Directly Probing Rapid Membrane Protein Dynamics with an Atomic Force Microscope: A Study of Light-Induced Conformational Alterations in Bacteriorhodopsin

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ABSTRACT This paper demonstrates that an atomic force microscope can be used to directly monitor rapid membrane protein dynamics. For this demonstration the membrane-bound proton pump, bacteriorhodopsin, has been investigated. It has been unequivocally shown that the light-induced dynamic alterations that have been observed do not arise from external artifacts such as heating of the sample by the incident light, but that these changes can be directly linked to the light-induced protein conformational alterations in this membrane. In essence, it has been shown that the light energy absorbed by bacteriorhodopsin is converted not only to chemical energy but also to mechanical energy. In summary a new ultrasensitive tool is described for monitoring the molecular dynamics of materials with wide applicability to fundamental and applied science.

INTRODUCTION

The atomic force microscope (Binning et al., 1986) has had a revolutionary impact in many areas of imaging and surface modification of a variety of structures (Quate, 1994; Muller et al., 1995). Although atomic resolution imaging has not been achieved in biology with this microscope (Hansma and Hoh, 1994), very high resolution images have been obtained on a crystalline membrane called the purple membrane of *Halobacterium salinarium* (Muller et al., 1995a,b). Nonetheless, there have been few investigations aimed at applying the unique qualities of this microscope to the investigation of physiologically important parameters in biological systems. One recent investigation in this vein probed the activity of an enzyme in solution during substrate digestion (Radmacher et al., 1995).

It is the aim of this report to demonstrate that a force microscope can be applied to probe rapid membrane protein dynamic changes with a time resolution that is sufficiently fast to monitor even light-induced alterations. This application of the atomic force microscope in biology with the combination of transient excitations such as light, sound, electrical stimulation, or other forms of external stimulation has wide and fundamental implications for biophysics. For example, we can foresee investigations in the vein of this paper that could span disciplines from photosynthesis to neurobiology. In terms of neurobiology, the growing availability of photolyzable neurotransmitters allows the approach taken in this paper to address fundamental questions concerning the molecular mechanisms that lie at the basis of neural activity.

During the past two decades numerous studies have been aimed at investigating the mechanism of action of the membrane-bound proton pump, bacteriorhodopsin (bR), which is found in the crystalline patches of the purple membrane of *Halobacterium salinarium* (Oesterhelt and Stoeckenius, 1971). The physiological function of bR is to convert light energy into chemical energy by generating a proton gradient across the bacterial cell membrane. The great interest generated in bR has arisen in part from the fact that proton pumps and proton gradients are the currency of exchange in biology (Stryer, 1981). Therefore, there have been two decades of effort to understand the structure (Henderson et al., 1990) and mechanism of action of this membrane protein (for recent reviews, see: Birge, 1990; Lanyi, 1993; Ebrey, 1993). Nonetheless, the detailed molecular mechanism of bR remains a fundamental unresolved problem in biophysics.

There are many studies defining the protein intermediate structures that are kinetically generated when bR absorbs light, and these investigations have been extensively reviewed. From these studies it is now well known that the photochemical event converts bR, which absorbs at 570 nm, to a species that is stable for 2 μs at room temperature and is characterized by a red shift in the absorption maximum of the protein. This species then decays through a series of additional thermally produced intermediates to finally regenerate the initial pigment form bR. The proton gradient is generated during this photocycle, which has a time scale of $\sim$10 ms at pH 7 and room temperature. In this photocycle the intermediate that accumulates with the largest concentration is a species called M, which absorbs in the blue at 412 nm and is produced on a time scale of tens of μs at pH 7 and decays with a half-life of 4 ms. All of the kinetic intermediates that lead to the formation of M can be reversed photochemically into the initial bR species.

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In the past there have been pioneering attempts at trying to indirectly elucidate the protein conformational changes that occur during the proton pumping cycle. One approach that is of specific relevance to the experiments reported in this paper is the investigation of volume changes that occur in the purple membrane during the bR photocycle. The initial investigation in this direction was by Ort and Parsons (Ort and Parson, 1978) who attempted to monitor volume changes in bR with a system based on a capacitance transducer microphone. For these experiments the suspension of purple membrane fragments was placed in a container enclosed by this microphone. In this system the flash induced volume changes detected in the suspension did not seem to be connected to the photocycle. Further results have recently been obtained from detecting volume changes in suspensions of membrane fragments containing bR using photoacoustic spectroscopy (Schulenberg et al., 1994,1995) and hydrostatic pressure measurements (Váro and Lanyi, 1995). These studies indicated that volume changes could be associated with light-induced alterations in this protein. Specifically, an increase in the volume was detected with the production of the M intermediate.

In this paper we demonstrate that combining light excitation with cantilevered near-field optical elements (Shalom et al., 1992; Lieberman et al., 1994) that can sensitively monitor surface forces allows the direct detection of the light-induced dynamic alterations in the membrane protein bacteriorhodopsin.

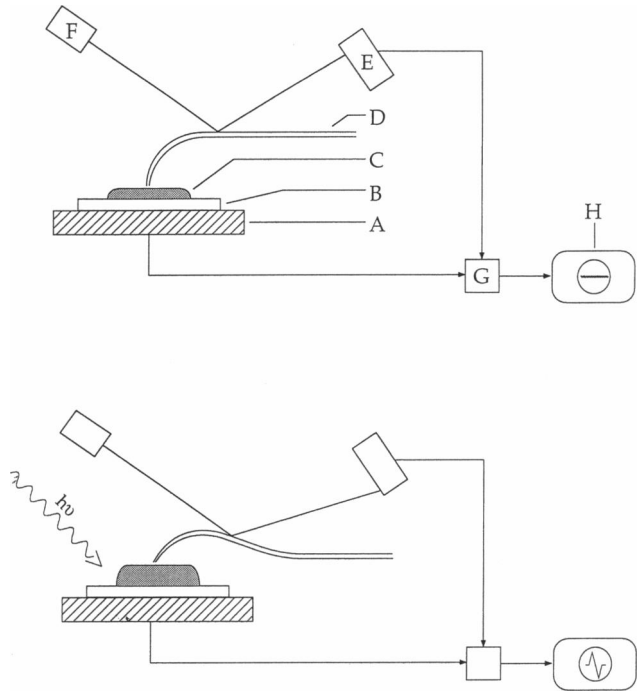

FIGURE 1 Schematic of the experimental apparatus showing the essential components and the effect of light. *A*) piezoelectric crystal, *B*) substrate, *C*) sample, *D*) cantilevered taped micropipette or fiber, *E*) position sensitive detector (PSD), *F*) diode laser, *G*) circuits for integration of PSD signal and for the imposition of voltage on the piezocrystal to alter sample *Z* position, and *H*) oscilloscope.

MATERIALS AND METHODS

A suspension at pH 7 of purple membrane fragments containing bacteriorhodopsin was prepared by the standard method (Oesterhelt and Stoeckenius, 1974). The films that were the object of this investigation were prepared by one of two methods. The purple membrane suspension was dried onto a glass slide using a mild vacuum (~15 mm Hg). For preparing membrane fragments with a defined orientation a previously published procedure was used in which membrane fragments were deposited onto SnO_2-covered glass slides in an electric field (Váro, 1982). Second harmonic generation (Huang and Lewis, 1989) was used to confirm the highly orientated character of these films. In each case the film had an approximate thickness of 1 μ when it was adsorbed onto a glass slide.

In our experiment we used a standard force microscope with a cantilevered optical fiber acting as the force tip (see Fig. 1) (Shalom et al., 1992; Lieberman et al., 1994). These cantilevered probes were prepared by pulling 125-μ diameter commercially available single-mode optical fibers under controlled heat (of a CO_2 laser), tension and cooling that was coordinated with microprocessor regulation. The resulting straight tapered optical fibers, which had a tip diameter of ~30 nm, were cantilevered ~50 μ from the tip using the same CO_2 laser beam and a micromanipulator (Narashige Ltd, Japan). These procedures have been described previously by Lieberman et al. (1994).

To measure the light induced dynamic alterations in this membrane protein, the tip of this cantilevered structure was brought in contact with the sample surface that was to be investigated. The reflection of a diode laser off the cantilever arm onto a position sensitive detector (see Fig. 1) is a sensitive monitor of the relative z position of the surface. Previous experiments with the cantilevered glass structures that were used in this experiment have shown that the technique is sensitive to alterations in z on a sub-Angstrom scale (Shalom et al., 1992). To measure the dynamic alterations in the membrane using this technique, the z changes, which are indicated by voltage alterations in the position sensitive detector with cantilever deflection, are directly monitiored. These time alterations in the position sensitive detector voltage were recorded using a digital oscilloscope (LeCroy Ltd, Switzerland; Model 6400A); thus, we were able to record the light-induced dynamics of the membrane.

The bacteriorhodopsin films were excited by an electronically controlled halogen lamp filtered with a 570-nm high pass cutoff filter. This excitation was transmitted to the membrane by an optical fiber, and for the double beam excitation experiments simultaneous irradiation through the same fiber with a HeCd laser ($\lambda_{emission}$ = 442 nm) was used.

RESULTS AND DISCUSSION

Fig. 2 shows the change in the voltage of the position sensitive detector (PSD) observed with the oscilloscope after irradiation of the sample with the 570-nm wavelength. To describe these results we have labeled five distinct regions in the time response of the signal. Regions *A* and *E* are the background when there is no light illumination. The noise that is seen is in the range of ±60 mV, which corresponds to a fluctuation in the cantilever of ~9.5 Å. This can be significantly improved with appropriate vibration isolation although for the present experiments it was not needed. Preliminary results indicate that, among other factors such as inhomogeneity of the films, the magnitude of the signal is dependent on the applied force of the cantilever tip on the membrane surface and thus, presently it is not possible to directly relate the deflection of the cantilever to specific expansions of the membrane.

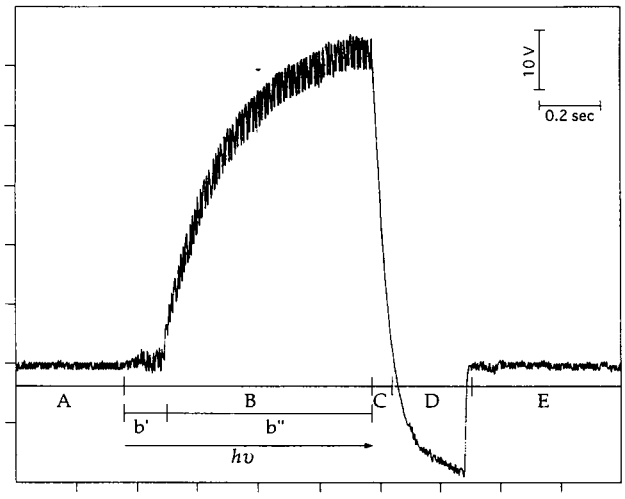

FIGURE 2 The observed PSD voltage signal as a result of bacteriorhodopsin illumination with 570-nm cutoff filter as a function of illumination and time.

At the start of region *B* the halogen lamp was turned on and there appeared to be at least two phases in the response of the PSD after turning on the lamp. The first of these regions is labeled as b′ in Fig. 2, and in this region the sample undergoes an oscillatory behavior with a period of ~10 ms. This leads to a sharply rising phase, labeled b″, in which a steady state is eventually achieved. The oscillation above the noise level continues throughout the period in which light is illuminating the sample. Similar results were obtained from the unidirectionally oriented films.

The first question that has to be addressed is whether these alterations in the signal with light are due to the bR photocycle. Several controls were completed. First, if the cantilever was placed in contact with a plate of mica and was illuminated as was done with the bR film, no signal was observed. Alternately, if the cantilever was taken out of contact with the surface and was illuminated as above, once again no signal was detected. In addition, a sample of β-carotene embedded in phosphatidylcholine vesicles was prepared. The cantilever was brought into contact with this preparation and was illuminated with white light or with white light and a 550-nm cutoff filter, and under all conditions no signal was observed.

A most crucial experiment in this series of controls that demonstrates the involvement of the photocycle in the observed signal is the simultaneous illumination of the sample with a HeCd laser with an emission at 442 nm. This laser wavelength is preferentially absorbed by the M intermediate and reduces the steady-state concentration of M. As is seen in Fig. 3 the effect of this laser is a decrease in the expansion of the bR film. This result rules out the possibility that the effects we are seeing are due to artifacts, such as heating, that are unconnected to the photochemical alterations in the purple membrane sample. We base this deduction on the fact that when the 442-nm light source irradiates the steady-state mixture produced by the simultaneous 570-nm illumination, the steady-state concentration of bR increases. Thus, if the effect monitored was due to heat induced by bR absorption, the signal should not decrease, and the dynamic response of the cantilever that has been observed is clearly dependent on the photochemically induced structural intermediates.

It is important to note that to obtain such results the cantilever production and mounting has to be carefully controlled. Specifically as noted in previous work from our laboratory, the glass cantilevers can be prepared with a variety of force constants and resonance frequencies. We have found that glass cantilevers with, for example, resonance frequencies between 300 and 400 kHz are the most appropriate for this experiment. Standard contact mode silicon cantilevers purchased from Digital Instruments, Inc (Santa Barbara, CA) were not sensitive to these conformational changes. One possible difference between the glass and silicon cantilevers is that the tip of the glass structure can be made to be very flexible and highly coupled to the cantilever and thus, this makes such cantilevers specially sensitive to the effects we are monitoring. Further studies will be required to fully understand the specific characteristics of these glass cantilevers needed to monitor such conformational changes.

After light illumination a delay of ~0.15 s is observed before the cantilever begins to rise to its steady-state position in a time scale of 0.8 s. These time scales are very similar to what has been observed in the steady-state photoelectrical signals of bR films of similar thickness (Haronian and Lewis, 1991). Even though the bR to M transition occurs on a time scale that is much faster than the accumulation of an M species, this accumulation is governed by the known back photoreactions that occur from the K and L

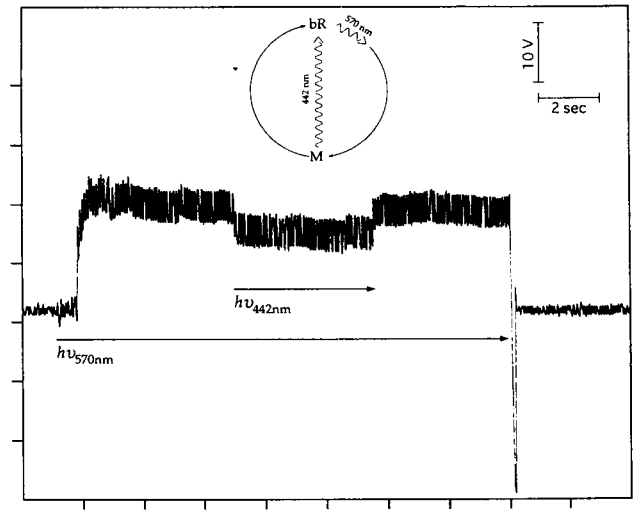

FIGURE 3 The effect on the amplitude of the steady-state PSD voltage signal as result of the simultaneous illumination of the sample with 570-nm irradiation absorbed by bR and 442-nm illumination absorbed by M.

intermediates that have strongly overlapping absorptions with bR.

When the light is turned off the expansion observed in the bR membrane decreases rapidly (region *C* in Fig. 2) and in a consistent fashion overshoots the background as seen in region *D* of Fig. 2. As the signal overshoots the background level a new oscillatory behavior appears. As is seen in Fig. 4 *A* the oscillation undergoes a change in amplitude and period until there is a rapid increase back to the background level. This is repeated over and over again in a totally consistent fashion (see Fig. 4 *B*). This pattern of oscillations is unique to the bR sample that is being investigated. For example, the pattern that is observed was different when the pH of the bR sample was altered from 7 to 9 and when a mutant in which aspartic acid 96 was replaced by asparagine (data not shown). These dark oscillations occur on a time scale that is much longer than those oscillations that are seen under illumination. The frequency of these dark oscillations may indicate that these protein conformational changes are unconnected with the photocycle. Unlike these dark oscillations, all of the oscillations detected when light is illuminating the sample arise from oscillatory behavior in the illuminating lamp. When a laser is used to illuminate the sample the oscillations disappear.

The rapid increase to the background level at the end of each expansion/contraction cycle seen in Figures 2–4 probably results from the method that was employed to monitor the relative changes in the membrane by way of the PSD voltage. Under the normal operating conditions of this atomic force microscope, the piezocrystal on which the sample is sitting adjusts to keep the *z* position of the membrane surface constant. The large magnitude of the positive expansion of the membrane disengages this feedback loop. Thus, when the light is turned off, an over shoot below background is observed in the PSD voltage. The rapid increase to the background level seen at the end of each expansion/contraction cycle is probably associated with the re-engagement of this feedback loop.

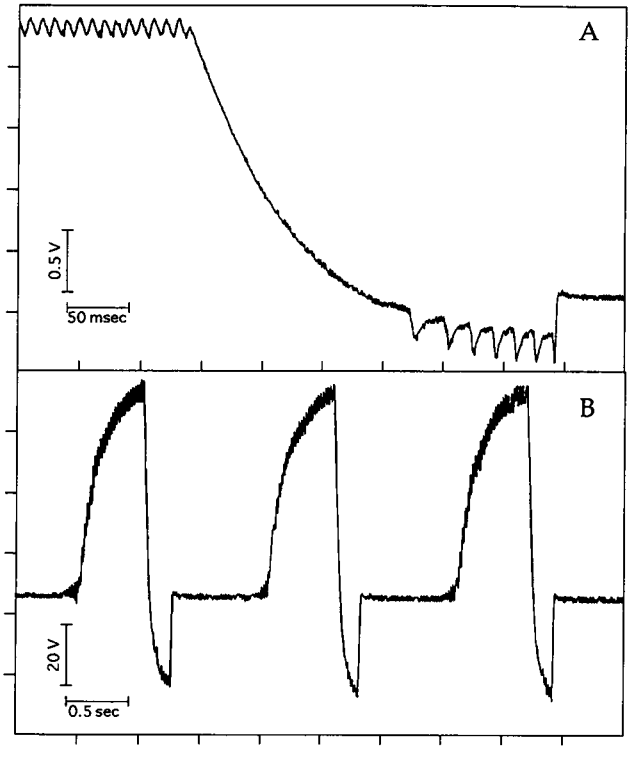

FIGURE 4 *A*) An expanded view of the dark oscillatory behavior after the 570-nm illumination is turned off. *B*) The reproducibility of the observed results including the dark oscillations.

The above results are of importance both in terms of bacteriorhodopsin and in terms of our demonstration of a new and exciting method for the investigation of membrane proteins in their diverse forms. Because there is such a wealth of information on the protein bacteriorhodopsin, the results that we have obtained with this new approach can be interpreted in terms of the known conformational alterations in this protein with light. In this regard the experiments with the simultaneous blue light illumination, which is preferentially absorbed by molecules in the M-intermediate state (Kalisky et al., 1977; Balashov and Litvin, 1981; Druckmann et al., 1992), demonstrates that the expansion of the membrane directly detected by the force cantilever is associated with the M-kinetic intermediate. Thus our results are in agreement with the reported data on photoacoustic spectroscopy, which indicated an expansion in the volume of the membrane in going from bR to M (Schulenberg et al., 1994,1995; Váro and Lanyi, 1995).

The similar results that we obtained with unidirectionally oriented membranes indicate that the volume expansion is isotropic. Such an isotropic change could possibly result from the known, significant tilting of the helices in this protein as M is produced (Subramaniam et al., 1993). In addition, alterations, water structure, and content could also be crucial factors in the observed volume changes. We know that our cantilever stiffness (defined as force/deflection) is on the order of a few Newtons/M. Based on this, cantilever stiffness forces of at least 1000 pN are required for 1-Å deflections of the cantilever position (Evans et al., 1995). The structural changes that result in such forces should be associated with weak covalent or ionic bonds in the protein. Further studies should help us define the specific nature of the changes that result in these various dynamic oscillations of the cantilever.

In summary, the directness of this methodology to monitor protein and membrane conformational changes and their dynamics in biological systems with time scales that are consistent with physiological function should open new avenues of experimentation not only in the biophysics of the purple membrane but also as a general tool in biology.

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