Laser Spectroscopy: Probing Biomolecular Functions

Biochemists want to know everything about the structure and function of biological molecules. This has proved difficult for some molecules because the processes in which they participate happen too quickly to be measured by conventional techniques; or because the molecules are very complicated; or because they are located in a complex matrix, such as a membrane, that is required for their function.

The development of lasers has given researchers a new tool with which to tackle these problems. Two techniques promise to be especially useful. They are picosecond spectroscopy, which can be used to follow very fast reactions, and laser resonance Raman spectroscopy, which can probe specific structural changes in complex molecules without interference from surrounding structures.

The main hindrance to application of these techniques—besides cost—is lack of commercial instruments. At present, investigators have to assemble their own experimental devices. Although the components are available commercially, a great deal of expertise in physics and engineering is still required. For this reason, the use of the techniques is now somewhat limited. The biochemical problems that could benefit from their application, however, are numerous.

Photosynthesis and vision are two examples of the kinds of problems that can be studied by picosecond spectroscopy. Among the investigators studying them are Peter Rentzepis and his colleagues at Bell Laboratories (Murray Hill, New Jersey) and Robert Alfano of the City College of New York. The Bell group is collaborating on the photosynthesis work with P. Leslie Dutton and John Leigh of the Johnson Research Foundation of the University of Pennsylvania.

In photosynthesis, visible light energy is converted to chemical energy. Despite much progress, the mechanism of this energy transduction is only partially understood. It is known that most of the light-absorbing pigments—the chlorophylls and accessory pigments—act as antennas. They absorb the light energy and transmit it to a specialized chlorophyll that acts as an energy trap. Excitation of this molecule causes it to lose an electron to the "primary acceptor," which has not yet been identified, and eventually results in the generation of chemical energy.

Investigators think that the lifetime of the excited state, that is, the one responsible for transfer of the electron, is very short—on the order of 10 picoseconds. Thus, in order to study the early events of photosynthesis, investigators need a light source that can generate pulses of light with a duration no longer than the events they wish to measure and also a clock that can measure in picoseconds. This is just what picosecond spectroscopy with lasers can accomplish.

The mode-locked laser is capable of generating extremely intense light pulses with a duration of picoseconds. Rentzepis, Dutton, and Leigh are using this type of laser to study light absorption by reaction centers isolated from the photosynthetic bacterium *Rhodopseudomonas sphaeroides*. Reaction centers are subcellular preparations containing the active site or energy trap of photosynthesis. The reaction centers from this bacterium contain bacteriochlorophyll, bacteriopheophytin, a ubiquinone, and a nonheme iron protein (a protein with an iron but not a heme moiety).

Measurement of Chlorophyll Bleaching

Energy transfer from the pheophytin to the chlorophyll excites the latter molecule. As a result the chlorophyll is bleached and it loses an absorption band at 865 nanometers. The Bell group has measured the rate at which this band disappears.

The light source for their experiments is a neodymium glass laser that emits light with a wavelength of 1060 nanometers; a process known as frequency doubling allows the generation of light with a wavelength of 530 nanometers. The investigators excite the pheophytin (pheophytin is an accessory pigment with an absorption band at 535 nanometers) in the reaction centers with a single pulse of the 530-nanometer light.

A clock capable of measuring in picoseconds is needed to measure the kinetics of the chlorophyll bleaching. The clock devised by the Bell investigators consists of a series of interrogating picosecond pulses with a wavelength of 864 nanometers. They generate the 864-nanometer light from a portion of the original 530-nanometer pulse by allowing the pulse to interact with water. Alfano and Stanley Shapiro, of the Los Alamos Scientific Laboratory, showed that this produces a pulse of light having a continuum of wavelengths. Light of the desired wavelength—here 864 nanometers—was filtered from the continuum.

This pulse was divided into a series of pulses by transmitting it through what Rentzepis calls an echelon. An echelon is a stepped delay consisting of a stack of plates, fibers, mirrors, cut glass, or metal blocks. The size of the steps, which are equal, determines the separation between pulses. This procedure provides a clock, the period of which can be varied from 1 picosecond to more than 50 picoseconds by varying the step size.

In order to measure the kinetics of chlorophyll bleaching, the Bell investigators adjusted the optical paths of the exciting and interrogating pulses so that arrival of the exciting pulse in the sample coincided with that of the seventh in the series of twelve interrogating pulses. The unexcited sample absorbed all of the 864-nanometer interrogating pulses, but the bleached sample transmitted pulses with that wavelength. Thus the first six interrogating pulses were absorbed; the seventh was partially transmitted, and the eighth was completely transmitted, as were the remaining four. Since each interrogating pulse segment lasted 6 picoseconds, chlorophyll bleaching required more than 6 picoseconds but less than 12.

The Bell investigators devised a variation of this procedure that allows them to measure the absolute change in the absorbance by molecules participating in very fast reactions. The variation involves splitting the train of interrogating pulses into two; one train passes through the sample where it may be absorbed; the other serves as a reference. The amount of light absorbed can be easily determined. With this system, it is possible to get structural information by measuring the change in absorbance at different wavelengths at a constant time.

For example, Rentzepis, Dutton, and Leigh observed changes in the absorption spectrum of excited bacterial reaction centers that indicated that both the chlorophyll and pheophytin play a role in the excited state. The persistence of the spectral changes, even when electron transfer to the primary acceptor was blocked, led the investigators to believe that they were observing one of the primary events of energy transduction. Under these conditions the participating molecules cannot progress to more advanced stages of the reaction.

Alfano, with William Yu and Philip Ho, also at City College, is investigating by picosecond spectroscopy the transfer of energy from antenna pigments to the reaction center chlorophyll of spinach chloroplasts. Higher plants have a more complicated photosynthetic machinery than do bacteria. The former have two photosystems, whereas the latter have only one. The two photosystems (PS1 and PS2)

have different functions, and each has its own reaction center.

These investigators also use a mode-locked neodymium glass laser as a source of picosecond light pulses. The laser beam is split into two parts. One undergoes frequency-doubling to give pulses of 530-nanometer light for excitation of fluorescence in chlorophyll. The 1060-nanometer pulses are used in conjunction with an optical Kerr gate to allow measurement of fluorescence at various times following excitation. The Kerr gate blocks transmission of the fluorescence from the sample to the detector until it is opened for 8 picoseconds by arrival of the 1060-nanometer pulse. The time of arrival can be adjusted as desired.

When Alfano and his colleagues measured the time dependence of the fluorescence emitted by a preparation of PS1 from spinach chloroplasts following excitation by a 5-picosecond pulse, they found that the emission had a lifetime of 60 picoseconds. The one emitted by a preparation of PS2 had a lifetime of 200 picoseconds. These values agree with those the investigators obtained in more recent experiments with whole chloroplasts. The fluorescence of the antenna pigments of the whole chloroplasts decayed in a double-exponential manner. The City College investigators again identified one component with a lifetime of 60 picoseconds and another with a lifetime of 200 picoseconds. Alfano thinks that these results are consistent with the hypothesis that excitation energy spreads through the pigments of the photosystem to the energy trap by means of a resonant dipole-dipole energy transfer.

The process of vision resembles photosynthesis in some ways. In vision, light energy is also absorbed by a pigment, rhodopsin; here, however, the light energy is converted to the electrochemical energy needed for a neural response. Rhodopsin, which consists of a chromophore (11-*cis* retinal) complexed with a protein (opsin), is located in highly organized membranous structures within the light-sensitive cells, the rods and cones. Because of the work of George Wald of Harvard University and many others, investigators know that, following excitation, the *cis* form of retinal isomerizes to a *trans* form, which eventually separates from the opsin. As a result of these changes, a neural response is generated. Neither the primary chemical events following excitation nor the links between the chemical events and the neural response are well understood.

Researchers studying vision have thought for some time that the first intermediate formed following excitation of rhodopsin is a compound called prelumirhodopsin. The evidence for its formation is the appearance of a new absorption band at 560 nanometers and disappearance of the 506-nanometer band characteristic of rhodopsin. Appearance of the 560-nanometer band is extremely rapid and, until recently, had only been studied at low temperatures. Now, evidence from both picosecond and resonance Raman spectroscopy appears to confirm the hypothesis that prelumirhodopsin is the first intermediate formed and also suggests that its formation is not due to a *cis-trans* isomerization. That apparently occurs later.

Picosecond spectroscopy permits observation of prelumirhodopsin formation at room temperature. Rentzepis found that the intermediate absorbing at 560 nanometers appears in 6 picoseconds or less after excitation and has a lifetime of about 30 nanoseconds. He says that the rapid appearance of prelumirhodopsin supports the hypothesis that this species is the primary product of visual excitation. Rentzepis thinks that the speed of prelumirhodopsin formation is incompatible with a major structural change such as the complete isomerization of the *cis* isomer to the *trans* isomer.

Resonance Raman Spectroscopy

Resonance Raman spectroscopy provides a method for gleaning structural information about biological molecules that contain chromophores. A regular Raman spectrum contains peaks of light scattered by molecules in the sample. The frequencies of the scattered light are shifted with regard to that of the incident light. Since the frequency shifts correspond to the vibrational frequencies of the scattering molecules, they are a source of information about the structure of the molecules.

When the wavelength of the illuminating light lies within an electronic absorption band of the scattering molecule, the intensities of some of the scattered bands will be greatly increased. This is resonance enhancement. Because enhancement requires an interaction between the vibrational modes and the electronic transitions, the technique is limited to molecules containing chromophores. Nevertheless it is both sensitive and selective and can be applied to the study of a large number of important proteins and enzymes—including hemoglobin, the cytochromes, photosynthetic reaction center proteins, and rhodopsin—that have such chromophores at their active sites. It is even possible to use the technique to study events in intact cells because water, the biological solvent, has a very weak Raman spectrum and does not interfere with the measurements. The availability of the tunable laser means that the investigator can select a wavelength that exactly coincides with an absorption band of the chromophore of interest.

Aaron Lewis of Cornell University says that his resonance Raman studies of rhodopsin suggest that the retinal is attached to a lysine residue of opsin through a protonated Schiff base. Moreover, the data provide information about the structural changes occurring in the retinylidene chromophore (the name for retinal when it is complexed with opsin) following excitation. They suggest that electron delocalization in the chromophore is a primary result of light absorption. After this delocalization, which occurs in picoseconds, there is a relocalization of the electron density within a millisecond. At the end of this process, the proton is released from the Schiff base. Lewis says that the time required for the proton loss is similar to that required for generation of the neural response.

Generation of neural responses can best be studied in intact photoreceptor cells. Lewis has obtained resonance Raman spectra of good quality from rods in the eyes of live albino rabbits. (Albinos must be used because normal animals have a layer of pigmented epithelium behind the retina that interferes with the measurements.) The results were similar to those from the previous experiments. By placing electrodes at appropriate locations on the animals, Lewis could record electroretinograms, which register the electrical activity of the retina, at the same time as he recorded the resonance Raman spectra. This showed that certain spectral changes occurred only when there was a physiological response.

Data obtained by Robert Callendar at the City College of New York support the hypothesis that the linkage between retinal and opsin is a protonated Schiff base. He found a peak in the resonance Raman spectrum of rod vesicles in the position expected for such a peak. Treatment of the vesicles with deuterium ions shifted the peak to the position predicted for it if a deuteron replaces the proton of the Schiff base. He also says that the data are inconsistent with the hypothesis that prelumirhodopsin formation occurs by means of a simple *cis-trans* isomerization.

Although this article has focused on investigations of photosynthesis and vision by laser picosecond spectroscopy and resonance Raman spectroscopy, the techniques should be equally valuable for studying other important biological problems. For example, Thomas Spiro of Princeton University is using resonance Raman spectroscopy to study hemoglobin and other heme proteins. As the instruments and methods become more available to biologists, they will no doubt shed new light on the functioning of biological molecules.

—JEAN L. MARX