Vol. 103, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 16, 1981 Pages 182-188

KINETIC RESONANCE RAMAN SPECTROSCOPY OF CAROTENOIDS: A SENSITIVE KINETIC MONITOR OF BACTERIORHODOPSIN MEDIATED MEMBRANE POTENTIAL CHANGES

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Received September 25, 1981

Abstract

A rapid (14 - 22 μs) light-induced, bacteriorhodopsin mediated membrane potential has been detected using the technique of kinetic resonance Raman spectroscopy and the model system of β-carotene incorporated into reconstituted vesicles containing bacteriorhodopsin. Our data demonstrate that the kinetic resonance Raman spectrum of β-carotene is an extremely sensitive monitor of kinetic alterations in membrane potential with micron spatial resolution in a highly scattering medium. In addition, our Raman results indicate that the potential sensitivity of β-carotene is an excited state property of the molecule, thus making it an electrochromic monitor of membrane potential. We feel the techniques illustrated in this paper have the advantage of being a native probe of kinetic membrane potential changes and will be applicable to a wide variety of biological systems without the perturbing side-effects which often accompany the use of non-biological, potential-sensitive dyes.

We describe in this paper a series of experiments which illustrate that the kinetic resonance Raman spectrum (KiRRS) of β-carotene is a sensitive monitor of kinetic changes in membrane potentials. Our system represents a well-defined functional model in which light interacts with bacteriorhodopsin to establish a proton gradient and a membrane potential. Our system includes purified bacteriorhodopsin reconstituted into liposomes prepared with β-carotene. The techniques of KiRRS (1) were then employed to monitor the changes in the β-carotene spectrum as the vesicles were exposed to the exciting laser beam for varying time intervals. Thus, the laser was both the exciting source for the bacteriorhodopsin proton pump and for the KiRRS of β-carotene.

In view of the ubiquitous nature of carotenoids in cellular membranes (neurons, [2,3] erythrocytes [4], photosynthetic reaction center preparations [5], lobster sarcoplasmic reticulum [6] and normal and transformed avian lymphocytes [7]) and our data indicating the sensitivity of its kinetic resonance Raman spectrum to

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transient changes in membrane potential in highly scattering media, we feel confident that KiRRS of carotenoids could become a kinetic, non-invasive, ubiquitous monitor of cellular membrane potentials with the spatial resolution of a laser beam.

β-carotene is one of the strongest resonance Raman scatterers that has been found. In fact, because of the intensity of its resonance Raman spectrum, β-carotene was the object of study even in pre-laser days. The first reported resonance Raman spectrum was by Euler in 1932 (8). The first laser Raman spectra of various carotenoids were obtained by Rimai, Gill and coworkers (9,10). These workers (11) together with Inagaki, Tasumi and Miyazawa (12) have completed a vibrational analysis of β-carotene. Jackson and Crofts (13) and Witt et. al. (14) clearly demonstrated the potential sensitivity of the carotenoid absorption transitions and more recently Carey et. al. (15) investigated the sensitivity to membrane potential of the resonance Raman spectrum of the carotenoid neurosporene in chromatophores of Rhodopseudomonas sphaeroides. However, no kinetic studies were attempted in any of these studies.

Our experiments have a dual purpose. First, to demonstrate the sensitivity of carotenoid kinetic resonance Raman spectra to kinetic alterations in membrane potential in a model system amenable to definitive controls, and secondly, to obtain the first time-resolved measurement of light-induced membrane potential formation by bacteriorhodopsin.

Materials and Methods

Halobacterium halobium S9 was cultured by standard procedures and purple membrane fragments containing bacteriorhodopsin were purified by the method of Kanner and Racker (16).

Acetone washed soybean phospholipids (Associated Concentrates) were diluted to 40 mg/ml in a solution of $CHCl_3$:CH_3OH (2:1 v/v) and 7 µl of a 20 mg β-carotene/ml CH_3Cl solution were added per ml of lipid solution. Three ml of the lipid solution were dried under a stream of nitrogen. After resuspension in diethylether and drying in vacuo twice, 3 ml of 150 mM KCl were added and the suspension was sonicated to optical clarity. One ml of bacteriorhodopsin (5 mg protein/ml) was added to the 3 ml suspension of liposomes. Octylglucoside (Calbiochem) was added to a final concentration of 1.5% and the total volume was brought to 6 ml with 150 mM KCl. Following a 3 min incubation at room temperature, 60 ml of 150 mM KCl were added and the vesicles were sedimented at 150,000 Xg for 15 min at 2°C. The supernatant was removed and the vesicles were resuspended in 15 ml of 150 mM KCl. The suspension was kept on ice prior to obtaining spectra.

Proton pumping was measured using the procedure of Racker and Stoeckenius (17). Valinomycin and nigericin were added to vesicle suspensions, where indicated, to final concentrations of 1 µg valinomycin/ml and 2 µg nigericin/ml.

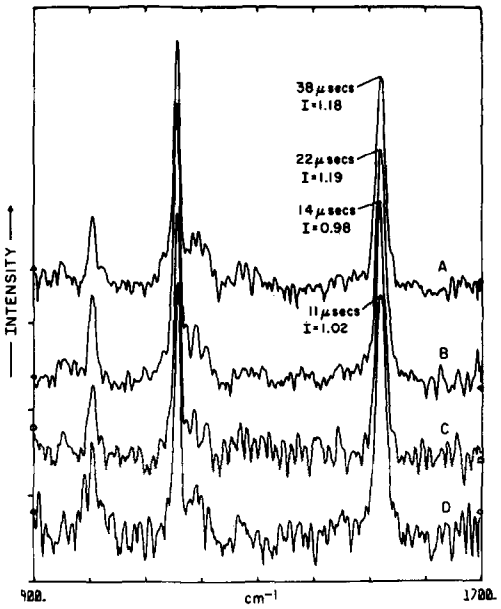

Figure 1 Kinetic resonance Raman spectra of β-carotene in reconstituted bacteriorhodopsin vesicles at various residence times. Spectra were obtained with 10 mW of 514.5 nm excitation, a 2 cm^{-1} stepping interval and a 5 s counting time per channel. I = Intensity ratio of the 1157 cm^{-1} band to the 1520 cm^{-1} band.

Kinetic resonance Raman measurements were made on vesicles placed into a recycling flow system driven by a variable speed pump. The reservoir containing the vesicles was cooled with water and the sample was circulated through the laser beam in a glass capillary tube. Measurements were made under aerobic and anaerobic conditions with no effect on the results. The 514.5 nm laser line of an argon ion laser was used for all the spectra. Raman scattering was collected through a spectrometer which has been previously described (1).

Results and Discussion

Resonance enhancement of the β-carotene Raman spectrum results from a $\pi\pi^*$ transition of the molecule when it encounters wavelengths of light close to its lowest energy electronic transition. Membrane potential changes have been shown to alter this electronic transition and these alterations are readily detected as perturbations of the resonance Raman spectrum (15). KiRRS of β-carotene incorporated into reconstituted bacteriorhodopsin vesicles are shown in Figure 1. Only vibrational modes of β-carotene are observed (10,11). Vibrational modes of bacteriorhodopsin (1) are absent. Vesicles exposed to the laser beam with 11 μs and 14 μs residence times were found to have I_{1157}/I_{1520} ratios of 1.02 and 0.98,

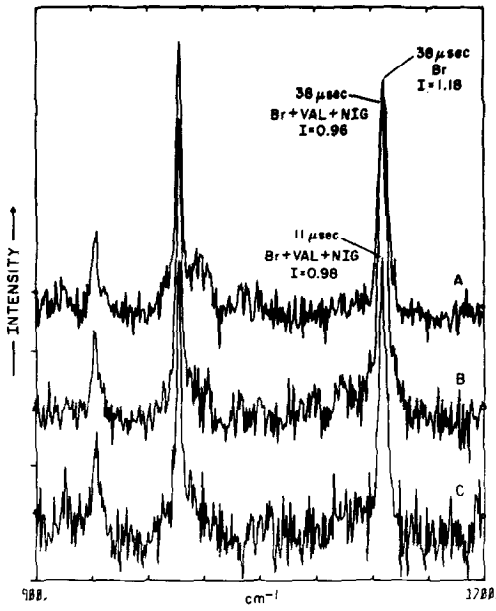

Figure 2 A comparison of the kinetic resonance Raman spectra of β-carotene in reconstituted bacteriorhodopsin vesicles, at 38 μs residence time without (A) and with (B) valinomycin and nigericin and at 11 μs with valinomycin and nigericin (C). Spectra were obtained under the same conditions and I has the same definition as in Figure 1.

respectively; whereas the ratios were 1.19 and 1.18 for 22 μs and 38 μs residence times. Based on these data, it appears that the resonance Raman spectrum of β-carotene is sensing a rapid light-induced membrane potential in these vesicles.

To test this hypothesis, valinomycin and nigericin were added to the vesicles to prevent membrane potential formation and spectra were again recorded at 38 μs. Data comparing vesicles without and with valinomycin and nigericin at 38 μs residence time are seen in Figures 2A and B. It is clear from these spectra that valinomycin and nigericin addition to the vesicles influences the I_{1157}/I_{1520} ratio. Furthermore, when spectra were obtained at 38 μs and 11 μs residence times with vesicles containing nigericin and valinomycin, the change in the I_{1157}/I_{1520} ratio was abolished (Figures 2B and C). These data indicate that the changes seen in Figure 1 reflect the establishment of a rapid light-induced membrane potential. It should be noted that variability in the absolute values of the I_{1157}/I_{1520} ratios were observed from different vesicle preparations. Similarly, vesicles

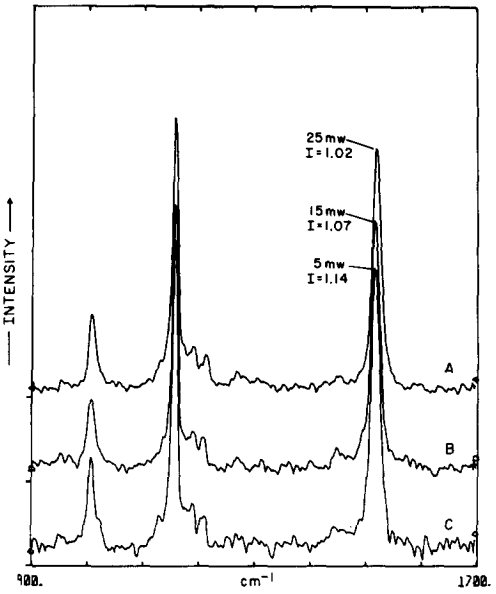

Figure 3 Kinetic resonance Raman spectra of β-carotene in reconstituted bacteriorhodopsin vesicles at 38 μs residence time as a function of laser intensity. Additional spectral conditions are the same as in Figure 1 and I has the same definition.

showed variability in magnitude of proton pumping between different bacteriorhodopsin preparations (range in these experiments was from 167 natoms H^+/mg protein to 204 natoms H^+/mg protein) but the direction of the changes in β-carotene bands as well as their time course was uneffected by this variability.

Finally, if bacteriorhodopsin were indeed responsible for the establishment of the potential, the photochemical back reactions of the proton pumping cycle (16) should be detectable by increasing the intensity of light (i.e., increasing laser power) and observing a decrease in the change of the I_{1157}/I_{1520} ratio at 38 μs residence time. Data in Figure 3 show that increasing laser power results in a decrease in the response of the β-carotene resonance Raman spectrum at 38 μs residence time in the beam indicating that increased light intensity results in a lower membrane potential. This effect is readily understandable if the intermediates in the proton pumping cycle absorb a photon and photochemically return to bR_{570}. Such photochemical back reactions have been documented for all intermediates in

the proton pumping cycle (18). Experiments are presently underway to quantitate the results.

In summary we have established that the resonance Raman spectrum of β-carotene in a well defined system is a sensitive monitor of kinetic membrane potential changes even in highly scattering suspensions. As we have demonstrated, the kinetic membrane potential changes caused alterations in the intensity of the 1157 cm^{-1} (=C-C=) vibrational mode and the 1520 cm^{-1} (-C=C-) vibrational mode. No changes in the frequency of these vibrational modes were observed. It is a well accepted fact (19) that frequency alterations reflect ground state conformational changes whereas intensity alterations are characteristic of excited state perturbations. Our data clearly demonstrate that the β-carotene potential-sensitive spectral changes originate as a result of excited state, electrochromic perturbations. Thus, the data provide good evidence that our measurements were not limited by the time response of β-carotene. Its response time should be <picoseconds (10^{-12} s) which is the approximate time scale of the resonance Raman scattering process. Therefore, because of the natural presence of β-carotene in a variety of cell membranes; kinetic resonance Raman spectra of β-carotene should become a ubiquitous, kinetic and spatially sensitive monitor of membrane potential changes in a variety of systems of interest in cell biology.

Acknowledgements

Professor E. Racker is thanked for his encouragement throughout the course of this investigation. This work was supported by a grant from the Solar Energy Research Institute and postdoctoral fellowship F32-CA06336 from NIH (J.H.J.). G.G. was also supported by an NIH postdoctoral fellowship (#08-F2GM07885A).

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