Time-Resolved Resonance Raman Spectroscopy of Photobiological and Photochemical Transients

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One of the fundamental topics in chemistry is the study of molecular structural changes which occur during the course of a chemical reaction. Much of the knowledge of the mechanisms involved in chemical change has come through the study of photochemical processes, using techniques such as flash photolysis. As was shown by Norrish and Porter[1] in the 1940s, by initiating a chemical process with a strong photolytic flash, and following the resulting changes with a weak probe, it was possible to obtain the optical absorption spectra of short-lived chemical transients and to determine rates of rapid processes. Short duration flashlamps available at that time allowed the study of chemical mechanisms with microsecond time resolution. The advent of pulsed lasers in the 1960s resulted in routine acquisition of kinetic data on the order of nanoseconds.[2] Laser modelocking and pulse compression presently allow investigations of events in the picosecond[3] and subpicosecond[4] timescales.

The strength of flash photolysis and related techniques, such as stopped flow[5] and temperature jump[6] kinetics (methods which provide transient absorption spectra) is the ability to derive kinetic rate information. The enormous potential of these techniques has been proven. A great deal of chemical information can be obtained from transient absorptions. Nonetheless, in many instances absorption bands are broad and diffuse with limited inherent structural information. Other spectroscopic techniques such as Raman and infrared absorption spectroscopy can produce high resolution vibrational spectra. Combining the principles of flash photolysis with Raman spectroscopy results in a powerful method for obtaining detailed information about changes in structure and bonding within a molecule. This technique is known as "time-resolved resonance Raman spectroscopy", by analogy to time-resolved absorption spectroscopy (flash photolysis).

The development of the laser revolutionized transient absorption spectroscopy allowed time-resolution that had been previously unobtainable. Among other spectroscopic techniques that felt a similar impact of the laser was Raman spectroscopy, which had been superceded by infrared absorption spectroscopy as the most popular form of vibrational spectroscopy after World War II, when commercial infrared absorption spectrometers became available. The properties of laser light make it the ideal excitation source for Raman spectroscopy. It is coherent, highly intense, monochromatic, and easily manipulated by simple optical components.

When the wavelength of the laser excitation is varied so that it coincides with an electronic absorption wavelength, the enormous signal intensity enhancement of the resonance Raman effect[7] is observed. This enhancement has been especially important for the study of biological samples in dilute aqueous solution, which are difficult to study by infrared absorption spectroscopy. Since resonance enhancement is localized to the chromophoric group, the vibrational spectrum is greatly simplified. Different parts of a complex molecule may be studied by changing the laser wavelength so that it coincides with electronic absorptions localized over different structural regions of the molecule. The resonance Raman enhancement effect is also advantageous for the study of chemical transients. Transients sometimes have very low concentrations, since full conversion from the initial reactants does not occur in many cases.

A time-resolved resonance Raman method[8] that is most reminiscent of the flash photolysis technique is a sequence of two laser pulses; the first photolyzes the sample, and the second acts as a resonance Raman probe.[9-14] Alternatively, a single laser pulse can be used

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to both photolyze the sample and simultaneously Raman scatter from whatever is present during the pulse duration.[15-24] Since Raman spectroscopy can be performed with the same pulsed lasers used for time-resolved (transient) absorption studies, Raman spectroscopy is capable of the same ultrafast time resolution as is realized with absorption techniques, though spectral resolution will be limited by the uncertainty principle. Time-resolved resonance Raman spectroscopy is now being performed on the picosecond timescale.[25-30]

The Ideal Time-Resolved Resonance Raman Experiment

As an example of an ideal time-resolved experiment, consider the three sequential processes in eq 1, where

$$A \xrightarrow{P(t_A)} B \xrightarrow{t_B} C \xrightarrow{t_C} \ldots \quad (1)$$

t_A, t_B, and t_C are characteristic times of the three transformations. The first transformation is induced by a sudden perturbing pulse of short duration compared to the characteristic times. The perturbing pulse of light induces the A → B change photochemically. The transients B and C can be an excited state of A or a new chemical species. Studies on transients, e.g., free radicals, anion and cation radicals, unstable isomers, intermediates in photobiological transformations, and excited states of organic and inorganic systems, constitute the largest fraction of systems studied so far.

The most ideal change to study by these techniques is that for which $t_A < t_B$ and t_C and for which the absorption wavelength, λ_i, is different for the different species i. A typical time-resolved resonance Raman experiment would thus involve two short laser pulses, a photolysis pulse, and a probe pulse. Ideally the separation of the pulses in time and frequency can be varied continuously and independently, and one records the time profile of each species by following its resonance Raman spectrum at its characteristic resonance absorption wavelength. Since our laboratory is not equipped with two tunable pulsed lasers, we have sometimes relied on a single continuous-wave or pulsed laser. By selecting systems where the parent and intermediates have overlapping strong absorption bands, the same laser can be used as the photolysis and the probe source.

In the following sections, we discuss different photobiological and photochemical systems and the type of techniques used to answer specific questions raised regarding each system.

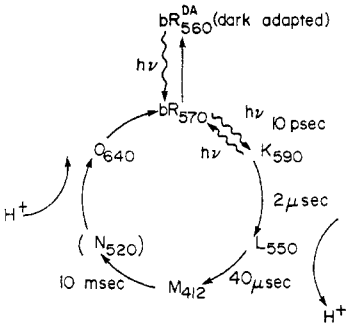

Figure 1. The photochemical cycle of bacteriorhodopsin, showing the predominant intermediates. Subscripts are absorption maxima in nanometers. The cyclic scheme is somewhat oversimplified as branching pathways occur. From ref 40.

Bacteriorhodopsin

Two photosynthetic mechanisms are known to exist in nature. The first, based on the various chlorophylls and accessory pigments, is familiar to everyone. The second is a purple retinal–protein complex, found in purple patches of the cell membrane (the purple membrane) of various halophilic bacteria.[31] Because of its similarity in structure to the visual pigment rhodopsin, it has been called "bacteriorhodopsin". Oesterhelt and Stoeckenius[32] reported that bacteriorhodopsin was able to convert light into chemical energy stored in the form of ATP. When light shines on the cell membrane, the bacteriorhodopsin pumps hydrogen ions from inside of the cell to the outside, setting up an electrochemical gradient that supplies the driving force for the synthesis of ATP, according to the chemiosmotic theory of Mitchell.[33] Extensive flash photolysis work has shown that the photolytic mechanism of bacteriorhodopsin involves a cyclic sequence of intermediates, as diagrammed in Figure 1. The pigment absorbs maximally at 560 nm when kept in the dark and is denoted bR_{560}^{DA} (DA = dark adapted). Upon exposure to light, the absorption maximum shifts to 570 nm; this species is denoted as bR_{570}. The photolytic intermediates of bR_{570} have been the most studied. The predominant notation for these intermediates uses the letters K, L, M, ..., etc. and a subscript which gives the absorption maximum (nanometers).

Resonance Raman spectroscopy had been shown to be able to provide important information about the structure of the retinal chromophore in rhodopsin,[34,35]

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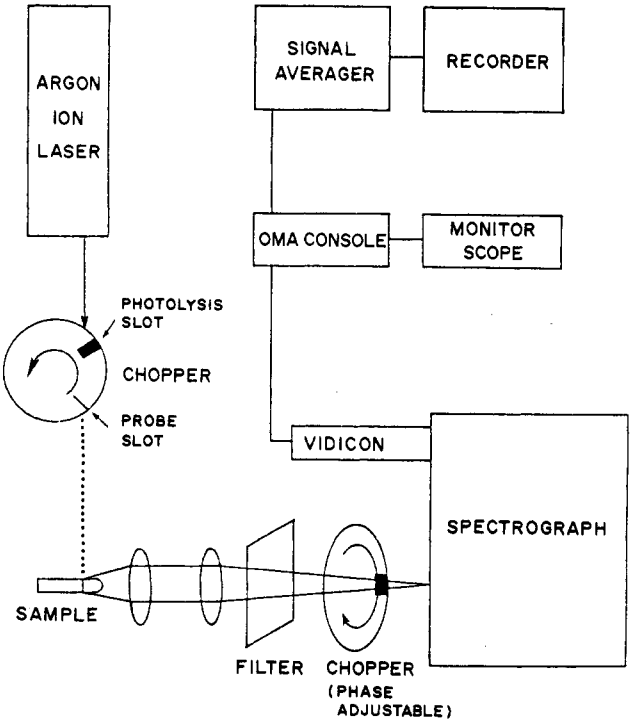

Figure 2. A diagram of a time-resolved resonance Raman apparatus that makes use of the flash and probe principle used in flash photolysis. Continuous laser light was modulated by a rotating light chopper into a repeating sequence of a strong photolytic flash followed by a weak probe pulse. Different delay times between the flash and probe were obtained by varying the spacing between the slots on the disk. A second chopper placed in front of the spectrograph was adjusted so as to block scattering from the photolytic flash but to allow scattered light from the probe pulse to reach the vidicon detector. From ref 9 (OMA – optical multichannel analyzer).

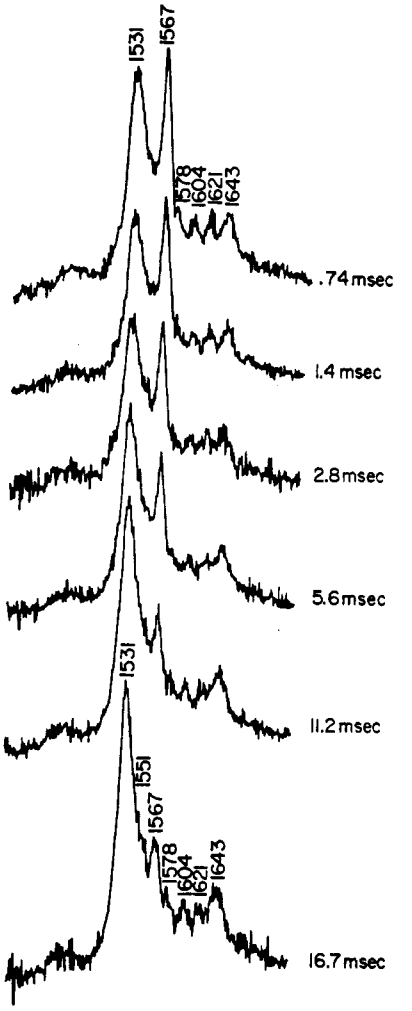

Figure 3. Time-resolved resonance Raman spectra of bacteriorhodopsin showing the decay of the M_{412} intermediate, using the apparatus of Figure 2. Major bands due to the M_{412} intermediate are at 1567 and 1621 cm^{-1} (457.9-nm excitation).[9]

and it was quickly recognized that the technique would also be quite useful for the study of the structurally related bacteriorhodopsin molecule[36] and its intermediates. Lewis et al.[37] showed that a sample of bacteriorhodopsin under intense continuous wave laser illumination would produce a large population of the M_{412} intermediate which could be subjected to resonance enhancement using dye laser excitation. In fact, for several of the longer lived intermediates, contributions to the resonance Raman spectrum can be seen when the sample is held stationary within a cuvette or melting point capillary.[38,39]

In order to assign the various resonance Raman bands to individual intermediates, it was necessary to deconvolute the superimposed resonance Raman spectra on the basis of the kinetic behavior of the individual resonance Raman bands. One approach utilised the flash and probe principle of flash photolysis.[9] Laser light from a continuous-wave laser was modulated with a rotating disk chopper (Figure 2). The disk had two slots, one wide and one narrow. As it rotated, continuous-wave laser light was chopped into a repeating sequence of an intense photolysis pulse followed by a weak probe pulse. The pulses were separated by a delay which was determined by the angular separation of the two slots as well as the rotational frequency of the chopper. The probe pulse intensity was adjusted to cause minimal photolysis, while still being sufficient to accumulate a Raman spectrum. A second chopper with only one slot, rotating synchronously with the first chopper, was placed in front of the entrance to the spectrometer and acted as a mechanical gate. The phase of this chopper was adjusted to block the photolysis flash from entering the spectrograph yet allowed the Raman scattering from the probe pulse to reach the detector.

By varying the delay between the photolytic and probe flashes, kinetic rates were obtained by plotting peak intensities vs. time. An example of the results (Figure 3) shows the decay of the ethylenic stretch (1567 cm^{-1}) of the M_{412} intermediate of bacteriorhodopsin with a half-life of 7 ms,[9] in agreement with the transient optical absorption measurements.[40]

The retinal chromophores of bacteriorhodopsin and rhodopsin are linked to a lysine residue on the polypeptide via a Schiff base linkage.[41] Resonance Raman

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spectroscopists have shown that the Schiff base linkages of the unphotolyzed pigments are protonated. The C=NH stretching vibration which occurs between 1640 and 1655 cm^{-1} shifts by approximately 20 cm^{-1} upon deuterium substitution.[37,42] The Schiff base deprotonates upon photolysis. Lewis et al.[37] observed that the corresponding vibration of the M_{412} intermediate of bacteriorhodopsin at 1622 cm^{-1} showed no shift upon deuterium exchange, implying nonprotonation of the Schiff base in this intermediate. The determination of the points of deprotonation and reprotonation in the bacteriorhodopsin cycle were of considerable interest because of theoretical investigations on the structure of the chromophore[43,44] and models for the mechanism of the primary photolytic step.[45]

A strong deuteration effect had been observed on the kinetic rates of formation of the first intermediates of both rhodopsin and bacteriorhodopsin, suggesting a deprotonation during the primary step.[46,47] The Schiff base proton was a strong candidate for the photoejection because it is the most readily exchangeable proton on the chromophore. On the other hand, correlation had been shown between the absorption maxima and protonation states[48] that suggested that the primary intermediates of rhodopsin and bacteriorhodopsin were both protonated. To resolve this question, it was important to monitor the C=N vibrational frequencies in the resonance Raman spectra of the various bacteriorhodopsin intermediates and especially of the primary photolytic intermediate K_{590}.

Rapid recirculation flow techniques have been used to obtain resonance Raman spectra of parent molecules that are photolabile[49,50] by minimizing the photolysis caused by the laser excitation. However, the resonance Raman spectra of specific photolytic products can be obtained[16,51-55] by using the same experimental method, if certain conditions are satisfied. These include a wavelength suitable for enhancement of the product, sufficient energy to cause photolysis of the parent, and a time interval chosen so as to favor a specific product over the others.

The above approach was used to obtain the resonance Raman spectrum of the K_{590} intermediate.[54] A continuous krypton ion laser beam was focused through a high powered microscope objective to a 1-μm spot on a fast flowing jet of bacteriorhodopsin in an aqueous suspension. With a jet velocity of 10 m/s, the residence time of the sample within the focused beam was approximately 100 ns. In essence the sample molecules experienced a 100-ns pulse, though the detector collected scattering from a continuous beam. The laser power could be kept low, minimizing damaging multiphoton effects and allowing minimal photolysis of the photolabile material. Longer lived intermediates that had rise times longer than 100 ns had mostly left the irradiated volume by the time they were formed and therefore contributed only minimally to the resonance Raman spectrum.

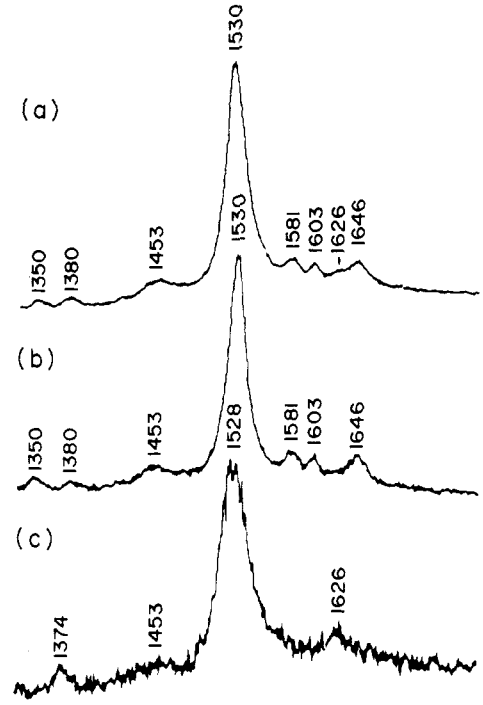

Figure 4. (a) Resonance Raman spectrum of a flowing jet of bacteriorhodopsin with a sample residence time in a focused laser beam of 100 ns. A high power of 50 mW gave a superposition of the K_{590} intermediate on the bR_{570} parent (552.3-nm excitation). (b) Low power (1-mW) resonance Raman spectrum of a flowing jet of bacteriorhodopsin giving a resonance Raman spectrum of unphotolyzed bR_{570} (552.3-nm excitation). (c) Difference of (a) minus (b) giving a resonance Raman spectrum of the K_{590} intermediate. The band at 1626 cm^{-1} was found to shift to 1616 cm^{-1} when the experiment was repeated in D_2O, indicating that the Schiff base of this intermediate is protonated. From ref 54.

The results of this experiment are shown in Figure 4. A resonance Raman spectrum obtained at very low incident laser power (Figure 4b) is that of essentially pure bR_{570} with no apparent photolysis. As the laser power is raised (Figure 4a), the bacteriorhodopsin sample becomes photolyzed. Resonance Raman peaks due to scattering from the K_{590} intermediate have become superimposed upon the resonance Raman spectrum of the unphotolyzed parent bR_{570}. In many chemical reactions, full conversion to the product is never reached because of equilibrium effects. Thus, it is sometimes impossible to observe pure samples of intermediates by either time resolution or low-temperature trapping. The photolytic cycle of bacteriorhodopsin is an example of such incomplete conversion, as the maximum quantum yield of photolysis is approximately 30%.[56] Moreover, some of the intermediates of bacterio-

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Table I.
Bacteriorhodopsin Schiff Base Vibrations (cm^{-1})

	H_2O	D_2O	
bR_{560}^{DA}	1644	1622	protonated
bR_{550}^{DA}	1644	1622	protonated
bR_{570}	1644	1622	protonated
K_{590}	1626	1616	protonated
L_{550}	1647	1619	protonated
M_{412}	1622	1622	not protonated
O_{640}	1630	1616	protonated

rhodopsin have absorptions that are close to or overlapping with each other and the parent bR_{570}, making selective enhancement by the resonance Raman effect somewhat difficult. Kinetic absorption spectroscopists have resorted to difference spectra, in cases were only partial conversion is attained, in order to monitor absorption spectral changes induced by the formation of intermediate states.[57]

To more clearly reveal the resonance Raman spectrum of the K_{590} intermediate of bacteriorhodopsin, a similar difference method was used. By computer subtracting the low power flow spectrum (Figure 4b), containing the resonance Raman spectrum of essentially only bR_{570}, from the high power flow spectrum (Figure 4a) containing the resonance Raman spectrum of K_{590} superimposed upon that of bR_{570}, a representation of the resonance Raman spectrum of K_{590} was obtained (Figure 4c). The M_{412} intermediate did not contribute to the spectrum significantly, since its absorption maximum is far removed from the excitation frequency. Its rise time is 40 μs, several times longer than the laser interaction time of 100 ns. The L_{550} intermediate did contribute slightly, but these bands were known from a parallel experiment[52] using a laser interaction time of 10 μs. Figure 4c shows a band at 1626 cm^{-1} in the resonance Raman spectrum of K_{590}, which was assignable to a protonated Schiff base since the frequency shifted to 1616 cm^{-1} when the experiment was repeated in D_2O suspension. Similar resonance Raman results on the K_{590} intermediate, as well as others, have been obtained by using spinning-cell and low-temperature trapping experiments.[58-60]

A summary of resonance Raman data for the C=N stretching frequency is given in Table I for the intermediates in the bacteriorhodopsin cycle.[54] When bacteriorhodopsin is suspended in D_2O, the proton on the Schiff base nitrogen exchanges with a deuteron, causing a frequency shift in the resonance Raman spectrum, if the Schiff base is protonated. All intermediates, with the exception of the M_{412} intermediate, exhibit a significant shift. Deprotonation was thus concluded to occur between the L_{550} to M_{412} steps, with reprotonation upon relaxation of M_{412} into the O_{640} intermediate.

The retinal Schiff base of the primary bacteriorhodopsin photointermediate K_{590} was therefore protonated.[54,60] Low-temperature resonance Raman studies show that the primary photointermediate of rhodopsin, bathorhodopsin, also has a protonated Schiff base.[61] The kinetic isotope effect of the picosecond absorption data thus implies that deprotonation occurs at a site within the protein other than the Schiff base, unless there has been a Schiff base deprotonation during formation of the electronically excited state that is restored upon relaxation to K_{590} or bathorhodopsin.

The resonance Raman spectrum of a picosecond transient[62] in the retinal conformation fingerprint region is different from that of the unphotolyzed bacteriorhodopsin. This suggests that the change of conformation takes place in a time shorter than 40–50 ps, the pulse width of the laser used. Also, an out-of-plane C–H vibration showed broad scattering for the picosecond transient, suggesting that the change in the retinal conformation takes place to a nonplanar unrelaxed form in the 0–40-ps time scale.

Hemoglobin Dynamics

It has been known for many years that liganded hemoglobin can be dissociated by light.[63] When the ligand bound to hemoglobin is released, the hemoglobin structure undergoes a reorganization resulting in a change in reactivity that is expressed as the cooperativity mechanism.[64] The quantum efficiency of photolysis of carboxyhemoglobin (HbCO) is very high, whereas the efficiency for oxyhemoglobin (HbO_2) is much lower[65] due to rapid geminate recombination. Because of its ease of photodeligation, many studies have used the photolysis of carboxyhemoglobin as a model for hemoglobin binding processes and cooperativity. Photolysis of carboxyhemoglobin is an efficient way to generate the transient unliganded hemoglobin state, R-state deoxyhemoglobin,[66] which is also known as *rapidly reacting hemoglobin*.[65]

Picosecond absorption studies have measured the rise time of the photolysis products of oxyhemoglobin and carboxyhemoglobin to be as fast as 0.5 ps.[67] Transient absorptions, produced upon photolysis of carboxyhemoglobin with a 10-ps laser pulse, are reminiscent of deoxyhemoglobin but with shifts in frequency, intensity, and bandwidth.[68] These absorptions evolve within microseconds to the absorptions of the R state of deoxyhemoglobin (rapidly reacting hemoglobin),[69] before final relaxation to the stable T-state deoxyhemoglobin. Picosecond absorption spectroscopy has been a powerful method for the elucidation of the kinetics and mechanisms of hemoglobin deligation.[70] Structural aspects of the short-lived hemoglobin structures that give rise to these absorptions were elucidated by picosecond resonance Raman spectroscopy.

Picosecond resonance Raman spectra of the photolysis product of carboxyhemoglobin[25,26] were obtained by using low-energy pulses (5 nJ, 30 ps, 1 MHz) that

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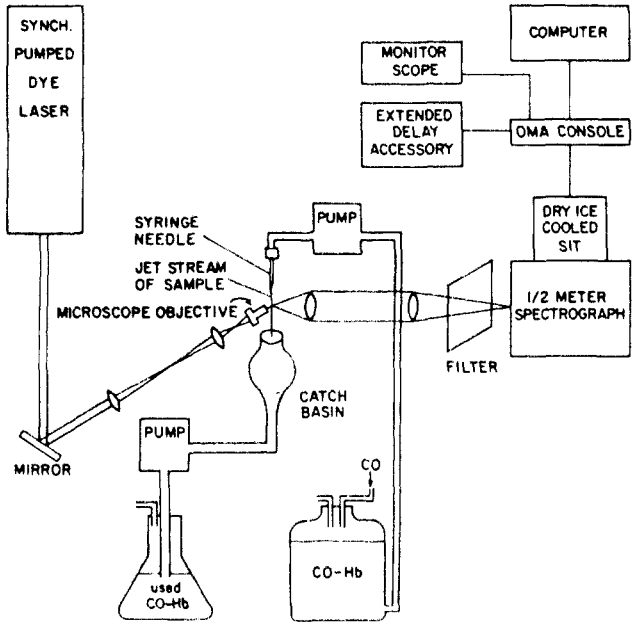

Figure 5. Experimental apparatus for picosecond resonance Raman spectroscopy of the photolysis of carboxyhemoglobin. Low powered (5-nJ) picosecond laser pulses (30 ps, 1-MHz repetition rate) were focused through a high powered microscope objective onto a flowing jet of carboxyhemoglobin. (SIT = silicon intensified target vidicon; OMA = optical multichannel analyzer). From ref 25 and 26.

were produced by a synchronously pumped mode-locked argon ion and cavity-dumped dye laser (Figure 5). The energies of the individual pulses from this laser system are low, avoiding destructive multiphoton and nonlinear processes that are frequently experienced when using picosecond lasers. Photolysis of the sample will not occur unless the laser pulses are highly focused. By focusing the picosecond pulses through a microscope objective to a one micron spot onto a rapidly flowing sample, sufficient photon flux was achieved to partially photolyze a sample with a low carboxyhemoglobin concentration. Slightly defocusing the beam resulted in a resonance Raman spectrum of essentially pure nonphotolyzed carboxyhemoglobin. Computer subtraction of a resonance Raman spectrum of unphotolyzed carboxyhemoglobin from a resonance Raman spectrum of partially photolyzed carboxyhemoglobin was used to produce the resonance Raman spectrum of the picosecond transient.

Picosecond resonance Raman spectra of the photolysis product of carboxyhemoglobin (Figure 6a) are those of a high-spin heme, resembling resonance Raman spectra of deoxyHb (Figure 6b) except for small downshifts in frequency, that persist when the pulse width is lengthened to 10 ns.[25,71] Correlations of these bands to porphyrin core size in high-spin hemes[72,73] imply that the transient state has an expanded porphyrin core that is greater than the already expanded core of deoxyhemoglobin. These frequencies are similar to those observed for a six-coordinate in-plane, high-spin Fe^{II} porphyrin.[25] The picosecond transient produced upon photolysis of carboxyhemoglobin thus appears to be a high-spin excited state ($S = 2$) of five-coordinate heme, with the Fe^{II} transiently closer to the heme plane than in stable deoxyhemoglobin.

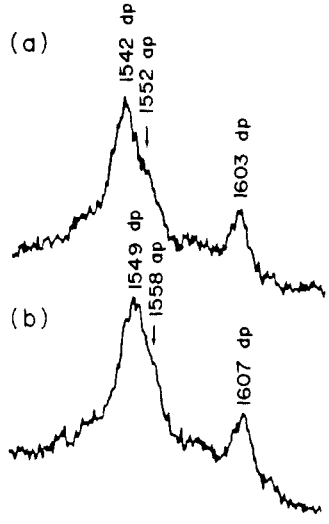

Figure 6. (a) Picosecond resonance Raman spectrum of the photolysis product of carboxyhemoglobin (30 ps, 576.0-nm excitation, 5 nJ), obtained with the apparatus of Figure 5. The resonance Raman spectrum of the picosecond photoproduct showed significant downshifts in frequencies relative to the resonance Raman spectrum of deoxyhemoglobin (b) giving evidence for an expanded porphyrin core. (b) Resonance Raman spectrum of deoxyhemoglobin with 576.0-nm continuous-wave excitation (10 mW). From ref 25.

Whereas picosecond absorption studies of carboxyhemoglobin have shown a single transient developed within 10 ps which persists for at least 680 ps,[68] two distinct picosecond transients have been reported for photolyzed oxyhemoglobin.[74] Within 90 ps the absorption of oxyhemoglobin is similar to the picosecond absorption of carboxyhemoglobin which in turn resembles the absorption of deoxyhemoglobin. However, within 10 ps the oxyhemoglobin absorption is broader and shifts in frequency.

Picosecond resonance Raman spectra of the photolysis products of oxyhemoglobin were obtained with the synchronously pumped argon ion and dye laser system described above,[75] as well as a mode-locked Nd:YAG laser.[27,29] With the 50-ps low-energy pulses of the synchronously pumped system, the resonance Raman spectra were similar to those of the picosecond photoproduct of carboxyhemoglobin.[25] However, with use of the higher energy pulses generated by the Nd:YAG laser,[29] the resonance Raman spectrum of the transient showed large downshifts, 10–15 cm^{-1} lower than the corresponding bands in the resonance Raman spectrum of deoxyhemoglobin. These shifts appeared to be too large for further porphyrin core expansion; but large downshifts had been observed,[76,77] reflecting electron donation into the lowest unoccupied porphyrin orbital, e_g^*. The early oxyhemoglobin phototransient therefore appears to be a π–π^* excited state, possibly a triplet state formed by dissociation of triplet O_2 from photoexcited oxyhemoglobin.

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The contribution of resonance Raman spectroscopy to the elucidation of the structure of the hemoglobin active site has been significant. Its application to the kinetic aspects of the hemoglobin cooperativity mechanism is growing rapidly. One area of interest is the dynamics of the iron–imidazole bonds of the α and β subunits.[78] These bonds have been observed to undergo significant changes in energy on the microsecond[55] and nanosecond[79] time scales. The iron–imidazole linkage appears to be how movement of the iron atom in and out of the porphyrin plane is communicated to the polypeptide. More work on this aspect of the cooperativity mechanism will appear in the future.

Additional Applications

Some of the early applications of time-resolved Raman involved studies of transient-free radicals generated by pulsed radiolysis.[80] Other studies used stopped-flow flash photolysis or electrochemistry. Species under study have included the *p*-terphenyl anion radical,[80] the phenoxyl radical,[81] the 1,4-dimethoxybenzene and diazabicyclooctane cation radicals,[82] and the $(SCN)^{-\cdot}$ radical anion.[83] The photoreaction of excited $Ru(bpy)_3^{2+}$ with methyl viologen dication to produce the methyl viologen cation radical[84] has interested solar energy researchers. Resonance Raman spectra of the metal-to-ligand charge-transfer state of $Ru(bpy)_3^{2+}$[85] give evidence for a free radical $[Ru^{III}(bpy)_2(bpy^{-\cdot})]^{2+}$ configuration.[86] Excited-state electron transfer has been observed from *fac*-$ClRe(CO)_3(bpy)$.[87]

The lifetimes of triplet states make them very amenable to study by time-resolved resonance Raman spectroscopy.[88] The resonance Raman spectra of some molecules in excited triplet states have been obtained in considerable detail. Examples include the lowest excited triplet states of anthracene,[89] diphenylamine,[90] acridine and anthracene,[91] naphthalene,[92] and *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.[93] The triplet state of β-carotene exhibits an anomalous lowering of C–C bond energies relative to the ground state.[94,95] Vibrational frequencies of the triplet state of chrysene have been observed in hexane[11,96] and aqueous micellar solutions.[97] Triplet quinoxaline has been observed to undergo a sequence of protonation reactions.[98]

In studies of reaction mechanisms, resonance Raman spectra of new intermediates not easily seen by transient absorption measurements have been observed during the photo-Fries isomerization of phenyl acetate.[99] The *trans*-stilbene radical anion has been identified as a short-lived intermediate in the photolysis of the *trans*-stilbene dianion[100] and has also been observed during electron-transfer quenching of singlet *trans*-stilbene by tertiary amines.[101] The S_1 state of *trans*-stilbene has been observed in a pulse-probe experiment.[14] Resonance Raman spectra have been reported for the intermediates formed in the reaction of $[Fe^{II}(EDTA)]^{2-}$ and $[Fe^{III}(EDTA)]^{-}$ with superoxide and hydrogen peroxide[102] and of the reaction of Cr(VI) with H_2O_2 in acid solution.[103]

Using mixing techniques, it has been possible to observe resonance Raman spectra of the enzyme substrate complex 4-amino-3-(nitrocinnamoyl)-α-chymotrypsin,[104] compound II of horseradish peroxidase,[105–108] and ferryl myoglobin.[109] Pulse radiolytic reduction of the alkaline form of cytochrome *c* has produced a transient state with a shifted stretching mode of the outer most C–C bonds in the porphyrin pyrrole rings.[110] In addition to time-resolved resonance Raman studies, flash photolysis with infrared detection has been used to obtain vibrational spectra transients. Examples are the reactions of metal carbonyls[111] and an excited singlet of methylene[112] as well as laser-initiated explosions in the gas phase.[113–115]

Picosecond resonance Raman spectra have been reported for the photoproducts of bacteriorhodopsin[116,117]

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and rhodopsin,[28] indicating that a retinal conformational change has already taken place within picoseconds. The resonance Raman spectrum of the triplet excited state of *all-trans*-retinal[118] shows evidence for increased π-electron delocalization as compared with the ground state and indicates the possibility of an all-trans or 9-cis configuration. A pulse and probe type of experiment has been used to study the isomerization of stilbene on the picosecond time scale.[30]

Concluding Remarks

Various types of vibrational spectroscopy such as Raman and infrared absorption have long been some of the principal methods for the determination of molecular structures. By monitoring vibrational spectra as a function of time, it is becoming possible to gain a direct picture of the changes that occur during the course of a chemical reaction. The selectivity and enhancement of the resonance Raman effect, and the time-resolution available through the use of laser excitation, have allowed the elucidation of details of photophysical and photochemical processes in complex chemical and biological systems on timescales as short as picoseconds. Future developments in the area of time-resolved resonance Raman spectroscopy will depend upon new innovations in technology as well as the ingenuity of the individual investigators. With the coming improvements in techniques and instrumentation, the contributions of time-resolved resonance Raman spectroscopy to all areas of chemistry should be considerable.

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