Fourier-transform Raman spectroscopy applied to photobiological systems

(resonance Raman spectroscopy/preresonant Raman scattering/Raman scattering cross section)

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ABSTRACT   Fluorescence and initiation of photoreactions are problems frequently encountered with resonance Raman spectroscopy of photobiological systems. These problems can be circumvented with Fourier-transform Raman spectroscopy by using the 1064-nm wavelength of a continuous wave neodymium–yttrium/aluminum-garnet laser as the probing beam. This wavelength is far from the absorption band of most pigments. Yet, the spectra of the investigated systems—bacteriorhodopsin, rhodopsin, and phycocyanin—show that these systems are still dominated by the chromophore, or that preresonant Raman scattering is still prevalent. Only for rhodopsin were contributions of the protein and the membrane discernible. The spectra of phycocyanin differ considerably from those obtained by excitation into the UV-absorption band. The results show the usefulness of this method and its wide applicability. In addition, analysis of the relative preresonant scattering cross sections may provide a detailed insight into the scattering mechanism.

Resonance Raman spectroscopy is a powerful technique for selectively studying chromophores in biological systems. By tuning the probing laser beam to the electronic transition of the chromophore(s) within the biological matrix (protein, lipids, etc.), the resonance-enhancement effect increases the scattering from the chromophore by several orders of magnitude. Thus, the spectrum contains only bands caused by the chromophore. The technique has contributed considerably to our understanding of the mechanism of retinal proteins and to the investigation of chromophore–protein interaction in proteins containing heme, bilin, chlorophyll, and carotenoid chromophores (1–6). There are only a few, but in some cases severe, drawbacks to this method, which can make its application difficult. Because the probing beam excites the chromophore into a higher electronic state, strong fluorescence may override the weaker spontaneous Raman scattering, making it undetectable against the fluorescence background. In active photobiological systems, the strong probing beam also initiates photoreaction(s), and special techniques must be applied to control the state of the system under investigation. This last point may become especially severe for systems in which the photoreaction is not reversible (i.e., systems that "bleach," such as vertebrate visual pigments) or in which the back-reaction is very slow (such as dark adaptation of bacteriorhodopsin or reversion of phytochrome). By pumping the sample through a capillary jet, the accumulation of the bleached state or of long-lived intermediates can be avoided, but large amounts of material are usually required.

For a long time, analyzing the Raman-scattered light by a Fourier-transform (FT) spectrophotometer, or FT-Raman spectroscopy, was thought to have disadvantages as compared with conventional Raman spectroscopy (7–9). However, about 2 years ago, it was realized that by using a near-infrared laser, such as a neodymium–yttrium/aluminum-garnet (Nd:YAG) laser, as the probing beam, the problem of fluorescence, a common problem of Raman spectroscopy, could, in many cases, be overcome (10–12). Also it was realized that, by combining a near-infrared laser with an FT-spectrophotometer, the high-wavenumber precision inherent in FT spectroscopy would be another advantage. The advantage of FT spectroscopy, the higher energy throughput, can, at least partially, compensate for the >16-fold-reduced Raman cross section by using a probing beam of about twice the absorption wavelength. [There is no multiplex advantage because the noise is photon-noise limited (9).] To avoid the necessity of too powerful lasers that could destroy the samples, the collecting optics have to be optimized, and very sensitive detectors must be used. To reduce the noise caused by unwanted scattering, such as the strong elastic and quasi-elastic Rayleigh scattering, effective filters have to be designed. By carefully considering the points discussed, much progress has been made during the last 2 years in implementing FT-Raman (12–15) and surface-enhanced FT-Raman (16) spectroscopy.

With a 1064-nm probing beam, the Raman scattering of most photobiological systems would be preresonant or even far from resonance with respect to the chromophore (17, 18). The question then arises whether or not the spectra would now also contain contributions from the protein. Especially, whether or not the many amide–I bands of the numerous peptide groups of a protein would now surpass the bands caused by a single chromophore. From the theory of resonance Raman scattering (17, 18), for bands caused by the chromophore the B term of the Raman cross section would be predicted to now exceed the A term. However, were the B term still much larger than the scattering cross sections of bands caused by the protein, the chromophore could be monitored without evoking fluorescence or a photoreaction. Some indication that bands caused by chromophores may indeed dominate the FT-Raman spectra was obtained from investigations of dye molecules embedded in Langmuir-Blodgett films (10). In this report we show that this result is also true for the FT-Raman spectra of (i) a strongly fluorescent system, such as phycocyanin; (ii) a system with very slow cyclic photoreaction, such as the dark adaptation of bacteriorhodopsin; and (iii) a system that bleaches upon absorption of light, such as rhodopsin.

Abbreviation: FT, Fourier transform.
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MATERIALS AND METHODS

Purple membranes from *Halobacterium halobium* were isolated from strain S9, as described (19). Bovine rod outer segments were prepared according to a published procedure (20). The preparation of phycocyanin has been described (21). The approximate optical densities of the sample solutions were 20 OD units/cm (λ = 570 nm) for bacteriorhodopsin, 8 OD units/cm (λ = 500 nm) for rhodopsin, and 40 OD units (λ = 615 nm) for phycocyanin. To obtain such a high phycocyanin concentration, a solution of known concentration was lyophilized, and the material was dissolved in the respective amounts of H_2O or ^2H_2O. Because the pH of the unbuffered phycocyanin solution was about pH 7, the solution at this high concentration can be assumed to contain essentially only trimers (22–24). Unless otherwise mentioned, the samples were measured in distilled water. A Bruker FT-Raman accessory and a Bruker IFS 66 FT-infrared spectrophotometer were used for the FT-Raman measurements. The laser power of the diode-laser pumped continuous wave Nd:YAG laser was ≈ 300 mW (the exact value is given in the figure legends). A 180° backscattering geometry was used for all the measurements. A 5-mm-pathlength quartz cuvette was used for bacteriorhodopsin and phycocyanin; a 1-mm capillary, normally used for melting point determination, was used for rhodopsin. The spectra were corrected by subtracting the spectra of the respective aqueous solvents.

RESULTS AND DISCUSSION

Fig. 1 shows the FT-Raman spectrum of nonilluminated bacteriorhodopsin (trace b) and of light-adapted bacteriorhodopsin (trace a). In trace c the weighted subtraction of the two spectra is shown, demonstrating the spectrum of 13-*cis* bacteriorhodopsin. The spectra compare very well with the corresponding resonance Raman spectra (25–28). The main difference is the lower intensity of the band at 801 cm^{-1} of 13-*cis* bacteriorhodopsin, which was assigned to the 14-hydrogen-out-of-plane mode of the retinal. From the strong intensity of this band, it is concluded that the terminal part of the chromophore is twisted (25). Because Raman scattering by the chromophore is dominated by the B term, this cross section must exhibit a very similar mode dependency as the A term, which governs the resonance Raman scattering. According to the theory of resonance Raman scattering, when large geometrical changes occur in the electronic excited state, even for the far-from-resonance case, the B term of the scattering cross section may depend similarly on the vibrational modes as the A term (18). In agreement with the theory, this relationship indicates that large geometrical changes occur upon excitation into the allowed electronic excited state. The lower intensity of the 14-hydrogen-out-of-plane band, however, cannot be explained at present.

In Fig. 2 the FT-Raman spectrum of disc membranes containing rhodopsin is shown (trace a). Only ≈ 10 μg of rhodopsin was needed for the experiment, and the sample could be recovered after the measurement. The less favorable geometry and the comparable low concentration yields a reduced Raman scattering. This result explains partly the increased noise in the spectrum. But, as will be shown later, the major part of the noise is from a much reduced Raman cross section of the chromophore of rhodopsin as compared with that of bacteriorhodopsin. The ethylenic mode (1545 cm^{-1}), the fingerprint modes (1270–1200 cm^{-1}), and the 11,12-hydrogen-out-of-plane mode (969.5 cm^{-1}) are in excellent agreement with the published resonance Raman spectrum (29, 30). In addition, however, Fig. 2 (trace a) shows a strong band at 1659 cm^{-1} and a broad and strong band at ≈ 1445 cm^{-1}. Both bands are, within the noise limit, not
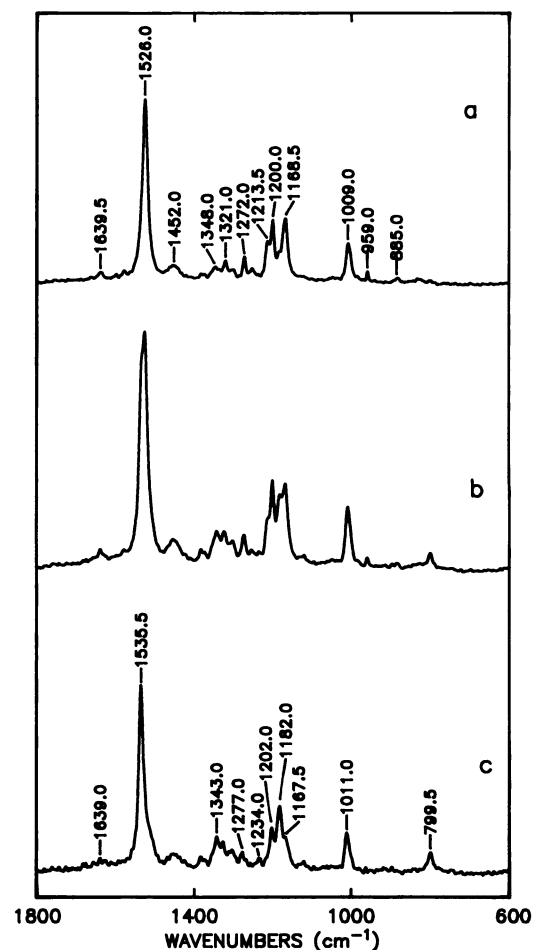

Fig. 1. FT-Raman spectra of bacteriorhodopsin in its light-adapted (trace a) and dark-adapted state (trace b). Trace c shows the spectrum of 13-*cis* bacteriorhodopsin, obtained by subtraction of a fraction of spectrum a from spectrum b. Laser power, 220 mW; number of scans, 1000, corresponding 42-min measuring time; resolution, 3 cm^{-1}.

influenced by illuminating the sample at room temperature (trace b). During the measuring time of 1 hr, the illumination produced a mixture of metarhodopsin-I, -II, -III, and retinal plus opsin. This result precludes that the former band is due to the C=N stretch of the protonated Schiff base of rhodopsin, which is located at the same position, and that the latter is due to a much weaker band at a similar position in the resonance Raman spectrum of rhodopsin. In the FT-Raman spectrum of the mixture of illumination products, the ethylenic mode is shifted up (1557 cm^{-1}), broadened, and reduced in intensity. In addition, the fingerprint modes show altered intensities and positions. The most straightforward interpretation is that the band at 1659 cm^{-1} is from the amide-I band of the protein and that the band around 1445 cm^{-1} is from the sum of the CH_2-bending vibrations and of the asymmetric CH_3-bending modes of aliphatic groups. Because the FT-Raman spectra were obtained from disc membranes that contain $\approx 50\%$ lipids by weight, this contribution is expected to be particularly strong. In the resonance Raman spectrum of rhodopsin, a band at 1000 cm^{-1} was assigned to the 13-methyl rocking mode (29, 31, 32), which is shifted down by the steric interaction with 10-H. Because the band at 1002 cm^{-1} in the FT-Raman spectrum is not influenced by illumination, it probably does not represent the same mode.

The molecular weight of rhodopsin is $\approx 40\%$ higher than that of bacteriorhodopsin. The lipid content of the rhodopsin-containing disc membrane is much greater than that of the bacteriorhodopsin-containing purple membrane. But also,
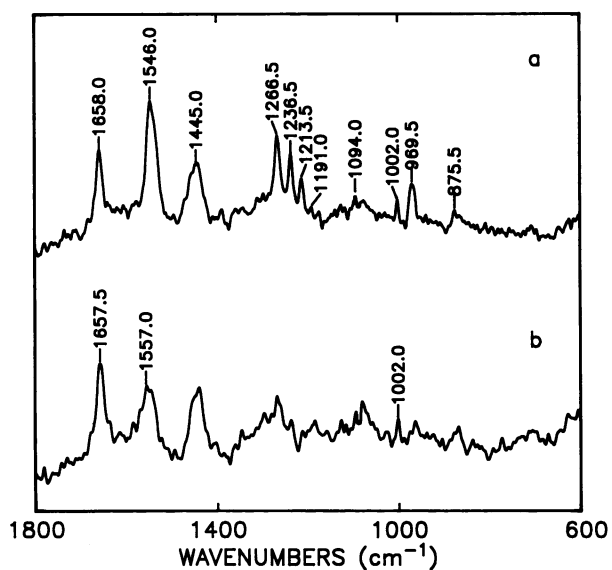
FIG. 2. FT-Raman spectra of rhodopsin in rod disc membranes. Solution contained 100 mM phosphate buffer, pH 5.5. Trace a represents unbleached rhodopsin, whereas trace b represents bleached rhodopsin. Laser power, 235 mW; other measuring conditions as for Fig. 1.

FIG. 3. FT-Raman spectra of phycocyanin in H_2O (trace a) and ^2H_2O (trace b). Measuring conditions were as for Fig. 2.

when these differences are taken into account, the large amide-I band in the FT-Raman spectra clearly shows the Raman cross section of the 11-*cis*-retinal chromophore of rhodopsin to be reduced at least by a factor of 20 as compared with that of the all-*trans*- or 13-*cis*-retinal chromophores of bacteriorhodopsin. This effect may partly be from the 70-nm blue shift of the absorption maximum, but the total integral intensity of the ethylenic bands in trace b of Fig. 2 is only reduced by a factor of ≈ 2 as compared with that of trace a. This fact shows that the further blue shift of the absorption maximum of the photoproducts does not reduce the Raman cross section to the same extent. Therefore, other mechanisms are required to explain the reduced Raman cross section of rhodopsin as compared with bacteriorhodopsin. Differences in homogeneous linewidths could play a role (17, 33). Loppnow and Mathies (34) concluded that rhodopsin has an unusual low-resonance Raman cross-section and explained this by a very large inhomogeneous absorption bandwidth. It is interesting that the same situation is seen under far-from-resonance conditions. Much better FT-Raman spectra could be obtained with higher rhodopsin concentration and with solubilized rhodopsin. In addition, optimizing the microsampling technique in the manner suggested by Schrader and Simon (15) also increases the efficiency. If necessary, bands not caused by the chromophore could be compensated for by subtracting a spectrum of opsin.

Fig. 3 shows the FT-Raman spectrum of phycocyanin in H_2O (trace a) and ^2H_2O (trace b). The spectrum compares well with the resonance Raman spectrum obtained by excitation into the visible absorption band (35), which was, however, difficult to measure because of the very strong fluorescence. A detailed comparison shows that the relative intensities of some bands are different. Note that in the FT-Raman spectra also, no carbonyl bands of the two lactam rings can be seen. However, considerable deviations are observed between the FT-Raman spectra and resonance Raman spectra, with excitation into the near-UV band (36, 37). This result shows that (*i*) during the measurements performed by Margulies and Toporowicz (35) no degradation of the chromophore occurred and (*ii*) care must be taken in comparing resonance Raman spectra of bile pigments obtained with excitation into different bands—e.g., the visible
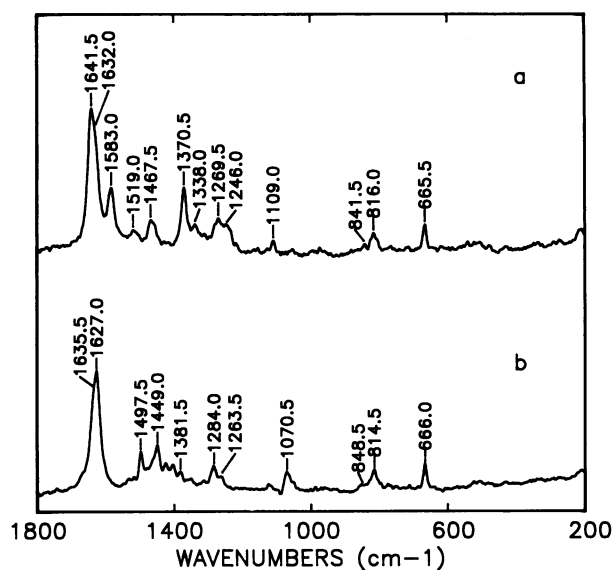
or UV absorption. Resonance-enhanced coherent anti-stokes Raman spectrum (CARS) obtained with a 640-nm pump beam also shows that different modes are enhanced (21) as compared with excitation into the UV absorption. The strong ^2H_2O effect on the band at 1583 cm^{-1} indicates that, as for phytochrome, the chromophore is protonated (38). Protonation of the chromophore was suggested by the absorption maximum and by refined structural data (39), which place an aspartic acid (Asp-87) near the nitrogen of ring C. Further support for a protonated chromophore is obtained from the high position of the C=C stretching modes (40). Comparing our data with those obtained by Margulies and Toporowicz (35), the differences in intensities of some bands may indicate that, for phycocyanin, the geometrical changes in the electronic excited state are not as pronounced as for bacteriorhodopsin.

Our data clearly show that FT-Raman spectroscopy is a useful tool for the study of chromophore(s)-containing biological systems. Because the spectra are still dominated by chromophore bands, the advantages mentioned in the introduction enable measurements difficult to perform with resonance Raman spectroscopy. In addition, it may also contribute to our understanding of the Raman scattering mechanism.

1. Spiro, T. G., ed. (1987) *Biological Applications of Raman Spectroscopy* (Wiley, Chichester, U.K.), Vol. 2.
2. Lutz, M. (1984) in *Advances in Infrared and Raman Spectroscopy*, eds. Clark, R. J. H. & Hester, R. E. (Wiley Heyden, Chichester, U.K.), Vol. 11.
3. Robert, B. & Lutz, M. (1985) *Biochim. Biophys. Acta* 807, 10–23.
4. Szalontai, B., Gombos, Z., Csizmadia, V., Csatorday, K. & Lutz, M. (1989) *Biochemistry* 28, 6467–6472.
5. Koyama, Y., Kanaji, M. & Shimamura, T. (1988) *Photochem. Photobiol.* 48, 107–114.
6. Koyama, Y., Umemoto, Y., Akamatsu, A., Uehara, K. & Tanaka, M. (1986) *J. Mol. Struct.* 146, 273–287.
7. Hirschfeld, T. & Schildkraut, E. R. (1974) in *Laser Raman Gas Diagnostics*, ed. Lapp, M. (Plenum, New York), pp. 379–388.
8. Hirschfeld, T. (1979) in *Fourier Transform Infrared Spectroscopy*, ed. Ferraro, E. (Academic, New York), p. 193.
9. Hirschfeld, T. (1976) *Appl. Spectrosc.* 30, 68–69.

10. Zimba, C. G., Hallmark, V. M., Swalen, J. D. & Rabolt, J. F. (1987) *Appl. Spectrosc.* **41**, 721–726.
11. Fujiwara, M., Hamaguchi, H. & Tasumi, M. (1986) *Appl. Spectrosc.* **40**, 137–139.
12. Chase, B. (1987) *Anal. Chem.* **59**, 881A–889A.
13. Hirschfeld, T. & Chase, B. (1986) *Appl. Spectrosc.* **40**, 133–137.
14. Bergin, F. J. & Shurvell, H. F. (1989) *Appl. Spectrosc.* **43**, 516–522.
15. Schrader, B. & Simon, A. (1988) *Mikrochim. Acta* **1988**, II, 227–230.
16. Angel, S. M., Katz, L. F., Archibald, D. D. & Honigs, D. E. (1989) *Appl. Spectrosc.* **43**, 367–372.
17. Myers, A. B. & Mathies, R. A. (1987) in *Biological Applications of Raman Spectroscopy*, ed. Spiro, T. G. (Wiley, New York), pp. 1–57.
18. Warshel, A. & Dauber, P. (1977) *J. Chem. Phys.* **66**, 5477–5488.
19. Gerwert, K. & Siebert, F. (1986) *EMBO J.* **5**, 805–811.
20. Siebert, F., Mäntele, W. & Gerwert, K. (1983) *Eur. J. Biochem.* **136**, 119–127.
21. Schneider, S., Baumann, F., Steiner, W., Fischer, R., Siebzehn-rübel, S. & Scheer, H. (1988) in *Photosynthetic Light-Harvesting Systems: Organization and Function*, eds. Scheer, H. & Schneider, S. (de Gruyter, Berlin), pp. 317–322.
22. Fischer, R., Siebzehnrübl, S. & Scheer, H. (1988) in *Photosynthetic Light Harvesting Systems: Organization and Function*, eds. Scheer, H. & Schneider, S. (de Gruyter, Berlin), pp. 71–76.
23. Rümbeli, R. & Zuber, H. (1988) in *Photosynthetic Light Harvesting Systems: Organization and Function*, eds. Scheer, H. & Schneider, S. (de Gruyter, Berlin), pp. 61–70.
24. Saxena, A. M. (1988) *J. Mol. Biol.* **200**, 579–591.
25. Smith, S. O., Pardoen, J. A., Lugtenburg, J. & Mathies, R. A. (1987) *J. Phys. Chem.* **91**, 804–819.
26. Smith, S. O., Braiman, M. S., Myers, A. B., Pardoen, J. A., Courtin, J. M. L., Winkel, C., Lugtenburg, J. & Mathies, R. A. (1987) *J. Am. Chem. Soc.* **109**, 3108–3125.
27. Stockburger, M., Klusmann, W., Gattermann, H., Massig, G. & Peters, R. (1979) *Biochemistry* **18**, 4886–4900.
28. Alshuth, T. & Stockburger, M. (1981) *Ber. Bunsenges. Phys. Chem.* **85**, 484–489.
29. Mathies, R. A., Smith, S. O. & Palings, I. (1987) in *Biological Application of Raman Spectrometry*, ed. Spiro, T. G. (Wiley, Chichester, U.K.), pp. 59–108.
30. Palings, I., Pardoen, J. A., Van den Berg, E., Winkel, C., Lugtenburg, J. & Mathies, R. A. (1987) *Biochemistry* **26**, 2544–2556.
31. Callender, R. H., Doukas, A., Crouch, R. K. & Nakanishi, K. (1976) *Biochemistry* **15**, 1621–1629.
32. Cookingham, R. & Lewis, A. (1978) *J. Mol. Biol.* **119**, 569–577.
33. Myers, A. B., Trulson, M. O., Pardoen, J. A., Heeremans, C., Lugtenburg, J. & Mathies, R. A. (1986) *J. Chem. Phys.* **84**, 633–640.
34. Loppnow, G. R. & Mathies, R. A. (1988) *Biophys. J.* **54**, 35–43.
35. Margulies, L. & Toporowicz, M. (1988) *J. Mol. Struct.* **175**, 61–66.
36. Szalontai, B., Gombos, Z., Csizmadia, V. & Lutz, M. (1987) *Biochim. Biophys. Acta* **893**, 296–304.
37. Szalontai, B., Csizmadia, V., Gombos, Z., Csatorday, K. & Lutz, M. (1988) in *Photosynthetic Light-Harvesting Systems: Organization and Function*, eds. Scheer, H. & Schneider, S. (de Gruyter, Berlin), pp. 307–315.
38. Fodor, S. P. A., Lagarias, J. C. & Mathies, R. A. (1988) *Photochem. Photobiol.* **48**, 129–136.
39. Schirmer, T., Bode, W. & Huber, R. (1987) *J. Mol. Biol.* **196**, 677–695.
40. Margulies, L. & Toporowicz, M. (1984) *J. Am. Chem. Soc.* **106**, 7331–7336.