BRIEF COMMUNICATIONS

RESONANCE RAMAN KINETIC SPECTROSCOPY OF BACTERIORHODOPSIN ON THE MICROSECOND TIME SCALE

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ABSTRACT Using a rotating disk with a slit of variable width, a continuous wave argon ion laser, and an Optical Multichannel Analyzer for detection, a new technique is reported which should, in principle, be capable of recording resonance Raman spectra with time resolution of 100 ns. The resonance Raman spectra of the intermediates of the photosynthetic cycle of bacteriorhodopsin are recorded on the microsecond time scale. Both the kinetic results and the resonance enhancement profile suggest that deprotonation results in an intermediate preceding bM_{412} that has an optical absorption maximum at a wavelength longer than that of bM_{412}.

INTRODUCTION

The purple membrane of *Halobacterium halobium* contains a single protein, bacteriorhodopsin. The chromophore of this protein is retinal, as in the visual pigment, and like vertebrate rhodopsin, bacteriorhodopsin is photoreactive. The functions of the two rhodopsins, however, are quite different. While vertebrate rhodopsin is involved in visual transduction, the role of bacteriorhodopsin is photosynthetic, or energy transducing. Upon illumination with visible light, protons are pumped across the cell membrane, resulting in a gradient which is thought to drive the phosphorylation of ADP (1).

Bacteriorhodopsin's photochemistry is completely reversible under moderate illumination levels, with the protein undergoing a cyclical series of conformational changes. The optical absorption maxima (in nanometers) and decay times of the various intermediates in the cycle have been measured by time-resolved optical spectroscopy and the cycle can be written (2) as:

$$\begin{array}{ccccc} bR_{570} & \overset{<10\text{ ps}}{\rightleftharpoons} & bK_{590} & \overset{2\,\mu s}{\rightarrow} & bL_{550} \\ \uparrow & & & & \downarrow 40\,\mu s \\ bO_{640} & \leftarrow & (bN_{520}) & \overset{10\text{ ms}}{\leftarrow} & bM_{412}. \end{array}$$

Lewis et al. (3), using resonance Raman spectroscopy, have shown that the Schiff base linkage which binds the retinal to bacterio-opsin is protonated in the bR_{570} species and unprotonated in the bM_{412} intermediate. Thus the primary photochemistry of bacteriorhodopsin results in the release of a proton during the first half of the cycle and a reprotonation during the second. These proton transfer reactions have been proposed to be responsible for the ultimate appearance of a proton gradient across the membrane (3).

Most of the kinetic data on bacteriorhodopsin have been obtained by optical flash techniques (2). Since optical spectra of many biological systems do not contain as much structural detail as Raman spectra, a need for the technique of time-resolved resonance Raman spectroscopy became urgent. This initiated the use of time-resolved Raman spectroscopy in the study of biological changes. We recently published (4) the first time-resolved Raman spectra of these systems (bacteriorhodopsin) on the nanosecond time scale, using a nanosecond pulsed laser. Marcus and Lewis (5) were the first to report studies in the microsecond time regime using variable speed flow techniques. In this communication, we apply a simple technique with time resolution in the microsecond (with potential for the nanosecond) range in which a continuous wave (cw) laser is converted to a pulsed laser of variable duration by using a rotating disk with a single slit of variable width. We recently described (6) a two-slit experiment, where one slit is used for photolysis and the second for Raman scattering, in which time resolution in the millisecond range is obtained by varying the separation between the two slits. We have obtained kinetic resonance Raman spectra of the C=C and C=N stretching vibration regions of bacteriorhodopsin which suggest that deprotonation occurs before the $bL_{550} \rightarrow bM_{412}$ step in the cycle. Our conclusion is in general agreement with that reached by Marcus and Lewis (5).

MATERIALS AND METHODS

Purple membrane fragments were extracted from *Halobacterium halobium* S-9, a mutant strain believed to contain few carotenoids, by the method of Becher and Cassim (7), and suspended ($\sim 5 \times 10^{-5}$M) in 4.3 M NaCl and 0.1 M $MgSO_4$.

The apparatus used for the kinetic resonance Raman experiments is shown in Fig. 1. Pulses of laser light were obtained by mechanically modulating a model 171 argon ion laser from Spectra-Physics, Inc. (Laser Products Div., Mountain View, Calif.). By focusing the laser (TEM_{00} mode) to a diffraction-limited spot onto a single slit of variable width in a rotating disk chopper (68 mm radius and driven with a 30-Hz synchronous motor purchased from Bodine Electric Co., Chicago, Ill.) we obtained rectangular pulses with durations from 10 μs to 5 ms. We encountered difficulties in achieving the rectangular pulse shapes at short times when we tried to use the laser multimode for higher power. This was due to the larger beam waist obtained at the focus of the lens, making the beam larger than the slit at this point, so all measurements were made with the TEM_{00} mode. The pulse widths were measured with a photodiode and oscilloscope to an estimated accuracy of 10%. The 30-Hz repetition rate

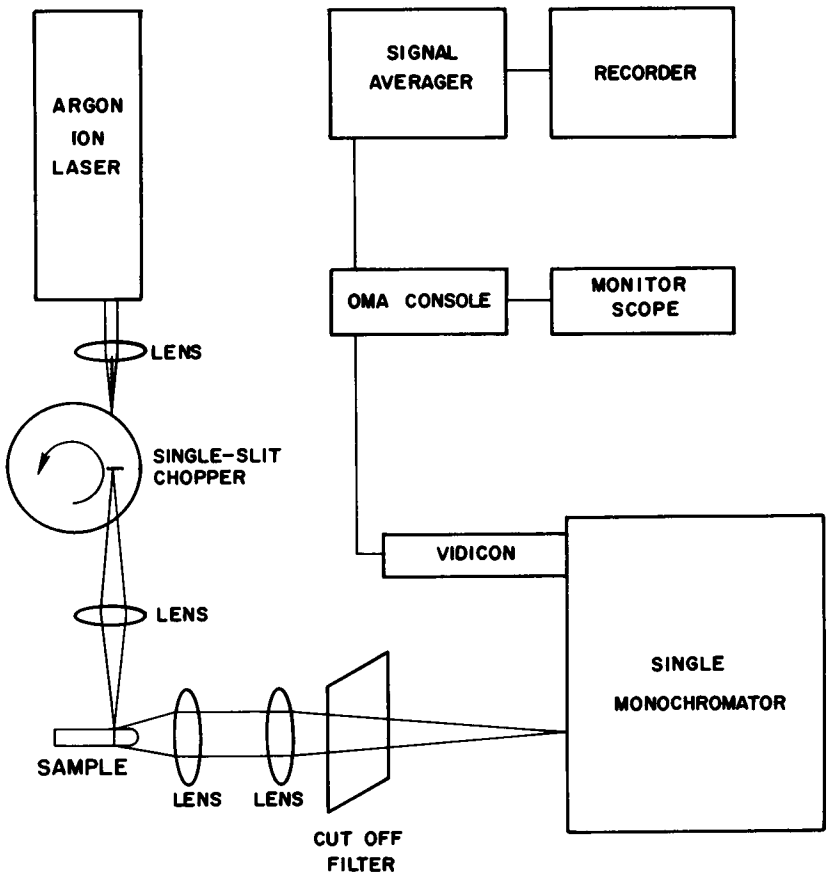

FIGURE 1 Block diagram of experimental setup for kinetic Raman spectroscopy using a chopped cw argon ion laser.

used is sufficiently slow to permit complete relaxation of the cycle between pulses at room temperature (~20°C).

The laser light was focused across a capillary tube containing the sample and the scattered Raman radiation collected and focused with *f*-matched optics onto the entrance slit of a Spex 1870 1/2 M spectrograph. A Corning cutoff filter (Corning Glass Works, Science Products Div., Corning, N.Y.) was placed in front of the slit to eliminate scattered Rayleigh light. The dispersed Raman scattered radiation was imaged onto the faceplate of an SIT vidicon detector which is part of an Optical Multichannel Analyzer (Princeton Applied Research Corp., Princeton, N.J.). This arrangement results in a spectral coverage of 200 Å at a resolution of 0.4 Å per channel.

Since in our method only one pulse is used to photolyze the sample as well as to probe the system, the resulting Raman spectrum reflects the average concentrations of intermediates present during the time of interaction with the light. As this time is lengthened, intermediates with longer rise-times will begin to contribute to the spec-

trum. If the appearance rates of two bands are very similar, and they show the same resonance enhancement profile, there is good evidence to assign them to the same intermediate. It is possible, in the case of sequential first-order reactions, that one intermediate might have a formation rate constant that is very fast compared to the width of the laser pulse. Under these conditions it would show an apparent rise-time that is the same as that of its precursor and thus, by using this technique, differences in observed resonance enhancement profile might differentiate these intermediates.

RESULTS AND DISCUSSION

The Deprotonation Process

Raman spectra from single-slit chopper experiments on the microsecond time scale are shown in Fig. 2 *a* (4,765 Å excitation) and Fig. 2 *b* (5,145 Å excitation). The 1,530, 1,554, and 1,570 cm^{-1} bands are the C = C stretching vibrations ($\nu_{C=C}$) of bR_{570}, bL_{550}, and bM_{412} species, respectively (3, 6, 8). The 1,604 cm^{-1} band is assigned to bR_{570} (6) and the 1,620 and 1,643 cm^{-1} bands have been previously assigned (3) as the C = N stretching ($\nu_{C=N}$) vibrations of the unprotonated and protonated Schiff bases, respectively.

Fig. 2 *a* (4,765 Å excitation) shows that the 1,620 cm^{-1} band appears to grow in at a rate similar to the appearance rate of the 1,570 cm^{-1} band of bM_{412}. This observation might suggest that deprotonation occurs as bL_{550} decays to form bM_{412}. As an alternative explanation, however, consider the possibility that deprotonation occurs earlier in the cycle. All of the intermediates that precede bM_{412} in the cycle have optical absorption maxima at wavelengths much longer than that of bM_{412}. If one assumes that the cycle deduced from the optical studies is correct, exciting with a longer wavelength laser line should preferentially enhance the vibrations of the earlier intermediates over those of bM_{412}. To investigate this possibility we carried out the one-slit experiment by using the 5,145-Å argon laser line. The results are shown in Fig. 2 *b*. There is clearly a band at 1,620 cm^{-1} that appears at an earlier time than the first appearance of the 1,570 cm^{-1} band of bM_{412}. We thus conclude that deprotonation occurs before the $bL_{550} \rightarrow bM_{412}$ step. In fact, the apparent appearance rate of this band is similar to that of a shoulder at 1,554 cm^{-1} that has been assigned to bL_{550} (6).[1] However, this result cannot show that deprotonation accompanies the formation of bL_{550} (as is suggested by the optical studies of Korenstein et al. [9] based upon kinetic isotope effects). In order to accomplish this, studies at shorter times using longer excitation wavelengths (to preferentially enhance bK_{590} vibrations) are required.

[1] Although a well-resolved shoulder is not observed, the combined width of the 1,530 and 1,554 cm^{-1} bands shows a smooth increase of 25% over the time scale of the experiment, indicating the increased concentration of bL_{550} (6).

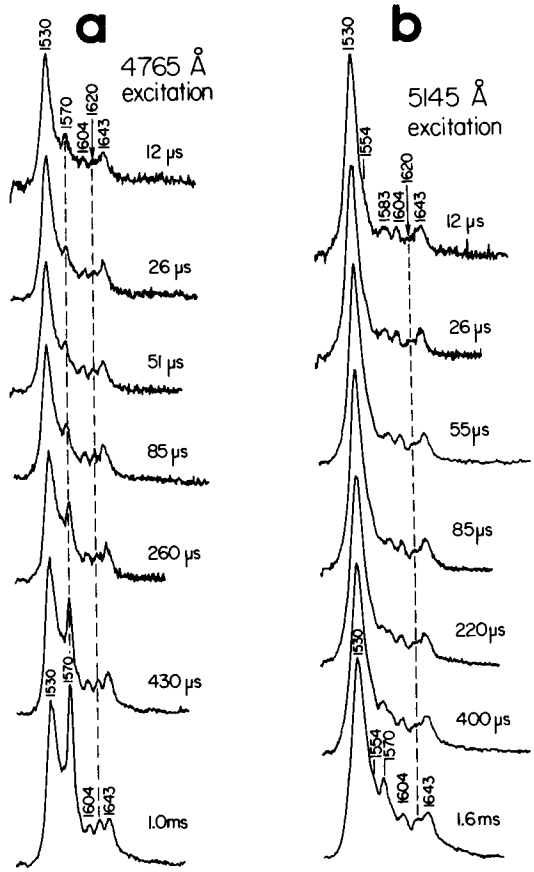

FIGURE 2 Kinetic resonance Raman spectra of bacteriorhodopsin taken with two excitation wavelengths: (*a*) 4,765 Å; (*b*) 5,145 Å; at room temperature (~20°C). The times are the measured laser pulse durations, determined in general by the slit width, speed, and diameter of the chopper. For this series only the slit width was varied. Peak incident laser power was ~2 W in all cases, with an average power (determined by the ratio of slit width to chopper circumference) ranging from 0.5 to 60 mW. Vibrational frequencies are given in reciprocal centimeters.

The conclusions concerning the observed time scale of the deprotonation process are in general agreement with those made recently by Marcus and Lewis (5) who used a quite different technique (a variable flow-rate method). However, there are a few rather important differences between our spectra and those reported by Marcus and Lewis: (*a*) we do not observe the peaks they observe in the region at frequencies higher than 1,650 cm^{-1}. These peaks appear to change frequency with time in a non-uniform manner and do not correspond to any peaks in our cw spectrum; (*b*) our base line is flat but theirs seems to change with frequency and flow rate; (*c*) the shoulder observed at lower frequency to the 1,530 cm^{-1} peak (and assigned to the bK_{590}) is not as well developed in our spectrum as it is in the spectrum of Marcus and Lewis (5).

Discussion of the Technique and Potential Applications

The ultimate time resolution obtainable in our technique is determined by the pulse duration, τ. In our experiment, τ is given by $W/2\pi fr$, where W is the slit width, r is the radius of the disk, and f is the frequency of the chopper. The limit on the slit width is set by the smallest size of the focused TEM_{00} mode of the cw laser used, which is $\sim 5\ \mu m$. To keep the pulses rectangular in time, a 10-μm slit is probably the smallest practical size for W. With the 10-μm slit, very short pulses can be obtained by using extremely fast motors and large disks. For example, with a 300-Hz motor and a disk of 20 cm radius, a pulse width of ~ 20 ns can be obtained. However, it must be emphasized that though short pulses can be obtained, there are important limitations on their utility. As the pulses become shorter, the amount of photolysis on each pulse decreases, unless the cw laser power can be increased. In addition the Raman signal intensity decreases, although this can be compensated for by using multiple slits on the disk, scanning longer, cooling the vidicon, increasing the concentration of the starting material, and using more efficient collection optics. Also, the chopping frequency is limited by the relaxation time of the process under study. With our current detection system, $\sim 10\ \mu s$ minimum resolution is S/N limited. We are improving the detector by cooling the SIT tube to dry ice temperatures, which should eliminate the dominant source of noise in our experiment. Thus we expect, on the basis of the strength of the signal we now observe, to be able to achieve ~ 100 ns time resolution.

It should be mentioned that this method could be adapted as well to study irreversible photochemical changes by flowing the sample past the laser beam. The flow rate would be adjusted so that the sample would not move during the pulse, but would be exchanged for a fresh sample between pulses. This should be feasible with typical duty cycles of $\sim 10^{-3}$ in these experiments. An alternative method would be to use a large reservoir of the sample which is well stirred to exchange continuously fresh sample between pulses in the region where the laser is focused.

Kinetic Raman spectroscopy taken with various exciting wavelengths can thus provide valuable qualitative information about the dynamics of photochemical processes. To obtain quantitative kinetic data by this technique, an internal standard (whose intensity is time-independent) should be developed for the frequency region of interest. This work is now in progress.

SUMMARY AND CONCLUSION

In conclusion, we have presented a simple method to study the kinetics of reversible photochemical changes with time resolution of a few microseconds by the use of the powerful tool of resonance Raman spectroscopy. We have suggested a modification of this technique that would make it applicable to irreversible changes, as well as the reversible one studied here, with time resolution of 100 ns. We are also working out the details necessary to extract quantitative information (absolute rate constants) from these kinds of experiments.

The results on bacteriorhodopsin suggest that the deprotonation process of the

Schiff base takes place on the microsecond time scale or earlier. As indicated previously (5), this is a shorter time scale than that of the appearance time of the protons outside the cell (10–12) in the proton pumping process. This could suggest that the Schiff base proton is transferred through one or more activated processes before it appears outside the cell; or else it could mean that the deprotonation of the Schiff base merely initiates a molecular mechanism for further protein conformational changes leading to deprotonation elsewhere.

The authors are grateful to Professor M. Nicol for the use of his facilities and to Dr. J. Lanyi for a culture of *Halobacterium halobium* S-9.

The authors would like to thank the United States Energy Research and Development Administration for their financial support. Alan Campion gratefully acknowledges IBM for a predoctoral fellowship. James Terner is a National Science Foundation National Needs trainee.

Received for publication 18 July 1977.

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