

Fourier Transform Raman Spectroscopy of Photoactive Proteins with Near-Infrared Excitation

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The Fourier transform (FT) Raman spectroscopic treatment of the photoactive proteins bacteriorhodopsin and the photosynthetic reaction center is reported, with excitation at 1.06 μm . Excitation at this wavelength circumvents the limitations on resonance Raman spectroscopy of these proteins imposed by their photolability and by the fluorescence of free pigments or impurities. The spectra are dominated by nonresonant Raman scattering by the protein-bound pigments retinal (in bacteriorhodopsin) and bacteriopheophytin, bacteriochlorophyll, and carotenoids (in reaction centers). The relative intensities of retinylidene modes in the spectrum for nonresonant FT Raman spectroscopy of bacteriorhodopsin are nearly identical to those observed in the resonance Raman spectrum of bacteriorhodopsin.

Index Headings: Raman spectroscopy; Fourier transform spectroscopy.

INTRODUCTION

Resonance Raman spectroscopy excited in the visible or near-ultraviolet regions of the spectrum has established itself as a revealing probe of the structure of chromophores in photoactive proteins such as rhodopsin, bacteriorhodopsin, and photosynthetic reaction centers.^{1,2} However, two significant problems are encountered in such studies. First, although the fluorescence quantum yield in photoactive proteins is in many cases low, due to efficient ultrafast processes,³ fluorescence may still pose formidable obstacles, due, in reaction centers for example, to fluorescent impurities or free chromophores. Second, the photolability of the proteins limits the average power that can be used to excite the Raman spectrum.^{4,5} The latter problem is especially severe if pulsed lasers are used.⁶

The advantages of near-infrared excitation of Raman scattering have been celebrated by a number of authors.⁷⁻¹¹ These advantages include the absence of fluorescence from sample or impurities absorbing in the visible region, the availability of cw near-IR lasers with high average power, and the reduction of photo-induced damage or decomposition of an absorbing sample. When combined with the multiplex and throughput advantages of Fourier transform (FT) Raman spectroscopy, near-IR excited Raman spectroscopy has proved to be a powerful technique.⁷⁻¹¹

We believe that a compelling application of this technique may be in the study of photoactive proteins, where the photolability limits the laser power that can be used for resonance excitation. In this paper, we report preliminary results on the application of near-IR FT Raman spectroscopy to two photoactive membrane proteins: bacteriorhodopsin (bR) and the reaction center (RC) of photosynthetic bacteria.

A significant question regarding the application of near-IR excitation to proteins with visible chromophores, however, is that of the importance of contributions from nonresonant Raman excitation of the chromophores relative to contributions from the polypeptide. An FT Raman study of cyanine dyes with long aliphatic appendages by near-IR excitation, for example, comprised contributions from both the cyanine chromophore and from the aliphatic side chains.¹² Near-IR excitation of synthetic polypeptides has been shown to be capable of generating detectable scattering from the polypeptide backbone.¹³ Thus, one might suspect that Raman scattering by polypeptide vibrations might severely congest, and perhaps swamp, the nonresonant scattering by protein pigments. In this study, however, we show that, on the contrary, the visible chromophores can make important and, at least in the proteins studied here, dominant contributions to the spectrum excited in the near-IR. We suggest that excitation of Raman scattering at 1.06 μm represents an especially attractive approach to both cw and time-resolved Raman studies of photoactive proteins.

EXPERIMENTAL METHOD

Raman spectra of the sample solutions were measured with a Bruker Instruments IFS 66 FT-IR spectrometer coupled to an FRA-106 FT Raman accessory, also from Bruker Instruments. The Rayleigh filter and the detector of the FT unit allowed measurement of a Raman shift range of 80 to 3500 cm^{-1} on the Stokes side. The sample was contained in standard near-IR quartz cuvettes (Wilmad Glass Company, Inc.) at room temperature. Raman scattering was excited by multimode 1.06- μm cw Nd:YAG laser radiation at 1.3 W for bR and 1 W for reaction centers, and collected in a 180° back-scattering geometry. Double-sided interferograms were acquired at 10 cm^{-1}

Received 16 February 1990.

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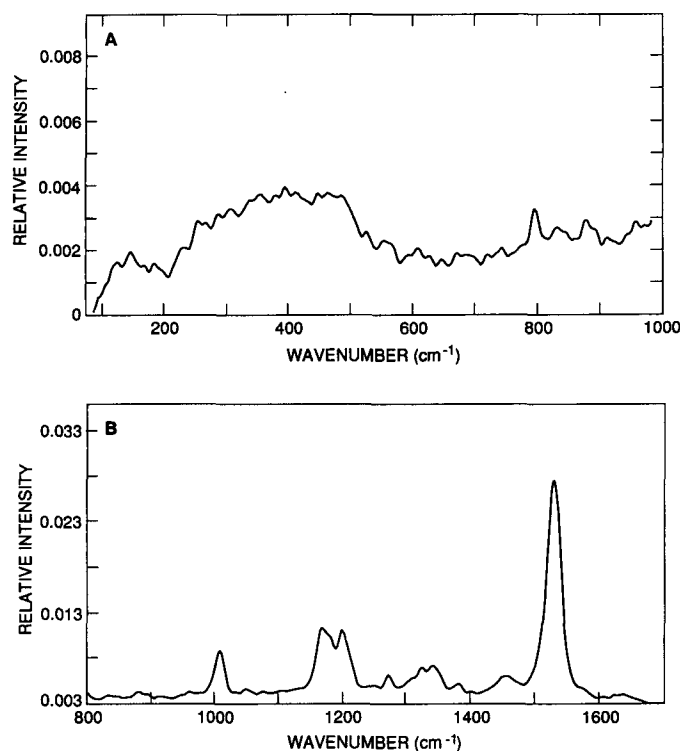


FIG. 1. Fourier transformed Raman spectrum of light-adapted bacteriorhodopsin, excited with 1.3 W at 1.06 μm ; 1500 scans at a resolution of 10 cm^{-1} were coadded. (A) 100 to 1000 cm^{-1} region. (B) 800–1700 cm^{-1} region.

resolution in both directions of travel of the interferometer moving mirror over a period of approximately 35 min. These were transformed to the corresponding power spectrum to eliminate the phase error.¹⁴ The Fourier transformed Raman spectrum of water was also measured and subtracted from the protein Raman spectra.

Bacteriorhodopsin was prepared from cultures of *Halobacterium halobium* according to standard techniques.¹⁵ The absorbance of the sample was approximately 1.3 at 570 nm in a 1-cm cell. The sample was light-adapted by light from a 14-W tungsten bulb for 45 min before being transferred to a cuvette for Raman analysis. Photosynthetic reaction centers of *Rhodobacter sphaeroides* were generously donated by Prof. Robert Blankenship (Arizona State University). The sample absorbance was approximately 1 to 1.5 at 800 nm in a 1-cm cell.

RESULTS AND DISCUSSION

Bacteriorhodopsin. The Fourier transformed Raman spectrum of light-adapted bR from 100 to 1700 cm^{-1} is shown in Fig. 1. (Note the change in intensity scale between Fig. 1A and Fig. 1B.) The near-IR excited Raman spectrum is dominated by vibrations of the retinylidene chromophore of bR,¹⁶ in particular by C=C (1528 cm^{-1}) and C-C stretches (1169 and 1200 cm^{-1}); CCH rocking modes (e.g., 1272, 1324, and 1341 cm^{-1}); and methyl rocking modes (1007, 1382, and 1456 cm^{-1}). Nearly all of the bands observed at wavenumber shifts above 700 cm^{-1} were observed in the resonance Raman spectrum of bR excited at 514.5 nm and have been assigned by Mathies and co-workers.¹⁶ A strong contribution from the poly-

peptide chain or membrane fragments themselves is conspicuously absent, although the broad shoulder at ~ 1650 cm^{-1} may be scattering by amide I vibrations of the polypeptide backbone.

A striking feature of the FT Raman spectrum of bR is that (with the exception of the 798- cm^{-1} band) the relative intensities follow closely those observed in the resonance Raman spectrum excited at 514.5 nm¹⁶ or in the preresonance spectrum excited at 752 nm.¹⁷ The similarity of the resonance Raman spectrum of bR excited in the visible and the near-IR Raman spectrum presented here suggests that, even though 1.06- μm excitation is not in resonance with bR, the mechanism of Raman enhancement is the same, i.e., that preresonance Raman enhancement¹⁸ dominates the spectrum observed in Fig. 1. Although the relative intensity of the band at 798 cm^{-1} is stronger in the Fourier transformed Raman spectrum than in the resonance Raman spectrum of light-adapted bR, this band is strong in dark-adapted bR.¹⁹ Thus, it may derive its intensity here because of incomplete light adaptation. A shoulder at 1179 cm^{-1} may also be due to dark-adapted bR.

Another conspicuous feature of the Raman spectrum of bR in Fig. 1 is the low-frequency vibrations in the 200–500 cm^{-1} region. Individual peaks in this region cannot be identified with certainty at this time due to the signal-to-noise level in this scan. However, a number of vibrations in this region (186, 257, 269, 398, 450, 527, and 555 cm^{-1}) were observed previously with 568-nm excitation.¹⁷ Other bands in this region could be low-frequency modes of the polypeptide backbone.

Reaction Centers. The reaction centers (RCs) of photosynthetic bacteria contain four bacteriochlorophyll (BChl) and two bacteriopheophytin (BPh) chromophores, as well as a carotene pigment.²⁰ BChl and BPh have absorptions in the visible (Soret and Q_x bands) and the near-IR (Q_y bands, 750–860 nm) regions. The absorption bands of several of these pigments overlap, particularly in the Soret regions of the BChl and BPh absorptions. As a result, the resonance Raman spectra of these species are complex when excited in the visible or near-UV regions.² The absorption bands of BPh, BChl, and the BChl dimer (the so-called special pair) are distinct in the near-IR Q_y region. However, resonance Raman excitation in this region is a difficult task, not only because of the laser wavelengths required but also because of interference from fluorescent impurities, which can only be eluded by extensive purification.²¹ Resonance Raman spectra of RCs have been reported with UV excitation in the Soret and Q_x bands of BChl and BPh.² Resonance Raman excitation at 752.8 nm, in resonance with the Q_y band of bacteriopheophytin, has also been reported.²¹

The FT Raman spectrum of the RC of *R. sphaeroides* is shown in Fig. 2. Particularly conspicuous is the broad and apparently congested band from 1300 to 1700 cm^{-1} in Fig. 2B. This is a region where a number of bands from BChl and BPh are expected.^{2,21} A similar broad feature was observed by Bocian *et al.*,²¹ although with less congestion, perhaps due to the higher resolution (3 cm^{-1}) of their spectrum, or the absence of contributions from pigments other than BPh. Several of the bands observed in Fig. 2B correlate well with previously ob-

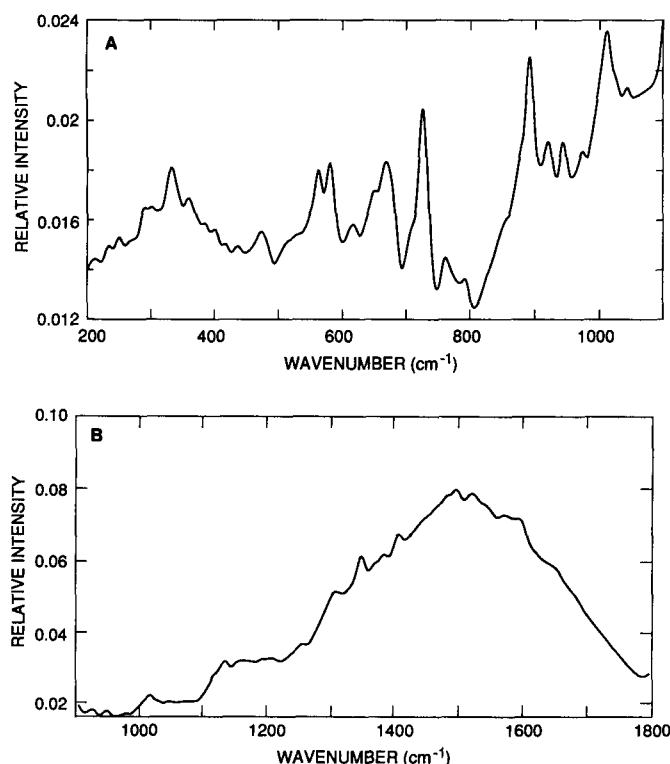


FIG. 2. Fourier transformed Raman spectrum of reaction centers of *R. sphaeroides* excited with 1.0 W at 1.06 μm ; 1500 scans at a resolution of 10 cm^{-1} were coadded. (A) 100–1000 cm^{-1} region. (B) 800–1700 cm^{-1} region.

served BPh bands.^{2,21,22} In addition, these regions may also contain overlapping contributions from BChl modes.^{2,22} Bands at 1013, 1167, and 1522 cm^{-1} can be assigned to carotenoid modes ν_3 , ν_2 , and ν_1 , respectively.² A shoulder around 1660 cm^{-1} may comprise amide I contributions, as well as keto or acetyl carbonyl vibrations of BChl and BPh. In contrast to bR, there is no clear and consistent correspondence in Fig. 2B with the relative intensities of Q_x , Q_y , or Soret-enhanced resonance Raman spectra of RCs, perhaps because of the existence of several visible and near-IR excited states in preresonance with 1.06 μm .

Figure 2A shows the low-frequency region of the FT Raman spectrum of the RC. This portion shows that near-IR FT Raman spectroscopy generates distinct spectra of the phorbins bending modes (500–800 cm^{-1}), as well as of lower-frequency vibrations. Most of these bands also correlate well with previously observed BPh or BChl modes.^{2,21,23} A more complete analysis of this spectrum will be published elsewhere.²⁴

CONCLUSIONS

Fourier transform Raman spectroscopy with 1.06- μm excitation is remarkably well suited to the study of photoactive proteins. Difficulties in dealing with sample photolability and fluorescence interference that accompany resonance excitation of Raman scattering of the protein-bound chromophores are circumvented. The Fourier transformed Raman spectrum of bacteriorhodopsin is of high quality, with vibrational bands principally of the chromophore retinal. Importantly, the relative intensi-

ties of the Raman bands closely resemble the relative intensities of the resonance Raman spectrum of bR excited in the visible region.

FT Raman spectroscopy of photosynthetic reaction centers produces a more congested spectrum, probably due to overlapping contributions from the chromophores in the RC. However, contributions from carotenoid, bacteriopheophytin, and perhaps bacteriochlorophyll appear in the Fourier transformed Raman spectrum. We anticipate that a higher-quality Raman spectrum of the RC can be generated with higher resolution and more extensive signal averaging.

In the Fourier transformed Raman spectra of both bR and the RC, a number of low-frequency modes are observed which, we feel, merit further study. Such low-frequency modes may find application in structural studies of chromophoric proteins.

The possibility of generating high-quality Raman spectra of the pigments contained in these proteins without serious interference from protein modes, and without inducing photochemistry due to the photolability of these proteins, is particularly attractive and should lead to further applications of FT Raman spectroscopy to their study. In particular, excitation at 1.06 μm should be significantly advantageous in time-resolved Raman studies.

ACKNOWLEDGMENTS

We thank Sarah Mounter and Martha West of the Biochemistry Research Service Laboratory for preparation of bacteriorhodopsin, and Prof. Robert Blankenship for his gift of the reaction sample. C.K.J. acknowledges support for this research from NIH (GM40071) and the USDA (88-37234-3404).

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