designs in the photosynthetic and the visual membranes.

Most of the functional data in bacteriorhodopsin literature were obtained by spectroscopic methods from purified purple membrane (aqueous) suspensions. An alternative functional approach is to monitor the light-induced electrical responses from a reconstituted model bacteriorhodopsin membrane. Although there has been an increasing number of reports based on this latter approach, there is a striking lack of consensus about the interpretation of electrical data. Our previous research based on an electrochemical concept of chemical capacitance has led to some clarification and is the basis of the present project. In the past year, we have made further progress both experimentally and theoretically. We were able to separate photoelectric signals that are originated from the light-induced charge movements inside the membrane as well as from the two membrane-solution interfaces. The obvious advantage of such a system is that it offers site-specific kinetic information in probing the membrane function in either chemically modified bacteriorhodopsin or modification via site-directed mutagenesis. Preliminary results on chemical modification will be described. We also plan to extend the study to bacteriorhodopsin reconstituted from vitamin A analogues. Along with the experimentation, we establish a new concept of local reaction conditions that may have a significant impact on future interpretation of spectroscopic data as well as photoelectric data. We also investigated an apparent paradox existing in the spectroscopic literature of bacteriorhodopsin. A theoretical analysis of the effect of internal electric field allowed us to resolve this apparent paradox. Finally, we propose a new mechanistic model of photosensory transduction in the visual membrane.

DETAILED PROGRESS REPORT:

(1) Concept of Local Reaction Conditions and Site-Specific Photoelectrokinetics in a pigment-containing membrane:

It has been known for almost a quarter century that light-induced rapid charge separation in the visual membrane leads to a weak electric signal that is physically distinct from bioelectric signals that are originated from electrodiffusion of aqueous ions. It is known as the early receptor potential (ERP) (Brown and Murakami, Nature 201:626, 1964), and is similar to the gating current in a squid axon (hence also referred to as a displacement photocurrent). The ERP has two components of opposite electric polarity: a faster R1 component is insensitive to temperature, and a slow R2 component is reversibly inhibited by

low temperature. Both components have microsecond relaxation times. Similar photoelectric signals were discovered by Montal and his colleagues (Trissl and Montal, Nature 266:655, 1977; Hong and Montal, Biophys. J. 25:465, 1979) and the two corresponding components were named B1 and B2. We have previously conducted a theoretical analysis of the light-induced charge separation in pigment-containing biomembranes (Hong, Bioelectrochem. Bioenerg. 5:425, 1978). We concluded that there are two physically distinct molecular mechanisms. Since the photopigment maintains an asymmetrical orientation in the membrane, pulsed light induced internal (intramolecular or intramembrane) charge separation and subsequent recombination will manifest as a displacement current. In addition, a heterogeneous light-induced charge transfer reaction between the membrane bound pigment and the aqueous charge donor or acceptor together with the subsequent back reaction can also lead to a displacement photocurrent (interfacial charge transfer mechanism). In either case, the photocurrent can be described as charging and discharging of a (chemical) capacitance. This latter capacitance is physically distinct from the ordinary membrane capacitance, mainly because the photocurrent source resides either inside the membrane or right at the membrane surface.

We have established in a reconstituted bacteriorhodopsin membrane that the B1 component is due to an intramembrane charge separation (oriented dipole mechanism) whereas the B2 component is due to light-induced proton uptake at the intracellular surface and its subsequent back reaction (interfacial proton transfer mechanism) (Okajima and Hong, Biophys. J. 50:901, 1986). In principle, two separate photosignals each representing the interfacial processes at the two membrane surfaces should be observable. In our model membranes reconstituted by the method of Trissl and Montal (1977), the process at the extracellular surface was suppressed because the oriented purple membrane was attached to a thin (6 um) Teflon membrane (as a mechanical support). This consideration leads us to wonder whether an additional B2′ component representing the extracellular proton release may be observable if the pigment is reconstituted in a bilayer configuration where both surfaces are exposed to water.

Our strategy is to treat the two interfacial proton transfer reactions as conventional bimolecular reactions across membrane-solution interfaces but otherwise obeying the law of mass action. An analysis of the existing data leads to a conclusion that the rate-determining step of proton transport across the purple membrane is the transmembrane step. An obvious corollary is that the two interfacial bimolecular reactions at the two separate surfaces can be treated as independent of each other on the microsecond time scale and that the reaction kinetics depends only on the reaction conditions of the adjacent aqueous phase but not those of the opposite aqueous phase (concept of local reaction conditions) (Publication #4).

We thus did the obvious experiment (which we shall refer to as a "differential" experiment) shown in Fig. 1 (Publication #5). The bacteriorhodopsin is reconstituted in a bilayer configuration according to the method of Drachev et al. starting with both aqueous phases buffered at pH 7, and both B1 and B2 components were recorded (signal 1). The intracellular pH was then lowered to zero, and the B2 component was reversibly suppressed (signal 2, B1 alone). The extracellular pH was subsequently also lowered to zero, and a negative component similar to B2 (except for an opposite pH dependence) appeared. This experiment shows opposite effects resulting from variation of the pH at opposite aqueous phases. It demonstrates the validity of the concept of local reactions conditions in the purple membrane. In addition, the experiment suggests that the negative component originates from the extracellular surface and

could well be the B2′ component. We thus established three separate components representing molecular processes at both surfaces and inside the membrane. We expect chemical modifications at the surface will affect only the surface components but not the B1 component. A preliminary experiment using fluorescamine as the surface modifying agent shows that this is indeed the case (Fig. 2; Publication #5). Another experiment using N-bromoacetamide as the surface modifying agent is more complicated. The effect of modification of the intracellular surface is primarily on the B2 component. However, if the light-induced response is preceded by another light pulse within a minute or less, the B1 component is also suppressed transiently (Fig. 3). A simple explanation is that the chemical modification has significantly increased the turnover time of the photoreactions from 10 ms in unmodified bacteriorhodopsin to about 2 minutes in modified one. We were unable to resolve beyond 25 seconds because it takes that long for our pulsed dye laser to become fully charged after a previous discharge. We acquired additional space and repaired a second laser recently. With two lasers to delivered two separate light pulses, we should be able to reduce the interval between the two light pulses to the microsecond range.

(2) Analysis of internal electric field in bacteriorhodopsin membranes:

It is known that the purple membrane contains negative surface charges on both sides. It is also known that an externally applied electric field profoundly affects the photocycle reaction kinetics of bacteriorhodopsin. Yet when the ionic strength of the aqueous phase is changed, there is virtually no change in the photocycle reaction kinetics. We analyzed the internal electric field arising from both the surface charges of phospholipid polar head groups as well as from light-induced proton transfer to or from bacteriorhodopsin (Publication #2). Several interesting results emerged. Based on a set of surface charge density data reported by Renthal and Cha (Biophys. J. 45:1001, 1984), the surface potentials were computed based on the Gouy-Chapman analysis. When the bathing solutions are changed from 1 mM KCl to 1 M KCl, the surface potentials are reduced by about 200 mV on both sides but the surface potential difference is altered by a mere 25 mV. Thus, there is actually very little change of the internal electric field as "seen" by the membrane-bound bacteriorhodopsin. This explains the apparent paradox mentioned above. Because both sides of the membrane carry negative charges, the ionic strength effect would have been much more dramatic if the two aqueous phases were controlled independently. This again speaks to the value of a "differential" experiment.

In our approach mentioned above, we treat the interfacial processe at the membrane surface as a bimolecular reaction. Specifically, the relaxation kinetics depends on the interfacial proton concentration (interfacial pH) rather than the bulk pH. Since the photoreactions lead to an increase of surface potential at the intracellular side (proton uptake) and to a corresponding decrease at the extracellular side (proton release), the internal electric field is modulated by the photoreactions. There are two separate effects of importance to the kinetic analysis. First, the progression of photoreactions could, in principle, alter the interfacial proton concentration and thus the relaxation time. A pseudo first order regime may be difficult to maintain even if the aqueous phases are pH buffered. Second, the internal electric field arising from combined action of proton uptake and proton release at opposite sides exerts an influence on the pigment itself, and could, in principle, alter the second order rate constant. All these effects will feedback to the interfacial pH, i.e., the light-induced change in the internal electric field will modulate the photoreaction. This complication may render isolation of an independent variable rather difficult if not impossible.

(3) An electrostatic regulatory mechanism of phototransduction in the visual membrane:

In addition to the above mentioned complication, the light-induced interfacial photoreactions could also provide an electrostatic regulatory mechanism at the membrane level (Publication #8). This is related to the elusive physiological function of the early receptor potential in vision. Although most investigators downplayed a possible physiological role of the ERP and regarded the ERP as an epiphenomenon (an evolutional vestige serving no physiological function), we think this conclusion premature because it was based on the absence of evidence.

It is known that the activation of transducin (G-protein), which precedes photoexcitation, occurs at the stage of metarhodopsin I to metarhodopsin II transition of the rhodopsin photochemical relaxation. The latter reaction coincides with the R2 component of the ERP and coincides with a rather significant surface potential change. This surface potential change leads a rapid change of interfacial concentration of physiologically important ions, and also exerts a rapidly varying internal electric field inside the membrane. It thus provides an ideal switching-on (triggering) mechanism for phototransduction in vision, because it is swift and highly localized (this is why it appears so small in magnitude when measured by conventional electrophysiological methodology). One further notices that during the inactivation of phototransduction a significant number of threonine and serine residues near the C-terminal of rhodopsin are phosphorylated. The resulting reduction of the surface potential at the cytosol side could provide a switching-off mechanism. Such a mechanistic model is viable only if the proton uptake accompanying the R2 component also occurs on the cytosol side of the membrane.

That the R2 component represents molecular process at the cytosol surface has been hinted at by myself but was based on indirect evident (Hong, 1978). The conclusion could be fortuitous. In a recent meeting at Szeged, Hungary (9th International Symposium on Bioelectrochemistry and Bioenergetics), I met Dr. M. A. Ostrovsky (Moscow State University) for the first time. He communicated to me a recent experiment of his group on reconstituted rhodopsin vesicles. Their work indicated that the proton uptake indeed occurs at the cytosol side. Furthermore, they found no proton release at the opposite (intradiscal) side. These findings together with the above analysis make an electrostatic regulatory mechanism of visual transduction eminently viable. Furthermore, the finding that there is only proton uptake at the cytosol side and no proton release at the intradiscal side does not support the evolutional argument that the early receptor potential is an epiphenomenon.

In the case of bacteriorhodopsin, light-induced proton transport is the major event, which requires both proton uptake and release at opposite surfaces. In the case of rhodopsin, there is no need to store the light energy by moving the proton across the membrane because photon acts as a trigger to stop the release energy previously stored as sodium ion gradient. The absence of a proton release at the intradiscal side is perfectly understandable. However, if the ERP were a useless evolutional vestige, why has nature bothered to delete the proton release while retain the proton uptake in rhodopsin? Maybe the light-induced rapid proton uptake in rhodopsin serves a subtle physiological function in visual transduction. This line of reasoning will help designs of experiments serving to prove or disprove the above described mechanistic model, and may enhance our insights into the phototransduction processes that may be in common to both energy conservation function and sensory function.

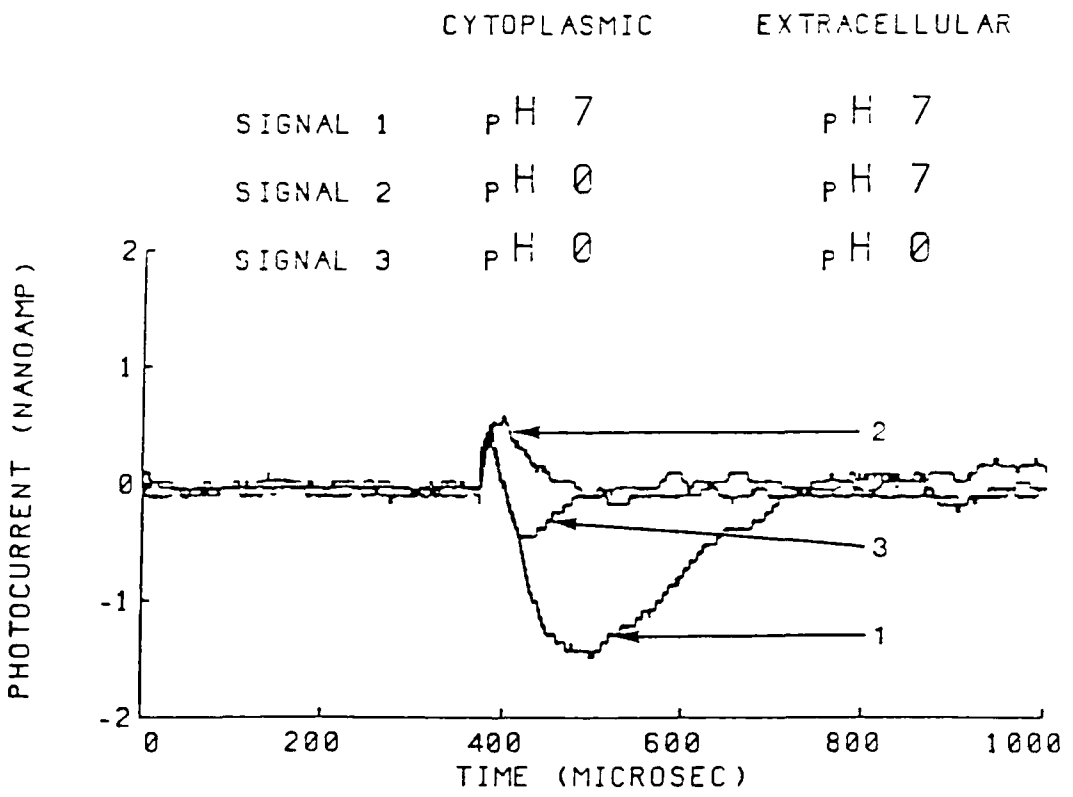

Fig. 1. A "differential" experiment showing the effect of varying the pH of the two aqueous phases independently. The membrane was reconstituted according to the method of Drachev et al. (FEBS Lett. 87:161, 1978) as modified by Dancshazy and Karvaly (FEBS Lett. 72:136, 1976). See text for explanation. The B1 component has a positive polarity, the B2 and B2′ component have negative polarities.

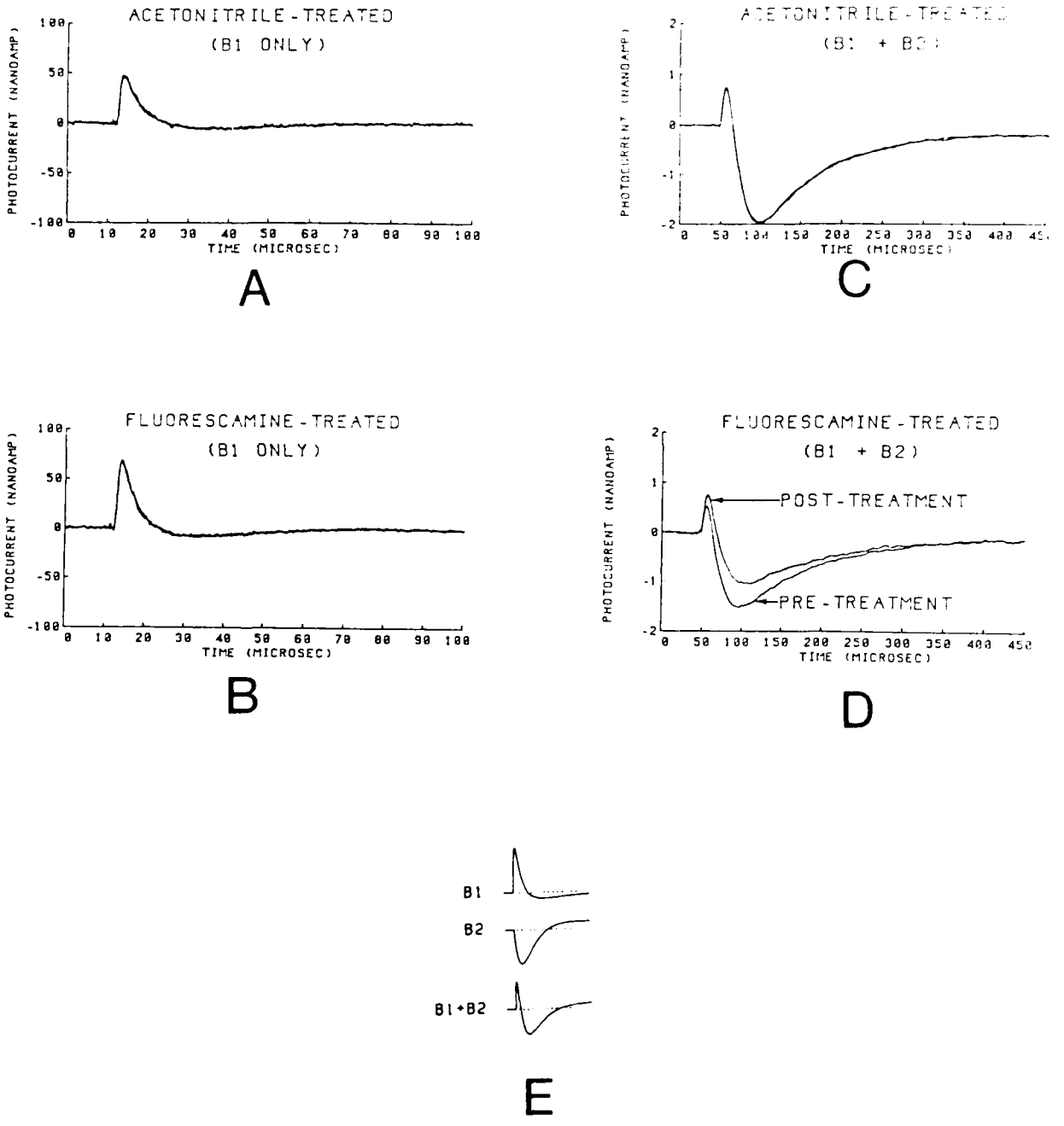

Fig. 2. Chemical modification with fluorescamine on the cytosol side of reconstituted bacteriorhodopsin membranes. Records A and B show pure B1 component whereas Records C and D show both B1 and B2. The reconstitution methods for obtaining pure B1 signals and for obtaining both components were described in Okajima and Hong, Biophys. J. 50:901 (1986). A stock solution of 0.5 % (w/v) fluorescamine in acetonitrile was added to the aqueous phase of the cytosol side (10 x dilution). Records A and C are solvent controls. Records B and D show effect of fluorescamine treatment. The slight increase of the positive peak in Record D does not mean an increase of B1 but is a consequence of B2 inhibition and the overlapping of B1 decay and B2 rise phases, as schematically explained in E.

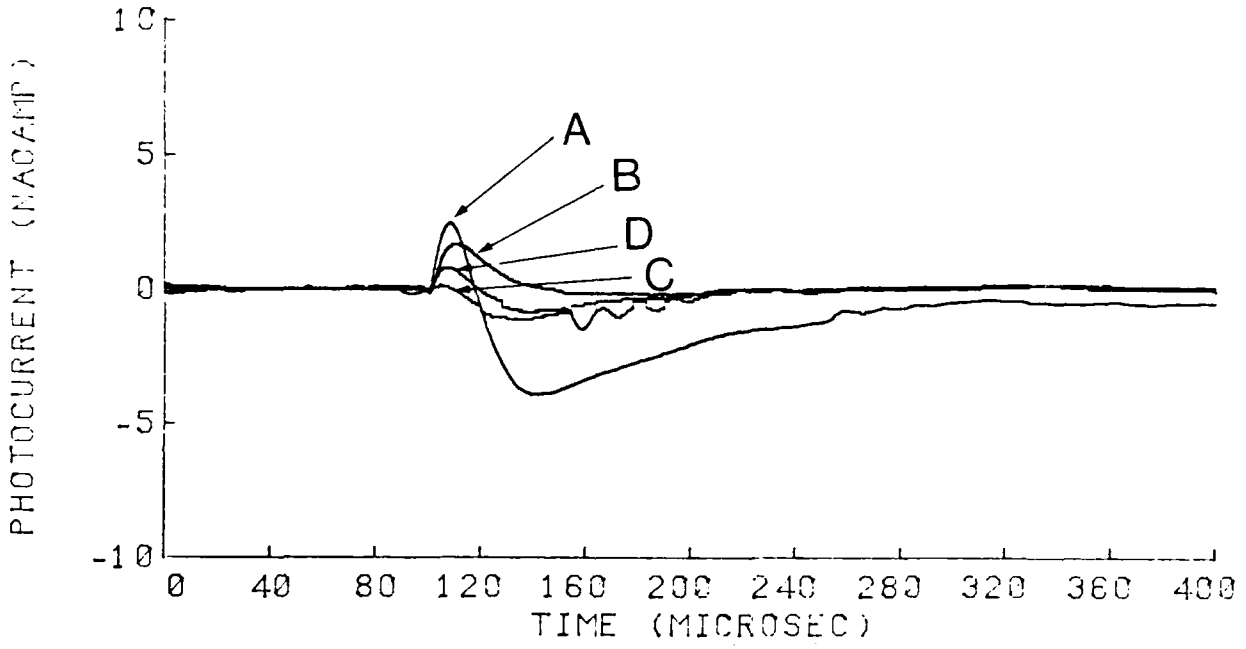

Fig. 3. Effect of N-bromoacetamide modification on the fast photosignals from a reconstituted bacteriorhodopsin membrane. The detailed methodology was described in Okajima and Hong (Biophys. J. 50:901, 1986). The B1 component has a positive polarity and the B2 component has a negative polarity. Signal A was the control before addition of N-bromoacetamide. Signal B was taken after addition of N-bromoacetamide to the cytosol side. Signals C and D were taken as in Signal B except a priming laser pulse was delivered 25 second and 50 seconds, respectively before the probing pulse was delivered. Photoresponses taken with a 2 minute (or longer) interval between the two pulses were indistinguishable from Signal B (showing full recovery).

PUBLICATIONS:

PUBLISHED

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5. Hong, F. T. and Okajima, T. L., Rapid Light-Induced Charge Displacements in Bacteriorhodopsin Membranes: An Electrochemical and Electrophysiological Study, in Biophysical Studies of Retinal Proteins, (T. G. Ebrey, H. Frauenfelder, B. Honig, and K. Nakanishi, Eds.), University of Illinois Press, Urbana-Champaign, in press (1988).

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SUBMITTED

8. Hong, F. T., Relevance of Light-Induced Charge Displacements in Molecular Electronics: Design Principles at the Supramolecular Level, submitted to Journal of Molecular Electronics.

9. Hong, F. T. and Okajima, T. L., A Bioelectrochemical Study of the Bacteriorhodopsin Membrane System, submitted to Bioelectrochemistry and Bioenergetics.

PUBLISHED ABSTRACTS:

1. Hong, F. T., Site-Specific Kinetics of Light-Induced Rapid Proton Movements Across A Bacteriorhodopsin Membrane and its Membrane-Water Interfaces, in Bioelectrochemistry Symposium, 193rd American Chemical Society National Meeting, Denver, Colorado, April 5-10 (1987).

2. Hong, F. T., Light-Induced Interfacial Charge Transfer in Biomembranes, 171st Electrochemical Society Meeting, Philadelphia, PA, May 10-15, 1987. Electrochem. Soc. Ext. Abstract Vol. 87-1, pp. 566-566, Abstr. No. 385 (1987).

OTHER ACTIVITIES:

Invited Visiting Scientist, Institute of Physical and Chemical Research (RIKEN), Wako, Saitama, Japan, February 23-March 2, 1987.

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