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Investigations of Ultrafast Dynamics of Photoexcited Heme Proteins and Iodomethane in Condensed Phase Using Transient Raman and Absorption Spectroscopies.

Huiping Zhu
Louisiana State University and Agricultural & Mechanical College

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Investigations of ultrafast dynamics of photoexcited hemeproteins and CH₃I in condensed phase using transient Raman and absorption spectroscopies

Zhu, Huiping, Ph.D.
The Louisiana State University and Agricultural and Mechanical Col., 1993
INVESTIGATIONS OF ULTRAFAST DYNAMICS OF PHOTOEXCITED HEMEPROTEINS AND CH$_3$I IN CONDENSED PHASE USING TRANSIENT RAMAN AND ABSORPTION SPECTROSCOPIES

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Chemistry

by

Huiping Zhu
B.S. Shandong University, P. R. China, 1982
M.S. Institute of Chemistry, Academia Sinica, P. R. China, 1985
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First of all, I am very grateful to God. Although he lets the bad thing happen in my body, I thank him for giving me a test and a lesson. In this way he lets me know his existence and believe in him so that I can open my heart and let the lord in. I am very happy to accept Jesus Christ as my savior. From then on I start to feel more peaceful, satisfactory, and joyful for my life. I will follow him and do as he pleases. I am praying that God will live with me and bless me forever.

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ABSTRACT

This dissertation describes the development and application of transient Raman and absorption spectroscopies using 8 ps laser pulses for the study of photoexcitation dynamics of hemeproteins and CH₃I in the condensed phase.

Transient Raman spectroscopy is a powerful ultrafast technique. Hemeproteins have been studied using this technique. The dynamics of vibrational cooling, geminate recombination, and structural change upon the photoexcitation of hemeproteins have been unambiguously separated. The vibrationally hot deoxyhemoglobin, oxyhemoglobin and photoproduct deoxy-liked hemoglobin have been directly detected. The deconvolved time constant of vibrational cooling for the species is about 2-5 ps. Direct evidence for the lack of geminate recombination and structural change occurring on a 2-100 ps time scale has been shown. The slow geminate recombination between the heme and ligand has been observed; the time constant is about 1000±500 ps. The reaction coordinate for ligand rebinding has been addressed.

Transient absorption spectroscopy is another useful technique. For the first time, CH₃I has been studied in solution using this technique. The dynamics following the photodissociation of CH₃I have been observed. The dynamics consist of two components. The fast recovery is assigned to the vibrational cooling of hot CH₃ and the deconvolved time constant is 14 ps, 25 ps, and 27 ps in cyclohexane, heptane, and hexane, respectively. The vibrationally hot CH₃ directly couples with
the lower-lying vibrational modes of the solvent molecules and the relaxation pathway is most likely by \( V \rightarrow V \). The geminate recombination between \( \text{CH}_3 \) and I is too fast to be detected using our 8 ps laser pulse. The geminate recombination of \( \text{CH}_3 \) and \( \text{I}^* \) is too slow to affect the observation of vibrational cooling dynamics of \( \text{CH}_3 \). Other reactions are responsible for the slow recovery dynamics.
1. INTRODUCTION

1.1 Ultrafast Phenomena in Condensed Phase

In this section, I will introduce some ultrafast phenomena, which are related to the experiments in our laboratory.

First, let us consider the simplest photochemical reaction as follows:

\[ A_2 \xrightarrow{h\nu} A + A \]  

(1.1)

The first question scientists will ask is how fast the photodissociation reaction is; the next question is what will happen to the two photofragments following the photolysis. The answer to these interesting questions is relevant for understanding microscopic solvent dynamics.

This kind of reaction in gas phase has been well-studied with the molecular beam technique. Theoretical studies have also established very good models which are in excellent agreement with the experimental results. However, if this reaction occurs in the liquid, what can be predicted in comparison with the gas phase? Can we understand how the liquid solvent molecules affect the reaction?

The high frequency of intermolecular collisions is one of the main features of a liquid. When a solute molecule is dissolved into the liquid, the first thing to occur is the collision between the solute molecule and the solvent molecules. There is no
doubt that the collision will strongly influence the chemical reaction. However, the question is how collisions affect the reaction.

One solute molecule is tightly surrounded by millions of solvent molecules. Thus, there is a potential formed between the solute and the solvent molecules. This potential will control the motion of the solute molecule. One factor of this potential is the so-called “solvent cage effect”, which is strongly dependent upon the viscosity, dimension, and mass of the solvent as well as the properties of the solute molecule. When a molecule $A_2$ absorbs some energy and dissociates into two fragments, $A + A$ in the solvent, the radicals can either stay in or escape from the solvent cage. The fate of radicals is contingent upon how strong the solvent cage is. The solvent cage is partially dependent on the viscosity of the solvent. The photofragments can usually travel less distance in a solvent of a high viscosity and therefore, have less probability for escaping the solvent cage in high viscosity solvent. Although there is no directly supporting experimental data, current thinking suggests that the translational energy carried by the radicals does not have much effect on the solvent cage because one or two collisions will quickly thermalize the translational energy of the radicals\(^{(1)}\). Therefore, the two radicals cannot travel much in the liquid and the solvent cage will force them to recombine in a very short time. This recombination is called “geminate recombination”, which usually occurs on an ultrafast time scale.

Vibrational energy relaxation is an important process in solution. With each solvent collision there is a certain probability that energy will exchange between
molecules. The process possibly involves a translational, electronic, vibrational, and/or rotational energy transfer between the solute and the solvent. How an electronically excited molecule transfers its excess energy to the solvent molecules is still not clear. A question which arises, then, is whether or not the molecule is vibrationally hot following the electronic cooling. If it is, what is the vibrational temperature? How fast does the vibrationally hot molecule transfer its excess energy to the solvent environment and by what mechanism does this transfer occur?

If a solute molecule dissociates in solution, another possibility is that the solvent molecules can quickly form a new solvent-solute bond. Following this the solvent molecule may reorient such that the bonding occurs with other functional groups of the solvent molecule. In this way, even more stable solvent-solute bonds can occur by rotation of the solvent molecule. Whether or not these phenomena exist is still waiting for the further evidence.

1.2 Ultrafast Spectroscopies and Applications

Ultrafast spectroscopies are powerful techniques for the study of ultrafast reaction dynamics in the condensed phase. Today, many of the ultrafast spectroscopists use transient absorption spectroscopy. This technique is based on the simple Lambert-Beer law. The major advantages of this technique are high sensitivity and easy application.

In a transient absorption experiment two different color laser beams are usually used, one of which is the pump beam and the other is the probe beam. The pump beam is used to provide energy to the solute molecule; the probe beam is used to
observe what will occur as soon as the molecule absorbs the energy. If the molecule dissociates or produces a new species, the absorption signal will change because the absorption coefficients at the probe wavelength for the parent molecule are different from those of the transient species. In the transient absorption spectrum the absorption band of the original molecule will show depletion and, at the same time, a new positive absorption band due to the transient will be detected. When the evolution of transient absorption with time is observed, the information about dynamics of the transient species can be obtained. The transient absorption studies of CH$_3$I will be shown in chapter 4.

There are many cases in which two or three processes can occur on the same time scale. For example, following the photoexcitation of oxyhemoglobin, ligand rebinding, vibrational cooling, and structural change may all occur on the picosecond time scale. In this case the transient absorption technique is at a disadvantage because it is difficult to separate these processes using absorption data alone. In order to solve this problem scientists use Raman spectroscopy. Raman scattering can directly give information on vibrational levels and is therefore, much more powerful than absorption spectroscopy. The transient Raman spectroscopy developed in our group provides an ideal tool for separating the various processes occurring on the same time scale. We can measure both Stokes and anti-Stokes spectra. In chapters 2 and 3, I will show the application of transient Raman spectroscopy in hemeproteins.
Transient Raman spectroscopy is also a powerful technique for the study of solute-solvent interaction. For example, solvation and solvent reorientation can be observed. This is because these processes can affect the electron cloud around the solute molecule which results in changes of vibrational frequency. The measurement of Raman shifts with time will give information about how fast the solvation and/or solvent reorientation take place. How much the Raman band shifts will show how strong the interaction between the solute and the solvent molecules is. The investigations of this interaction between solute and solvent molecules have been shown in our Cr(CO)₆ work.²⁻³

1.3 Raman Spectroscopy

1.3.1 Raman Scattering

The Raman effect arises from the interaction of the incident light with the electrons in the illuminated molecule. In nonresonance Raman scattering, the energy of the incident light is not sufficient to excite the molecule to a higher electronic level. Instead, Raman scattering results in changing the molecule from its initial vibrational state to a different vibrational state (Figure 1.1).

In order for a molecule to exhibit the Raman effect, the incident light must induce a change in polarizability. The change in polarizability can be qualitatively visualized as a change in the shape of the electron cloud. An electron with certain
Figure 1.1: The energy diagram of Raman scattering.
vibrational modes can couple with the incident photon and lead to scattered pho­
tons with altered frequency. Most of the scattered light is simply Rayleigh scatter­
ing. Only a small fraction of photons are scattered by Raman scattering, so Raman
lines are usually very weak (only $10^{-6}$ of the intensity of the Rayleigh line). When
photons interact with a molecule, some of the energy can be converted into various
modes of vibrations of the molecule. As seen in Figure 1.1, the scattered light loses
energy equivalent to the energy given to molecular vibrations. This is the Stokes
Raman effect. If the energy is transferred to the scattering light from a molecule,
then the scattered light has more energy than the original incident light. This is the
anti-Stokes Raman effect which requires that the scattering molecule already be in
an excited state. Because most of the population is generally in the $v = 0$ level,
Stokes Raman scattering is much stronger than anti-Stokes scattering.

Raman scattering in Stokes and anti-Stokes has been described qualitatively
above; the theoretical result is presented here in brief. If a molecule interacts with
light, the electric field of photons will exert oppositely directed forces on the elec­
trons and the nuclei. As a result, the electrons will be displaced relative to the
nuclei, and the polarized molecule will have an induced dipole moment caused by
the external field. The induced dipole moment $P$ is proportional to the electric field
$E_0$ and the polarizability $\alpha$.

$$P = \alpha E_0 \cos 2\pi \nu_0 t$$  \hspace{1cm} (1.2)
In this equation, $\nu_0$ is the frequency of the incident light and $t$ the time. The polarizability $\alpha$ depends upon the position of the nuclei in the molecule. Instantaneous positions of the nuclei can therefore be expressed relative to their equilibrium positions in terms of the normal coordinate $Q_i$, where $i = 1, 2, ..., 3N-6$. Considering a diatomic molecule with the single normal coordinate $Q_1$, the dependency of $\alpha$ on $Q_1$ is expressed as a series expansion

$$\alpha = \alpha_0 + (\partial \alpha / \partial Q_1)_0 + ... \quad (1.3)$$

where $\alpha_0$ is the equilibrium value of the polarizability. The position of the nuclei is time dependent because the molecule is vibrating with frequency $\nu_1$. Information on the frequency of vibration can be obtained from knowledge of the forces between the vibrating nuclei, and the application of the classical mechanics of small vibrations. This motion can be expressed as

$$Q_1 = Q_1^0 \cos 2\pi \nu_1 t \quad (1.4)$$

where $Q_1^0$ is the maximum vibrational amplitude. It is seen, therefore, that $\alpha$ also oscillates at the frequency $\nu_1$. Finally we have

$$P = \alpha_0 E_0 \cos 2\pi \nu_0 t + 1/2 E_0 Q_1^0 (\partial \alpha / \partial Q_1)_0 [\cos 2\pi t(\nu_0 + \nu_1) + \cos 2\pi t(\nu_0 - \nu_1)] \quad (1.5)$$
This classical derivation for a diatomic molecule predicts three basic light-scattering modes due to the induced dipole moment $P$ oscillating at frequency $\nu_1$:

1. The $\alpha_0$ term produces scattered light unshifted in frequency (Rayleigh scattering).

2. If $\partial \alpha / \partial Q \neq 0$, Raman scattering occurs, and incident light of frequency $\nu_0$ is shifted to scattered light with higher frequency $\nu_0 + \nu_1$ (anti-Stokes) and lower frequency $\nu_0 - \nu_1$ (Stokes).

For a nonlinear polyatomic molecule containing $N$ atoms, there are $3N-6$ vibrational modes. In this case, the induced dipole is given by

$$P = E_0 \alpha_0 \cos 2\pi \nu_0 t + 1/2 \sum [E_0 Q_i^0 (\partial \alpha / \partial Q_i)_0] [\cos 2\pi (\nu_0 + \nu_i) t + \cos 2\pi (\nu_0 - \nu_i) t]$$

(1.6)

The first term refers to Rayleigh scattering (elastic), the second term to anti-Stokes lines, and the third term to Stokes lines. The fundamental difference between Raman and infrared spectroscopy is that $\partial \alpha / \partial Q$ does not equal zero for Raman scattering. This means that a molecule must have a change in its polarizability as it vibrates in order to be Raman active.
1.3.2 Resonance Raman Spectroscopy

When a molecule is excited with incident light whose frequency is within an electronic absorption band, the intensity of some Raman spectral lines is greatly enhanced. The effect is called resonance Raman enhancement, and is due to a coupling of electronic and vibrational transitions.

The resonance Raman effect is a very important spectral phenomenon. The enhancement strongly affects the Raman spectrum and therefore, must be considered in the analysis of the spectrum. In chapter 2 and 3, I will show that oxyhemoglobin (oxyHb), deoxyhemoglobin (deoxyHb), and carbonylhemoglobin (carbonylHb) have different resonance Raman enhancement at several different vibrational bands. The different resonance Raman effect for the three hemeproteins strongly influences the observed dynamics.

The theoretical description of resonance Raman effect is well understood\(^{(4-6)}\). The following is a qualitative description:

In resonance Raman scattering, the energy of the exciting laser beam coincides with that of an electronic transition. When the energy of the laser is close to, but not higher than an electronic excitation level of a molecule, the process is called pre-resonance Raman scattering. In normal resonance Raman scattering, the energy level of the virtual state falls far below that of the electronic transition.

The components \(\alpha_{qr}\) of the polarizability tensor can be calculated by a semiclassical treatment of the interaction of the molecule and the incident-radiation
Figure 1.2: Diagrams of resonance Raman scattering. In the resonance Raman effect, the energy of the excitation wavelength reaches the energy level of the excited electronic state.
field. The reason for the enhancement can be seen in a theoretical quantum-mechanical expression for the Raman-scattering tensor elements $\alpha_{qr}$, which are proportional to the scattered-light intensity. The quantum-mechanical expression for the $\alpha_{qr}$ is

$$\alpha_{qr} = \frac{1}{h} \sum \left[ \frac{(M_r)_{me}(M_q)_{en}}{(\nu_e - \nu_0 + i\Gamma_e)} + \frac{(M_q)_{me}(M_r)_{en}}{(\nu_e + \nu_s + i\Gamma_e)} \right]$$ (1.7)

where q and r refer to the coordinate directions x, y, or z, $\nu_0$ is the frequency of incident radiation, $\nu_e$ is the frequency of the transition between the ground and the excited electronic state, $\nu_s$ is the scattered (Raman) frequency, and $i\Gamma_e$ is a damping term related to the width of the excited state $\nu_e$ (the bandwidth of the electronic transition). The initial and the final states of a molecule are represented by m and n, respectively, and the $(M_r)_{me}$ and $(M_q)_{en}$ are electric dipole transition moments along the directions of r and q from the initial state of m to an excited state e and from e to the final state n. The term $h$ is Plank's constant. The first term of the equation describes a transition involving an absorbed photon and subsequently an emitted photon, whereas the second term describes the emission of a scattered photon followed by absorption of a photon from the laser light field. The first term is the resonant term. When the incident laser frequency $\nu_0$ is chosen near the excited electronic transition $\nu_e$, the denominator of this term $(\nu_e - \nu_0 + i\Gamma_e)$ becomes very small, so that the polarizability element becomes very large. This is why the resonance Raman effect can effectively result in an increase of the Raman cross
section by many orders of magnitude. From this equation it can also been seen that the changes of vibrational frequency $\nu_s$ can make $\alpha_{qr}$ larger or smaller. Thus, the enhancement depends on the vibrational mode.

Because of the resonance Raman effect, some of the vibrational modes of the chromophore or adjacent groups of atoms are selectively enhanced. In this way detailed selective information about the chromophore can be obtained. For instance, in the heme protein experiments described in chapter 3 we can selectively observe the dynamics of oxyHb and photoproduct deoxyHb due to the fact that oxyHb and deoxyHb have different resonance Raman enhancements at $\nu_4$ and $\nu_{16}$.

The resonance Raman enhancement is dependent on the absorption spectrum of the molecule and excitation wavelength. As the excitation wavelength comes close to the absorption maximum, the intensity of the resonance Raman line increases. Generally speaking, to achieve a maximum enhancement the laser excitation wavelength should be close to the absorption maximum. In the heme protein experiment we used 355 nm and 532 nm laser lines, which are close to the N-band and Q-absorption band, respectively. Therefore, we can detect the very strong transient Raman signal from the heme proteins. The effects of resonance Raman enhancement on the heme proteins will be described in detail in chapter 3.

1.3.3 Transient Raman Spectroscopy

As mentioned above, the Raman signal is very weak in comparison with the absorption signal. Typical cross sections\(^{(4)}\) for nonresonant Raman scattering are on the order of $10^{-13}$ Å\(^2\). On the other hand, a moderate visible absorption cross
section would be on the order of $10^{-2}$ Å$^2$ (molar extinction coefficient of $10^3$). The difference in cross sections clearly illustrates that Raman spectra are much more difficult to obtain than absorption spectra. Furthermore, it seems to be impossible to obtain the normal Raman spectrum for the very small concentrations of transient species. However, it is possible to obtain such a spectrum by using resonance Raman effect, which is typically orders of magnitude stronger than non-resonant Raman.\(^{(4)}\) The strong resonant enhancement is necessary to observe the Raman signal from a low concentration of excited states.

Although a transient absorption signal is much stronger than Raman scattering, absorption data cannot distinguish vibrational processes. Therefore, many beautiful absorption data have been misinterpreted. The example of the iodine photodissociation and geminate recombination reaction has been studied in our group recently\(^{(7)}\). This reaction was first studied in solution by picosecond absorption spectroscopy\(^{(8)}\). For several years the data were interpreted as slow (\(\approx 100\) ps) recombination of the iodine radicals produced by photodissociation. Actually, the hundred picosecond event is slow vibrational relaxation\(^{(9)}\). This example shows that absorption spectroscopy cannot easily discriminate excited electronic dynamics from either excited vibrational dynamics or from molecular conformational dynamics even for the simplest condensed phase system! On the other hand, the advantage of transient Raman spectroscopy over absorption spectroscopy is that Raman spectroscopy provides direct information about vibrations, hence structure. A given
Raman shift is directly related to a vibrational motion in the molecule. The Raman band position corresponds directly to a vibrational energy spacing for one of the modes of the molecule, identifying more specifically the quantum state under observation. An absorption spectrum of oxyHb is shown in Figure 2.3. The visible absorption spectrum shown here is due only to the 73 atom heme molecule (a porphyrin ring with an iron atom in the center). The heme is the functional subunit of hemoglobin. These absorption bands do contain information about the vibrational levels of the porphyrin molecule, but that information is hopelessly lost in the smooth features of the electronic absorption spectrum. In contrast, the ground state Raman bands can be seen in Figures 3.5 and 3.6. The vibrational levels are clearly resolved in the Raman spectrum in solution. Raman spectroscopy on a picosecond time scale can directly observe processes involving the vibrational levels of the molecule. Thus, Raman spectroscopy can be used to separate the vibrational processes from other processes.

The nonlinear broadening effect is a big problem when high peak power laser pulses are used\(^{10}\). In order to obtain Raman spectra, high average laser powers are required. If the peak power is decreased, the repetition rate must be increased. This is the key point for a successful picosecond Raman spectroscopy in solution. The laser system used in these experiments\(^{11}\) was developed in order to be able to produce picosecond pulses at kiloHertz repetition rates. This enables us to get the photon flux at the sample high enough to collect good data, without driving nonlinear processes. Most previous experiments have been done at 10 to 20 Hz repetition
rates. We work at 1 to 2 kHz repetition rates. Section 2.3.3 specifically compares the powers used in our experiment with that used in a previous femtosecond Raman experiment by other workers\(^{(12)}\). Nonlinear phenomena associated with high peak powers include both resonant and nonresonant multiphoton absorption and artificial broadening\(^{(13)}\) mechanisms. High laser repetition rates are the only way to deliver the high average power necessary to do a Raman experiment in a feasible span of time while avoiding nonlinear effects. The weak Raman signals often require many hours of signal averaging. Tens of Hertz versus kiloHertz repetition rates represent the difference between hours of signal averaging to collect one spectrum versus weeks of signal averaging to collect one spectrum!

### 1.4 Intensity of Raman Scattering and Vibrational Temperature

The Stokes line has a higher intensity than the anti-Stokes line at room temperature. The Stokes line originates when a molecule at a low vibrational energy is elevated to higher energy \(h\nu_1 (\Delta\nu\text{ in Figure 1.3})\) by interacting with the incident light, whose energy is equal to \(h\nu_0\). On the other hand, the molecule at a higher vibrational-energy level gives up the energy \(h\nu_1\). The molecule becomes lower in vibrational energy, and the scattered light increases in energy by \(h\nu_1\). At low temperature (or at room temperature), more of the molecules have population in lower vibrational-energy levels (Figure 1.3). Thus, a larger fraction of molecules will have Stokes-type transitions than anti-Stokes transitions. As a result, the Stokes line will have higher intensity than the anti-Stokes line. This can be seen by examining the
Boltzmann distribution law, which states that the relative population of molecules with higher energy increases as the temperature is increased.

\[
\frac{N_i}{N_0} = e^{-\Delta E/kT}
\]  \hspace{1cm} (1.8)

where \(N_i\) is the number of molecules at energy state \(E_i\) and \(N_0\) is the number of molecules at energy state \(E_0\); \(N_i/N_0\) is the fraction of molecules at the energy state \(E_i\); \(k\) is the Boltzmann constant; \(T\) is the temperature in Kelvin scale; and \(\Delta E\) is the energy difference between energy state \(E_i\) and \(E_0\). Therefore, we can see that as the temperature is elevated, the Stokes line intensity decreases and the anti-Stokes line intensity increases. The ratio of the anti-Stokes to the Stokes line is directly related to the fraction of molecules at higher vibrational-energy levels.

It is obvious that a depletion will be observed in the difference Raman spectrum when the Stokes Raman spectrum at low temperature is subtracted from the Stokes spectrum at high temperature. On the other hand, the anti-Stokes difference Raman spectrum will show a positive band if the cold Raman spectrum is subtracted from the hot one. The negative difference signal in the Stokes spectra complemented by the positive spectra in the anti-Stokes spectra show a net result, which is related to the number of molecules excited to higher vibrational-energy levels.
At low temperature — The population of molecules at energy level \( V=0 \) is more than that of \( V=1 \).

At higher temperature — The population of molecules at energy level \( V=1 \) increases relative to that of energy \( V=0 \).

Figure 1.3: Intensity difference of Stokes and anti-Stokes effects. The relative intensity of Stokes and anti-Stokes lines varies depending on temperature.
1.5 Laser System and Raman Spectrometer

The goal of the work reported in this dissertation has been to develop uses for picosecond transient Raman spectroscopy, which has been made possible by previous laser advances in this laboratory\(^{11,14-15}\). A diagram of the apparatus is found in Figure 1.4. The laser system\(^{11,16}\) is based on a high repetition rate, chirped pulse, Nd:YAG regenerative amplifier which provides 1 mJ pulses with an 8 ps pulsewidth at 1.064 \(\mu\)m. A mode-locked cw Nd:YAG laser (output pulse is 80 ps FWHM) in combination with a single-mode fiber optic is used to produce the seed pulse to the regenerative amplifier. Self phase modulation\(^{17}\) in the fiber broadens the bandwidth of the 1.064 \(\mu\)m laser pulse; the frequency dependent index of refraction temporally broadens the pulse so that the longer wavelengths travel at the leading edge of the pulse. After amplification, the laser beam is reflected off of a grating pair. The grating pair geometry is designed to delay the longer wavelength components of the pulse while providing a shorter path length for the shorter wavelength components. The net result is that the different frequencies “pile up on top of each other,” giving a more narrow pulse than originally produced by the mode-locked laser.\(^{18}\) The process described above involves broadening the frequency bandwidth of the pulse while narrowing the time width, in accordance with the reciprocal relationship \(\Delta t \Delta \nu \sim 1\).

The configuration of the laser system reported in the references cited above has changed in two ways. A “pulse picker” using a Pockel’s cell and polarization sensitive optics has been placed between the fiber and the regenerative amplifier.
Figure 1.4: LSU picosecond Raman apparatus.
A Pockels cell is an electro-optic device which will rotate the polarization of linearly polarized light when a high-voltage pulse is applied. The pulse picker is arranged such that the linearly polarized laser pulse which enters the pulse picker will be normally lost to a beam dump. A pulse can only escape the pulse picker when high voltage is applied to the Pockels cell; in that case, the laser pulse will be reflected out of the pulse picker by the polarization sensitive optics to seed the regenerative amplifier. Pulse picking is helpful because the repetition rates of the modelocked YAG ($\approx 100$ MHz) and the YAG regenerative amplifier ($\approx 2$ kHz) differ.

The second difference is that Q-switching\(^{(19)}\) in the regenerative amplifier is now accomplished by an acousto-optic modulator; the amplified pulse is still switched out of the amplifier cavity by a Pockels cell and polarization sensitive optics. Q-switching refers to the process in which the gain of the cavity is purposely and temporarily spoiled in order to give the laser rod time to store up energy. The output of a Q-switched laser consists of intense pulses at a repetition rate determined by the Q-switching frequency. The timing between the pulse picker and the Q-switch must be carefully controlled so that the injected seed pulse arrives at the cavity just as the Q-switch is opening. As with any state-of-the-art system, this laser system needs frequent attention. Therefore, developing an ability to troubleshoot and fix the laser, and associated timing electronics is necessary for the successful graduate student.

The YAG harmonics of 532, 355, 266, and 213 nm have been used in experiments described in this dissertation. Typically, one YAG harmonic is used as the
pump wavelength, while another is used as the probe. The hemeproteins experiments were done by pumping with the 532 nm green photons and probing with the 355 nm blue photon. In the transient absorption experiment, we use 266 nm as the pump beam and 213 nm as the probe beam. The laser harmonics are generated in KDP, KTP, or BBO crystals. After harmonic generation, the colors are separated by a beamsplitter which is anti-reflection coated for one wavelength but high-reflection coated for the other. Then one color is sent down an optical delay line. Referring to Figure 1.4, we see that the delay line is simply a reflector where we can bounce the beam down and back over a carefully measured distance. Practically, this is controlled by computer using a stepper motor to drive a lead screw to which the reflector is attached. A travel distance of one mm corresponds to a travel time of 3.33 ps at the speed of light. Thus this simple method of controlling the path length traveled by the two pulses is practical for delay times from fractions of a picosecond up to several nanoseconds. For delay line distances beyond those corresponding to several nanoseconds, beam divergence becomes a prohibitive problem.

Various methods have proven practical for temporally overlapping the pulses at various wavelengths. In all of these techniques, the Raman spectrometer is used to observe a signal which a) depends on the cross correlation of the pump and probe pulses, and b) is believed to have a rise time which is practically instantaneous as compared to the laser pulsewidth. When using 532 nm as pump and 355 nm as probe, the timing between pulses can be measured experimentally using stimulated emission from the laser dye coumarin 500A in methanol. First the two beams are
overlapped on the coumarin 500A sample which might be in a spinning cell or a flowing liquid jet. This technique consists of pumping the fluorescent coumarin dye with the 355 nm or 266 nm pulse and observing the fluorescence with the monochromator set at \( \approx 4500 \) nm while the 532 nm beam is blocked. If the 532 nm beam is then allowed to hit the sample under the condition that each 532 nm pulse is arriving at the sample before the 355 nm, then there will be no change in the observed fluorescence intensity. If, however, the 532 nm pulses are arriving after the 355 nm pump pulses, then the 532 nm photons will stimulate emission at 532 nm directionally along the path of the incident 532 nm beam. This will steal intensity from the previously isotropic fluorescence and result in a reduced fluorescence signal. By scanning the time delay between the pulses, the fluorescence intensity can be traced out and time zero found. Another technique we have used is to monitor the transient absorbance of the ground state of a molecule at one color due to pumping at another color. The monochromator is usually set to observe the Rayleigh line in this method, since the transient absorbance in the sample will affect the intensity of the Rayleigh scattering.

A PDP 11/73 computer is used for data collection, monochromator control, delay stage control, and for sensing which laser colors are hitting the sample on a given laser shot. All software for data collection and experiment control and most software for data analysis were written by Prof. J. B. Hopkins in Fortran and assembly language.
Figure 1.4 also illustrates that the two laser beams which are incident on the sample can be mechanically chopped. By chopping the pump and probe beams at several hundred Hertz, we can collect four spectra in one sweep of the monochromator: pump-probe, pump-only, probe-only, and background. The pump-probe spectrum is collected when both laser beams are incident on the sample (with the desired time delay). The probe-only spectrum is the Raman spectrum observed in the vicinity of the probe wavelength when only the probe wavelength is incident on the sample. The pump-only spectrum is the spectrum which is observed in the vicinity of the probe wavelength due to the presence of the pump beam only. The background spectrum is for the case where both laser beams are blocked by the choppers. The phase of the chopper is sensed by a photodiode which "looks at" a small piece of each chopped beam. This information is used by the computer to sort the emission data from each laser shot into bins corresponding to the color combinations mentioned above. Since the chopper will sometimes partially block the beams, the computer sets thresholds for determining whether or not to use the data on a given laser shot. If one beam is partially blocked, then data from that laser shot is discarded.

The data collected when the pump-probe combination illuminates the sample may contain contributions from a) weak Raman spectra of excited states generated by the pump pulse, b) weak Raman spectra of excited states which were both generated and probed by the probe pulse, c) strong ground state Raman bands of solvent or solute molecules from the probe pulse, d) fluorescence background from the
probe pulse, e) fluorescence background from the pump pulse, and f) background noise due to the photomultiplier or electronics. The chopping and subtraction (with sufficient signal-to-noise) allow us to isolate the weak Raman spectra of excited states generated by the pump pulse and detected at the desired time delay by the probe pulse.

Most generally, we subtract the pump-only and the probe-only, and the background spectra from the pump-probe spectrum to get the transient spectrum. Frequently, we find that it is not necessary to chop the probe beam (i.e. it is not necessary to collect the pump-only spectrum). This is particularly true if we pump to the red of the Raman probe wavelength. Pumping to the red of the Raman probe wavelength is advantageous because this eliminates fluorescence from the pump wavelength in the region where we are looking for Raman scattering.* In those cases, we collect two spectra and subtract (The experiments on Cr(CO)$_6$ mentioned in chapter 5 were performed with 266 nm pump and 266 nm probe photons. Those experiments clearly required chopping both pump and probe beams.)

A pure transient Raman spectrum is obtained by using this difference spectrum technique. This is a crucial aspect of the experiment because the transient spectrum is isolated from the large signal resulting from unexcited molecules. The (pump-probe minus probe-only) spectrum is referred to as the subtracted or transient spectrum. Any feature appearing beyond the noise level in this spectrum is due to a transient species created by the pump photons and is characteristic of the

* This statement assumes that our pump intensity is below the threshold for significant multiphoton absorption.
time delay between pump and probe pulses. If photoexcitation bleaches a ground state Raman band, then the subtraction procedure will yield a negative transient band. If the pump photon creates a new Raman band or increases the intensity of an existing band, then subtraction will yield a positive transient band. It is often useful to describe a transient band by its magnitude relative to the magnitude of the corresponding probe-only band.

Notice that the time delay in this type of measurement is determined solely by the delay time between the arrival of the pump and probe pulses. If that time delay is 20 ps, then all Raman scattering in the subtracted, or transient, spectrum is due to molecules which have been relaxing for 20 ps since photoexcitation. Thus we collect one time-delay data point per laser shot. If we successively sweep through different time delays, then we can map out the time-dependence of the Raman intensity for a given spectral feature. It is not necessary to have picosecond electronics to study picosecond phenomena! Only picosecond laser pulses are necessary.

We typically focus the laser beams to spot diameters at the sample which range from ≈ 400 to 1000 μm. Beam focusing is critical to the success of an experiment. As a rule, we put as much light into the pump beam as possible and focus as tightly as possible, being careful not to create a plasma and multiphoton absorption affect. Tight pump beam focusing is desirable because this produces the maximum transient species concentration. Focusing the 355 or 266 nm laser spots too tightly can easily result in plasma production in the sample. This is evident by the onset of white light emission from the sample. The multiphoton absorption by the target
molecules can also result from the too tight focus which gives too high photon fluence on that spot. This multiphoton effect can be detected from the fluorescence, which appears at higher frequency than the laser pulse. A second general principle is that we want the probe beam spot at the sample to be the same size as, or a little smaller than, the pump spot. We do this in order to maximize the ratio of excited molecules to unexcited molecules in the probe volume.

Good spatial overlap must be achieved for the pump and probe spots. The ability to successfully overlap the beams usually takes much practice to develop. The precise time delay between pulses must be determined in order to calibrate the optical delay. The delay can be obtained to an accuracy of a few hundred picoseconds by carefully measuring the optical paths with fishing line. Then the zero of time must be found precisely as described earlier. Data and specific discussions about laser intensities can be found in the reports on individual experiments in the following chapters.

The monochromator for these experiments is a 1 meter, dispersive, double monochromator. The dispersed light is imaged onto a photomultiplier tube (PMT) at the exit slit. The PMT signal is processed by a gated integrator and stored in the computer. The sorting of the data according to which sequence of laser pulses was incident on the sample has already been described. Both frequency and time-resolved data are typically collected in many five minute scans and then added up by the computer to signal average. Each frequency or time data point typically consists of 4000 to 6000 laser shots. The total data collection time for one spectrum
is typically on the order of hours. Thus we average out shot-to-shot fluctuations of the laser pulses in addition to the slower fluctuations in laser power which may occur over the course of many minutes.
2. STUDY OF VER IN DEOXYHEMOGLOBIN

2.1 Introduction to Hemoglobins

Hemoglobin (Hb) is a well-known biomolecule. It has the remarkable ability to assume the dual role of collecting oxygen in the lungs and delivering it efficiently to the distant tissues. To accomplish this, in addition to having a variety of special properties, the molecule must bind oxygen efficiently at ambient pressures and transport it efficiently to the lower-pressure tissue regions. It is the famous sigmoid shaped relation between the degree of saturation and the oxygen pressure, and its sensitivity to pH which makes such behavior possible. The protein binds or delivers oxygen at a rate that depends on how much is already bound: this is called the cooperative binding of oxygen.

Hemoglobin\(^{(20)}\) is a 65000 molecular weight protein consisting four subunits, which are the two alpha-chains and two beta-chains. The each subunit forms a nest for one heme and each heme can bind one oxygen.

The heme is an iron porphyrin consisting of a central iron atom and 4 pyrrole rings; the 4 pyrrole rings connect each other through a -\(\text{CH}_2\)- group; one nitrogen atom from each pyrrole molecule binds with the cental iron atom; thus, the central iron atom and the four pyrrole rings together form the heme plane.

The heme is a very important unit in biochemical reaction and it also is the chromophore in photochemistry. In this experiment it is due to the heme that absorbs the photon energy that we can study the photophysical and photochemical processes of the hemeproteins using spectroscopies. The vibrational transitions
Figure 2.1: **A model of hemoglobin at low resolution.** The α chains in this model are light, the β chains darker and the heme group round dark plates. View (A) is at right angles to view (B).
Figure 2.2: A model of heme. Model of the ligand-binding site in myoglobin showing the heme group, the proximal histidine (F8), and the distal histidine (E7).
involved in resonant scattering of visible and near ultraviolet light correspond to porphyrin skeletal motions and to ligand-porphyrin motions which influence the \( \pi \)-electron structure. These in-plane vibrational modes of the heme are very sensitive to the photochemical reaction and structural changes in the hemeproteins. Therefore, we can study the hemeproteins using transient Raman spectroscopy.

In this experiment three hemeproteins, deoxyhemoglobin (deoxyHb), oxyhemoglobin (oxyHb) and carbonylhemoglobin (carbonylHb) are studied and therefore, it is necessary to point out the difference between them. (see Figure 2.2) DeoxyHb has the paramagnetic center \( \text{Fe}^{II} \) atom in the heme and one histidine ligand called the proximal histidine. However, oxyHb or carbonylHb has the sixth ligand \( \text{O}_2 \) or \( \text{CO} \) binding to the high spin center \( \text{Fe}^{III} \) atom besides those in deoxyHb. These differences in structure result in their differences in electronic states and vibrational levels. We can see these differences from their absorption spectra (Figure 3.1) and Raman spectra (Figure 3.5 and 3.6). One of the most important differences among these three hemeproteins is in photochemistry. When deoxyHb absorbs one visible photon (for example, 532 nm), no photochemistry occurs; however, the ligand \( \text{O}_2 \) or \( \text{CO} \) will be blown out following the photoexcitation of oxyHb or carbonylHb. With the photodeligation many ultrafast processes could be occur. These processes are ligand rebinding, energy transfer and structural change. Distinction of these three processes is the main purpose of our experiments.

The x-ray diffraction results for deoxyHb and the fully oxygenated form, \( \text{HbO}_2 \) show that the two structures have significant differences. This fact suggested the so-
called two-state model of hemoglobin\(^{21}\) in which the kinetics of oxygen binding is modeled by assuming the existence of just two basic protein structures, R and T, each of which can bind oxygen and whose various partially oxygenated forms can interconvert. The T form is considered to have lower affinity for O\(_2\) than the R form. The structural differences between the equilibrium Hb and HbO\(_2\) systems, which are generally regarded to have the T and R forms, involve mainly a change in the way the various components of the proteins are bound. The structural change of the T and R forms is the so-called quaternary structural change.

### 2.2 Rationale for this Experiment

Understanding the flow of vibrational energy in a protein-bound porphyrin chromophore is of both practical and fundamental significance. A spectroscopist must account for the effect of vibrational energy relaxation (VER)* in order to interpret correctly ultrafast emission or absorption data. The abundance of pico- and femtosecond spectroscopy of heme proteins in the last decade underscores the need to understand vibrational population relaxation in these systems. VER data in protein-chromophore systems may also help explain the dynamics of electron transfer in photosynthetic reaction centers. The initial electron transfer step in these membrane-bound proteins begins with absorption of a photon by a Mg-porphyrin.\(^{23}\) The rate of vibrational relaxation following photoexcitation in these systems

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* Dlott\(^{(22)}\) reserves the term VER for a transition from vibrational level to vibrational level in the molecule. Dlott then refers to the total process whereby the molecule rids itself of excess mechanical energy as vibrational cooling. Using this terminology, vibrational cooling involves many steps of VER which have the overall effect of transporting energy from the excited molecule to the heat bath. While recognizing this distinction, we often use the term VER to refer to either of the processes described above.
systems is likely similar to the electron transfer rate and thus may be influencing the dynamics. Measurements of vibrational cooling are also of fundamental interest to test computer simulations of energy flow in macromolecules. Our experimental work applies picosecond Raman spectroscopy to study photoexcited deoxyhemoglobin (deoxyHb). We directly measure the time scale for vibrational cooling of the porphyrin chromophore following absorption of a green photon.

This study is designed to investigate the dissipation of residual vibrational energy under the simplest conditions. Photolysis of ligated heme proteins can give rise to conformational changes in both the heme chromophore, the local protein monomer, and the quaternary structure of the hemoglobin tetramer. The choice of studying deoxyHb allows comparison with other workers while eliminating many spectral complications due to these conformational dynamics. Since there is no ligand, only electronic and vibrational relaxation processes occur in photoexcited deoxy hemes.

Picosecond Raman spectroscopy is the ideal technique for the study of VER. Two color pump-probe Raman spectroscopy is very sensitive to small changes in the Stokes and anti-Stokes Raman spectra caused by the presence of the pump pulse. Stokes and anti-Stokes bands come in pairs.* For a given vibrational mode, Stokes Raman data provide population information on the vibrationless level while anti-Stokes data yield population information for the low-lying excited vibrations. A vibrationally hot heme should have Stokes Raman bands which are weaker than

* see introduction for further explanation
those of an unexcited heme. Similarly, the anti-Stokes bands of a vibrationally hot heme will be more intense than those of an unexcited heme. In the transient (hot-cold) spectrum, the population changes result in a negative Stokes transient band together with a positive anti-Stokes transient band. For a given mode this is the unambiguous spectral signature of VER. This example illustrates how excess vibrational energy can clearly be distinguished from conformational changes. If a conformational change in a molecule eliminates a certain vibrational mode, then both the Stokes and anti-Stokes bands for that mode must appear as negative features in the transient spectrum of the photoexcited species. In picosecond Raman spectroscopy, only vibrational excitation will produce a negative transient on a Stokes band and a positive transient on the corresponding anti-Stokes band.

Previous work from our group\(^{(2-3,24)}\) has demonstrated the above assertion that transient Stokes and anti-Stokes Raman data provide an unambiguous spectral signature of VER in small and medium size molecules. Our picosecond transient Raman technique also has the advantage of resolving the differing vibrational dynamics of individual modes.\(^{(24)}\) In this chapter, we present specific dynamical information for a high-frequency porphyrin skeletal mode, a low-frequency skeletal mode, and a high-frequency vinyl side chain mode. The vinyl modes have been a subject of interest because the side chain orientations are fixed by specific globin contacts. Thus these modes may provide information on the heme-pocket interaction.\(^{(25-26)}\) We are able to specifically monitor the vibrational dynamics of vinyl modes and
to compare the dynamics of vinyl modes with the dynamics of porphyrin skeletal modes.

There have been many recent experiments which indirectly measure VER in the condensed phase. These experiments have greatly increased our understanding of condensed phase photophysics. However, direct studies of vibrationally excited states in solution remain few in number. Intermolecular transfer of vibrational energy from large photoexcited molecules to the solvent has been investigated by the detection of excess fluorescence. Data from that type of experiment provide indirect information about VER. For large fluorescent molecules, these experiments have yielded time constants in the range of 9 to 11 ps for mechanical energy transfer to the solvent.

Recent direct experiments have shown that VER in condensed phase media cannot be assumed to be so fast as to be negligible when interpreting electronic absorption spectra. Well-known studies of I2 vibrational relaxation in solution have shown that a low frequency oscillator can retain its vibrational energy in the condensed phase for long times on the order of hundreds of picoseconds. This is reasonably well understood and was in fact predicted by theory. Picosecond Raman studies of the polyatomic molecule Cr(CO)6 in solution prove that the Cr(CO)5 photoproduct loses the majority of its vibrational energy on a 20 to 50 ps time scale, while retaining some vibrational energy in bottleneck states for 200 ps. The recent observation that a polyatomic molecule can retain mechanical energy in solution on this time scale has been somewhat surprising.
However, it is not at all clear how to extend our knowledge of VER times in gas, solution, and molecular crystal phases to the case of a protein-bound chromophore. The rate of VER in a protein-bound porphyrin provides information about coupling between the heme and the protein matrix. Genberg et al.\textsuperscript{(32)} assert that the heme-pocket interaction effectively models a heme in liquid solution. A comparison has been made between heme-protein coupling and chromophore-molecular crystal coupling.\textsuperscript{(22)} Molecular dynamics (MD) simulations\textsuperscript{(33)} have modeled the vibrational cooling process based on the current understanding of this coupling. These ideas need to be tested by definitive experimental study of the time scale of VER.

Several noteworthy Raman studies of heme protein cooling have been carried out on the picosecond and femtosecond time scale. The pioneering work of Petrich et al.\textsuperscript{(12)} used femtosecond pump-probe Raman spectroscopy to study shifts in the oxidation state marker band of a ligated hemoglobin. Conclusions about vibrational cooling in the low frequency modes of the porphyrin were drawn from an analysis of these shifts. However, that study did not attempt to observe anti-Stokes scattering and remains an indirect measurement, relying on the applicability of a theoretical interpretation of the band shifts. In fact, their interpretation of the observed band shifts has been recently questioned by Schomacker and Champion.\textsuperscript{(34)} Alden et al.\textsuperscript{(35)} suggest that nonlinear broadening due to probe peak powers in excess of $10^{11}$ W/cm\textsuperscript{2} may invalidate the interpretation of Petrich et al.\textsuperscript{(12)}
This chapter reports the results of a picosecond transient Raman study of deoxyHb designed to detect hot vibrations following visible excitation of the chromophore. The dynamics of both Stokes and anti-Stokes vibrational spectra have been obtained to measure unambiguously the dynamics of VER. We directly observe hot vibrations in photoexcited deoxyHb which decay with a time constant of 2 to 5 ps. The anti-Stokes data for different modes enable us to estimate the porphyrin internal temperature at time zero.

2.3 Experimental

2.3.1 Some Details of this Experiment

The experimental apparatus has been previously described in section 1.5. We have studied deoxyHb by exciting at 532 nm and probing at 355 nm. The pure transient Raman spectrum is produced by subtracting the background probe-only components from the spectrum obtained with the pump-probe sequence.\(^{(24)}\) We do not record the pump-only spectrum, because the monochromator is scanning wavelengths which are \(\approx 9000\) cm\(^{-1}\) to the blue of the 532 nm pump pulses. No Raman scattering of the green pump pulses is possible at these near-UV observation wavelengths. In fact, no background signal is observed from 532 nm pump pulses.

In the deoxyHb experiments, laser excitation occurs in an o-ring sealed spinning cell. The spinning cell prevents multiple probing of the same sample spot. Collinear pump and probe laser pulses enter through a suprasil window, and the Raman scattering is collected through the same window at \(\approx 135^\circ\) with respect to
the laser pulse direction. The cell was tested for air-tightness by certifying that no leakage occurred when the fully loaded cell was placed under vacuum. The position of the \( \nu_4 \) oxidation state marker band is sensitive to the ligation state of the heme. The position of this band was also used to check for oxygen leakage into the cell. We observed no shifting of this band over a period of two days.

Large (2 mm) monochromator slits were used for increased throughput of light. The instrument response function as measured by the width of the 354.7 nm Rayleigh line is then 16 cm\(^{-1}\) and is well approximated by a Gaussian function. The width of the 982 cm\(^{-1}\) sulfate band is instrument-limited at \( \approx 16 \) cm\(^{-1}\). The width of the deoxyHb \( \nu_4 \) Raman band fit to a Gaussian function is \( \approx 23 \) cm\(^{-1}\). Deconvolution of the instrument resolution from the \( \nu_4 \) band width yields a true \( \nu_4 \) width of \( \approx 16.5 \) cm\(^{-1}\). The typical scan time for dynamics data at a single Raman frequency is three hours. The typical scan time for an anti-Stokes frequency spectrum at one time delay is 12 hours. In all cases, these data are recorded by taking repetitive scans of 5 to 10 minute duration and adding the results using a computer. Dynamics are obtained by varying the optical delay between the pump and probe pulses as discussed previously. The difference spectrum technique discussed previously is used for both frequency-resolved and time-resolved data.

2.3.2 Sample Preparation

Hemoglobin samples were prepared from both fresh and five-day old human red cells. No difference in the absorption spectra were noted for samples prepared from the five day old cells versus the fresh cells. The red cells were washed in
0.85% NaCl solution four times to remove anti-coagulants and centrifuged to remove residual organic material. The red cells were then lysed in distilled water and centrifuged to remove cell membrane ghosts. The resulting hemoglobin solution was dialyzed in distilled water for at least 24 hours. Lastly the hemoglobin was passed through a mixed-bed ion-exchange resin (Bio-Rad AG501-X8) two or three times, yielding a final heme concentration of 3 mM. No peak due to methemoglobin (metHb) could be detected in the visible absorption spectrum.

Oxyhemoglobin (oxyHb) was stored in liquid nitrogen until needed. A UV-vis absorption spectrum of oxyHb is shown in figure 2.3A. We used a 100 mM sodium phosphate buffer solution at pH 7.5. Deoxygenation was accomplished by stirring an oxyHb sample in an argon environment for at least four hours. A UV-vis absorption spectrum of deoxyHb is also shown in figure 2.3A. The final deoxyHb sample was prepared by diluting the heme concentration to 1.5 mM with buffer and loading the spinning cell in the argon environment.

Na$_2$SO$_4$ was used in one experiment to calibrate transient absorbance in the sample. Sample degradation results if the Na$_2$SO$_4$ concentration is near saturation. Due to the high absorbance of the heme solution, it is necessary to keep the heme concentration low in order to see the sulfate non-resonant Raman band. The concentrations necessary to bring out the sulfate band were 100 $\mu$M heme, 100 mM sodium phosphate buffer, and 150 mM Na$_2$SO$_4$.

The sample was checked for cumulative effects due to long exposure of the sample to laser radiation. Figure 2.3B shows two absorption spectra of deoxyHb.
Figure 2.3: **Effect of Photolysis on Absorption Spectrum.** A) Static absorption spectrum of deoxyHb (solid curve) overlaid with the spectrum of oxyHb (dotted curve). B) Static absorption spectra of deoxyHb before (solid curve) and after (dotted curve) 24 hours of laser excitation.
The solid curve is a spectrum of freshly deoxygenated deoxyHb. The dotted curve is the absorption spectrum of the same sample taken after 24 hours of pump-probe laser spectroscopy. The spectra are virtually identical. The position of the $\nu_4$ band is sensitive to the oxidation state of the heme. Oxidation of ferrous deoxyHb to ferric metHb is easily detected as a shift to higher frequency of the $\nu_4$ band position from 1359 cm$^{-1}$ to 1375 cm$^{-1}$. Oxidation of the iron atom is also accompanied by a threefold increase in $\nu_4$ band intensity. Therefore the position and intensity of the $\nu_4$ band were used as checks to assure that these data come from deoxyHb and not metHb.

2.3.3 Laser Powers and Nonlinear Artifacts

We excite deoxyHb with the YAG second harmonic (532 nm) and probe the Raman spectrum of the excited sample at the third harmonic wavelength (355 nm). This pump photon energy falls in the deoxyHb Q absorption band which peaks at 555 nm and has a millimolar extinction coefficient of 8.5 at 532 nm. Using a pump beam radius of $\approx 250$ $\mu$m and a pump intensity of $6 \times 10^{13}$ photons/pulse, we estimate that we photoexcite 20-25% of the heme chromophores in the probe volume. We excite deoxyHb at 532 nm with 2 kHz repetition rate and an average power of 50 mW. Thus we deliver 25 $\mu$J in an 8 ps pulse over a spot of radius $\approx 250$ $\mu$m for a peak intensity of less than $5 \times 10^9$ W/cm$^2$ at the pump wavelength.

The probe wavelength of this experiment is 355 nm. This wavelength is near the maximum of the N absorption band which has its origin in the $\pi \rightarrow \pi^*$ transitions of the porphyrin ring ($e_{mM} = 32.5$). Our probe intensity is somewhat
smaller than our pump intensity at approximately $2 \times 10^9 \text{ W/cm}^2$. Hochstrasser and Johnson\(^{(10)}\) assert that the peak power density should be less than $\approx 3 \times 10^{10} \text{ W/cm}^2$ to avoid photodamage. Both the pump and probe peak power densities used in this experiment are below this limit.

The B or Soret band absorption peaks at 430 nm with a millimolar extinction coefficient of 133.\(^{(37)}\) Recently Alden et al. found power-dependent broadening of the $\nu_4$ resonant Raman band in deoxyHb for excitation within the Soret absorption band.\(^{(35)}\) That phenomenon has been explained as nonlinear Rabi broadening. Theoretical analysis\(^{(35)}\) indicates that the nonlinear artifact requires both the very intense resonance of the Soret transition and the high resonant Raman scattering cross section of $\nu_4$ in particular. Thus other high-frequency modes in that study did not exhibit significant broadening.\(^{(35)}\) Alden et al. found that as the probe laser power was increased from $\approx 10^8 \text{ W/cm}^2$ to $\geq 10^9 \text{ W/cm}^2$, the width of the $\nu_4$ band broadened, primarily on the low frequency side of the band. The apparent threshold for the nonlinear broadening of Alden et al. can be scaled upward to account for the lower extinction coefficient in the N absorption band. Alden et al. detect broadening of $\nu_4$ at a laser fluence of $5 \times 10^8 \text{ W/cm}^2$ at a probe laser wavelength of 416 nm. The extinction coefficient of deoxyHb at 416 nm is 2.7 times higher than at 355 nm. Therefore we multiply the above laser fluence by 2.7 to estimate the corresponding power density at which we might expect to see nonlinear broadening with 355nm pulses. In this simple estimate, we expect nonlinear effects using
355 nm excitation at $\approx 10^9$ W/cm$^2$. The probe laser power in our work is approximately twice this value. Nevertheless, we observe no broadening. Our $\nu_4$ band width of $\approx 16.5$ cm$^{-1}$ is equal to the $\nu_4$ width measured by Alden et al. for laser powers in the linear range. Apparently the threshold for nonlinear broadening in the N absorption band is significantly higher than our simple scaling estimate would indicate.

Several additional arguments indicate that nonlinear artifacts do not interfere with the results presented in this chapter. The subtraction procedure will remove all probe-only features from the transient spectrum, whether those are linear or nonlinear effects. The bands in the transient spectra of these experiments are due strictly to changes in the 355 nm Raman spectrum caused by the absorption of a 532 nm pump photon. Furthermore, the dynamics spectra of figures 2.4 and 2.5 monitor these changes as a function of delay time between 532 nm pump and 355 nm probe pulses. The time dependence of these transient Raman signals is due strictly to the delay time between pump and probe pulses, so that broadening of a Raman band by the 355 nm peak power can have no effect on the dynamics. We specifically note that Alden et al. studied the effect of spatially and temporally overlapping $\approx 10^{10}$ W/cm$^2$ 532 nm pulses with $\approx 10^8$ W/cm$^2$ 436 nm probe pulses to determine if the broadening could be induced by simultaneous excitation in the Q absorption band. The very intense 532 nm pulse had no influence on the bandwidth of $\nu_4$ probed at 436 nm. Thus we can be confident that our 532 nm pump pulses (which
are a factor of 2 less intense than those tested in Alden et al.) do not contribute any nonlinear artifact to either the raw pump-probe spectra or to the transient spectra.

Multiple absorption effects are seen in photoacoustic spectroscopy if $\geq 25\%$ of the sample is photoexcited.\(^{(38)}\) Our estimate of 20-25\% photoexcitation of the sample indicates that the transient species in our experiment are predominantly singly-excited heme chromophores.

2.4 Analysis of Raman Spectra

The ground state and transient Raman spectra are assigned and discussed in detail in this section. A summary of these transient features and their interpretations are provided immediately prior to the DISCUSSION section. Absolute Stokes band positions were calibrated against the laser Rayleigh line at 354.7 nm and agree very well with literature values. Anti-Stokes band positions were read directly from the monochromator.

2.4.1 Assignment of Unexcited Stokes Bands

Figures 2.6A and 2.7A show the ground state Raman bands observed by probing at 355 nm. The spectrum shown in figure 2.6A has two features which are also present in the spectrum of the suprasil window and buffer solution without deoxyHb. These quartz bands are the steeply sloping background on the left edge of the spectrum and the broad band peaked at $\approx 790 \text{ cm}^{-1}$. The band at 672 cm$^{-1}$ is assigned to the $\nu_7$ band of deoxyHb and the band at 755 cm$^{-1}$ is assigned to the $\nu_{16}$ band of deoxyHb.\(^{(13,39)}\) Friedman and co-workers\(^{(13)}\) also see the $\nu_{32}$ band
Figure 2.4: **Complementary dynamics of deoxyHb $\nu_2$ mode.** The Stokes and anti-Stokes transient Raman intensity at 1563 cm$^{-1}$ is plotted as a function of delay time between pump and probe pulses. This position corresponds approximately to the $\nu_2$ band is referred to as such. A) The transient Stokes Raman intensity for $\nu_2$. These dynamics are assigned to the recovery of population in the vibrationless level in the ground electronic state. B) The transient anti-Stokes Raman intensity for the $\nu_2$ band. These dynamics are assigned to population movement through the lowest excited vibrational levels of $\nu_2$ on the ground electronic surface. The circles are the experimental data points. The solid line is the fit to the dynamics. This fit includes a single exponential rise time constant, a single exponential decay time constant, and convolution with the laser pulse. The deconvolved decay time constant is 2 to 5 ps for both dynamics.
Figure 2.4: Complementary dynamics of deoxyHb $\nu_2$ mode.
Figure 2.5: **DeoxyHb anti-Stokes dynamics.** A) Anti-Stokes dynamics of the $\nu_7$ band of deoxyHb at -672 cm$^{-1}$. B) Anti-Stokes dynamics at -1424 cm$^{-1}$ of the $\delta_2$ (\textasciitilde CH$_2$) symmetric scissoring mode of the vinyl side chains at -1424 cm$^{-1}$. C) Anti-Stokes dynamics at -1563 cm$^{-1}$ (primarily $\nu_2$). The circles are the experimental data points. The solid lines are the fits to the dynamics. Each fit includes a single exponential rise time constant, a single exponential decay time constant, and convolution with the laser pulse. Within the S/N of these data, the three curves are identical. With deconvolution of the laser pulse width, all three curves have rise time constants of 0 to 2 ps; all three decay with the time constant 2 to 5 ps.
Figure 2.5: DeoxyHb anti-Stokes dynamics.
at 790 cm$^{-1}$. We cannot resolve this band from the broad silica band centered at 790 cm$^{-1}$ in the Stokes spectrum; however we do observe the positive transient of this mode in the anti-Stokes spectrum, which is mentioned below.

Figure 2.7A displays the resonant Raman bands of deoxyHb between 1240 and 1640 cm$^{-1}$. These are the well-studied oxidation state and core size marker bands. Prominent in the spectrum is the $\nu_4$ oxidation state marker band centered at 1359 cm$^{-1}$. The position of this feature is sensitive to the $\pi$ electron density on the tetrapyrrole ring, which is determined by the oxidation state of the iron atom.$^{(40)}$ The low value of the $\nu_4$ Raman shift in this spectrum corroborates that we are indeed studying ferrous deoxyHb as opposed to ferric metHb. The broad feature with the largest intensity is likely a superposition of many bands.$^{(12,41)}$ Two bands which can be individually resolved are the core sensitive markers $\nu_2$ at 1569 cm$^{-1}$ and $\nu_{37}$ at 1593 cm$^{-1}$.$^{(42)}$ The $\nu_{11}$ mode at $\approx$1545 cm$^{-1}$ and a vinyl C=C stretch at $\approx$1620 cm$^{-1}$ are also evident in this group of bands.$^{(42)}$ These Raman shifts are necessarily approximate since we cannot fit this congested part of the spectrum. Another core marker $\nu_3$ is seen at 1475 cm$^{-1}$.$^{(42)}$ The weak band at 1390 cm$^{-1}$ is assigned to the skeletal mode $\nu_{20}$ and/or $\nu_{29}$.$^{(26)}$

The relatively strong band at 1430 cm$^{-1}$ in figure 2.7 corresponds to a symmetric scissoring mode of the terminal =CH$_2$ on the two vinyl substituent groups of protoporphyrin IX, the heme variety found in biological systems.$^{(23)}$ In the absence of strong electronic interactions with another chromophore, the vinyl C=C absorption would peak at $\approx$ 180 nm.$^{(43)}$ However as a side chain on a porphyrin ring, the
Figure 2.6: **Low frequency Stokes Raman spectra.** A) Stokes Raman spectrum of unexcited deoxyHb probed at 355 nm. The $\nu_7$ and $\nu_{16}$ bands are located at 672 cm$^{-1}$ and 755 cm$^{-1}$. The broad feature centered at 790 cm$^{-1}$ is due to the suprasil quartz window. B) Transient spectrum at time zero. All bands in this spectrum are due to the effect of the 532 nm pump pulse. DeoxyHb transient bands are negative due to removal of population from the vibrationless level of the ground electronic state by the optical excitation. The quartz window bands are absent in the subtracted spectrum. The unexcited spectrum of frame A) and the pump-probe spectrum of frame B) were obtained by summing many single scans. Each sweep of the monochromator recorded both pump-probe and probe-only spectra using mechanical chopping of the pump beam. The transient spectrum has been smoothed with a five-point Fourier transform filter. Frequency units are cm$^{-1}$. 
Figure 2.6: Low frequency Stokes Raman spectra.
Figure 2.7: **High frequency Stokes Raman spectra.** A) The 355 nm probe-only spectrum of unexcited deoxyHb. The mode assignments are printed on the figure for clarity. The $\nu_4$ band at 1359 cm$^{-1}$, $\nu_{20}$ and/or $\nu_{29}$ at 1390 cm$^{-1}$, $\nu_3$ at 1475 cm$^{-1}$, $\nu_2$ at 1569 cm$^{-1}$, and $\nu_{37}$ at 1593 cm$^{-1}$ are skeletal modes of the porphyrin ring. The strong band at 1430 cm$^{-1}$ is a symmetric scissoring vibration of the vinyl group $=\text{CH}_2$. B) Time-zero transient spectrum of deoxyHb following absorption of a 532 nm photon. All Raman bands shown in frame A) display negative transients in frame B), indicating that 532 nm excitation bleaches ground state deoxyHb bands. The depletion is $\approx6\%$ when measured as a percentage of the band intensities in frame A). The transient spectrum has been smoothed with a three-point Fourier transform filter. Frequency units are cm$^{-1}$. 
Figure 2.7: High frequency Stokes Raman spectra.
vinyl group double bond \( \pi \) orbitals conjugate into the delocalized porphyrin \( \pi \) system resulting in resonant Raman enhancement for modes which otherwise would not appear in the spectrum. Another example is the vinyl C=C stretching mode at 1620 cm\(^{-1}\) which appears as a shoulder on the congested band from 1500 to 1640 cm\(^{-1}\).

Figure 2.8A shows a Stokes Raman spectrum of deoxyHb plus Na\(_2\)SO\(_4\). Band assignments are given in the figure caption. The deoxyHb bands at 1119 cm\(^{-1}\) and 1175 cm\(^{-1}\) involve \( C_b \)-substituent stretches and are among those heme bands most strongly perturbed by heme-protein coupling.\(^{26}\) The band at 1119 cm\(^{-1}\) is probably the \( \nu_{44} \) band observed at 1117 cm\(^{-1}\) in deoxymyoglobin (deoxyMb).\(^{26}\) This band is primarily a \( C_b \)-vinyl stretch. The \( \nu_{44} \) mode is inactive in the resonant Raman spectra of protein-free complexes but has been observed in the resonant Raman spectra of metmyoglobin and deoxyMb. Raman activity in the myoglobins is likely induced by a symmetry-lowering effect of the myoglobin pocket.\(^{26}\) The \( \nu_{44} \) band was not previously observed\(^{26}\) in hemoglobin (metHb fluoride) when probing with excitation into the Soret or Q bands. Our experiment probes in the N absorption band and looks at a ferrous five-coordinate heme. Either of these differences may explain the appearance of this band in our spectrum.

### 2.4.2 Assignment of Unexcited Anti-Stokes Bands

Figures 2.9A and 2.10A show resonant Raman scattering in the anti-Stokes region. These bands all have counterparts in the Stokes region of the spectrum.
Figure 2.8: DeoxyHb Stokes Raman spectra with sulfate. The 355 nm Stokes Raman spectra of deoxyHb with Na$_2$SO$_4$ added to measure transient absorption which might result from 532 nm excitation. A) The unexcited spectrum. The most prominent feature is the 982 cm$^{-1}$ Raman band of the sulfate ion. The broad feature from 1020 to 1090 cm$^{-1}$ is due to scattering from the quartz window. All features to higher frequency are due to Raman scattering from deoxyHb: $\nu_{44}$ at 1119 cm$^{-1}$, $\nu_{30}$ at 1175 cm$^{-1}$, possibly $\nu_{13}$ at 1225 cm$^{-1}$, $\nu_4$ at 1359 cm$^{-1}$, $\nu_{29}$ at 1390 cm$^{-1}$, the vinyl side chain $\delta_s (=\text{CH}_2)$ at 1430 cm$^{-1}$, and $\nu_3$ at 1475 cm$^{-1}$. B) The transient spectrum at time zero. The vertical dashed lines are provided to mark corresponding features in frames A) and B). The deoxyHb bands marked with dashed lines visibly deplete. For example, the negative transients of $\nu_4$ and $\nu_3$ shown in frame B) are 6 to 7% of the band height in frame A). The sulfate ion Raman band shows no measurable transient feature. This indicates that heme Raman band transients are due to pure vibrational dynamics, not transient absorption. The transient spectrum has been smoothed with a five-point Fourier transform filter. Frequency units are cm$^{-1}$. 
Figure 2.8: DeoxyHb Stokes Raman spectra with sulfate.
Clearly these bands correspond to the same vibrational modes and are assigned as such.

2.4.3 Transient Spectra at Fixed Time Delay

All transient bands for deoxyHb have corresponding bands in the probe-only spectrum. The Stokes spectrum of figure 2.6 shows negative transients corresponding to all ground state Raman bands in the lower frequency region when the 532 and 355 nm pulses are temporally overlapped. The anti-Stokes spectrum in figure 2.9 shows positive transient features at time zero for the same lower frequency region as figure 2.6. The magnitudes of the negative Stokes transient bands shown in figure 2.6B are about 6% relative to the corresponding bands in the probe-only spectrum. The positive anti-Stokes transient on $\nu_7$ shown in figure 2.9B, however, is $\approx 17\%$ of the corresponding unexcited band intensity shown in figure 2.9A. The prominent quartz band centered at 790 cm$^{-1}$ in figures 2.6A and 2.9A is absent in the transient spectra due to the subtraction procedure.

The Stokes and anti-Stokes transient spectra for the higher frequency region are shown in figures 2.7B and 2.10B. The pattern of transient features in this frequency region is the same as that in the lower frequency region. The negative Stokes transient bands are 8% of the unexcited Stokes band heights. The positive anti-Stokes transients are about 40% of the unexcited anti-Stokes band intensities.
Figure 2.9: **DeoxyHb low frequency anti-Stokes Raman spectrum.** A) Anti-Stokes spectrum of unexcited deoxyHb probed at 355 nm. These bands correspond directly to $\nu_7$ at 671 cm$^{-1}$, $\nu_{16}$ at 755 cm$^{-1}$, and the quartz bands assigned in figure 2.6A. The assignments are thus identical with those of the Stokes spectrum and are labeled as such in the figure. B) Time zero anti-Stokes transient spectrum taken simultaneously with frame A). All deoxyHb Raman modes observed in the unexcited spectrum show positive transients. The quartz band is absent in the transient spectrum. The transient feature at 790 to 800 cm$^{-1}$ corresponds to $\nu_{32}$ which is obscured in the probe-only spectrum due to the quartz band. The transient at the $\nu_7$ position is $\approx$17% of the unexcited band height in frame A). The positive transients are interpreted as vibrational heating on the ground electronic surface due to photoexcitation at 532 nm. The transient spectrum has been smoothed with a three-point Fourier transform filter. Frequency units are cm$^{-1}$. 
Figure 2.9: DeoxyHb low frequency anti-Stokes Raman spectrum.
Figure 2.10: **DeoxyHb high frequency anti-Stokes spectrum.** A) Anti-Stokes spectrum of unexcited deoxyHb probed at 355 nm. These bands correspond directly to the \( \nu_4 \), vinyl \( \delta_s \) mode, \( \nu_3 \), and the conglomeration band assigned in figure 2.7A. The assignments are thus identical with those of the Stokes spectrum and are labeled as such in the figure. B) Time zero anti-Stokes transient spectrum taken simultaneously with frame A). The transient difference spectrum of frame B) is the result of subtracting out the unexcited probe-only Raman features of frame A) from the (532 nm pump + 355 nm probe) spectrum (not shown). All deoxyHb Raman modes observed in the unexcited spectrum show positive transients. The transient at the \( \nu_4 \) position is \( \approx 43\% \) of the unexcited band height in frame A). The positive transients are interpreted as excess vibrational energy on the ground electronic surface. Both the unexcited and transient spectra have been smoothed with a three-point Fourier transform filter. Frequency units are cm\(^{-1}\).
Figure 2.10: DeoxyHb high frequency anti-Stokes spectrum.
2.4.4 Dynamics Spectra of Specific Raman Bands

In order to determine precisely the time scale for the transient features observed in the frequency scans, we have monitored specific Raman band transient signals while varying the time delay between pump and probe pulses. Figure 2.4 shows the complementary dynamics of the Stokes and anti-Stokes scattering at 1563 cm\(^{-1}\). This position is within the broad overlapping feature centered at 1584 cm\(^{-1}\) shown in figure 2.7 and is approximately at the position of \(\nu_2\). The laser pulse width can be deconvolved from the experimentally measured decay curve to yield the molecular response time. The measured dynamics are only slightly wider than our laser pulse. The data were fit with curves of the form 
\[ c_1 \left[ -\exp(-k_{\text{rise}}t) + \exp(-k_{\text{decay}}t) \right] + c_2. \]
Within our uncertainty, both the negative Stokes transient and the positive anti-Stokes transient develop with 0 to 2 ps time constant, and both features decay with 2 to 5 ps time constant. Although the dynamics are well modeled by exponential functions, we would be very hesitant to attach any significance to this observation. When the dynamics being measured are shorter than the laser pulse width, then variations in the temporal profile of the pulse will strongly affect the shape of the rise and fall.
2.5 Interpretation

2.5.1 Absorption Effects on the Raman Spectrum

Transient changes in the optical density of the solution can affect the trans­
sient Raman spectrum. We have checked for and eliminated this possibility by
the looking for a transient feature associated with the sulfate ion Raman band.
The picosecond Raman data presented above show that all heme Stokes Raman
bands are negative in the transient spectrum due to photoexcitation, while all heme
anti-Stokes Raman bands show positive transients due to photoexcitation. These
transient bands correspond to the known vibrations of the ground electronic state
of deoxyHb. Raman bands in the ground electronic state could change intensity
due to a simple transient absorbance change in the sample. Transient absorption
might arise from an electronically excited minority species such as those seen in
model porphyrins\(^{44-45}\) or from vibrational heating. The Raman probe light and/or
the Raman scattered light might be more strongly or less strongly absorbed by the
sample after 532 nm photoexcitation.

The data of figure 2.8 show that picosecond transient absorption effects in this
wavelength region are not responsible for the transient Raman bands of figures 2.6,
2.7, 2.9, and 2.10. The intensity of 355 nm Raman scattering from the sulfate ion
could only change under 532 nm excitation due to transient absorption in deoxyHb.
A time zero transient spectrum including the 982 cm\(^{-1}\) sulfate band is shown in
Figure 2.8B. The spectrum is somewhat noisy due to the low heme concentration
necessary to bring out the sulfate band (see Sample Preparation section). Nonetheless the following observation is clearly made. No transient band can be seen in the spectrum at the sulfate band position. Within the signal-to-noise (S/N) of the scan, any transient feature at the sulfate band position in figure 2.8B is limited to ±0.7% of the probe-only unexcited sulfate band of figure 2.8A. In contrast, the negative transient of the $\nu_4$ heme band in figure 2.8B is 6.3% of the unexcited $\nu_4$ band intensity in figure 2.8A. This is a factor of nine greater than the limit set for the sulfate band. The positive deoxyHb transient Raman bands in the high frequency anti-Stokes region are $\approx$40% of the unexcited anti-Stokes band heights. Any picosecond absorbance change in the sample is therefore too small to account for the prominent negative transients due to bleaching of the heme bands at 1359 cm$^{-1}$, 1430 cm$^{-1}$, and 1475 cm$^{-1}$ in figure 2.8. Negative transients on the smaller heme bands are less obvious at this S/N level but still discernible in the spectrum.

We have also checked the temperature dependence of the static absorption spectrum of deoxyHb. The change in 355 nm absorbance at 15 K above room temperature is $\approx$0.2%. The protein pocket is much less significantly heated in a picosecond laser experiment compared to a static absorption experiment. Therefore this measurement does not exactly mimic the picosecond experiment. It does suggest, however, that the 355 nm region is not so sensitive to temperature.
2.5.2 Transient Heating in the Heme

Having eliminated transient absorption as the explanation for the data of figures 2.6, 2.7, 2.9, and 2.10, the Raman transients are assigned to vibrationally hot deoxyHb in the ground electronic state. The complementary negative Stokes transient bands and positive anti-Stokes transient bands shown in figures 2.11 and 2.12 are the required spectral signature of hot vibrations. These transient bands are plotted together in figures 2.11 and 2.12 to emphasize the striking opposite behavior of the Stokes and anti-Stokes transient bands.

A positive transient feature indicates that a vibrational mode is more populated following photoexcitation than in the unexcited molecule. A negative feature indicates that population has been removed from a vibrational level by the pump photon. Our subtraction procedure removes all contribution of Stokes and anti-Stokes scattering from levels thermally populated at room temperature. Clearly, negative Stokes transient bands indicate removal of population from the lowest vibrational levels. Similarly, positive anti-Stokes transient bands indicate that levels with \( v > 0 \) are more populated following photoexcitation than they were at room temperature.

*Negative transient Stokes Raman bands complemented by positive transient anti-Stokes Raman bands provide direct and strong evidence of excess vibrational energy in the chromophore.* This unambiguous spectral signature of VER is not subject to complications arising from structural rearrangements or the unknown resonant Raman enhancements in the chromophore. The resulting picture is clear. Internal conversion from the electronically excited state leaves the heme in a highly
Figure 2.11: **Low frequency transient spectra.** A) Transient Stokes Raman spectrum at time zero of deoxyHb. B) Transient anti-Stokes Raman spectrum at time zero of deoxyHb. These scans illustrate the opposite and complementary behavior of the transient Stokes and anti-Stokes Raman spectra. This is a clear and unambiguous indication of vibrational heating in the ground electronic state over the time scale of the 8 ps laser pulses. Both the unexcited and transient spectra have been smoothed with a six-point Fourier transform filter. The band labels on the figure mark the positions of the modes contributing to the transient spectra. Frequency units are cm$^{-1}$.
Figure 2.12: High frequency transient spectra. A) Transient Stokes Raman spectrum at time zero. B) Transient anti-Stokes Raman spectrum at time zero. These scans emphasize the complementary nature of the transient Stokes and anti-Stokes data which is the spectral signature of vibrational heating. Both the unexcited and transient spectra have been smoothed with a four-point Fourier transform filter. The band labels on the figure mark the positions of the modes contributing to the transient spectra. Frequency units are cm⁻¹.
excited vibrational state of the $^5T_2$ ground electronic state. Vibrational cooling then proceeds until the porphyrin reaches thermal equilibrium with the protein pocket. The cooling process in the ground state is observed in our spectra without reference to the indirect analyses which have been necessary in previous ultrafast Raman experiments.$^{12-13}$

2.5.3 Dynamics of Individual Raman Bands

The data of figure 2.4 beautifully illustrate the complementary nature of transient Stokes and anti-Stokes dynamics data as a probe of VER. The bleaching of the high frequency $\nu_2$ Stokes Raman band represents the instantaneous removal of population from the vibrationless level of the ground electronic state by the absorption of a green laser photon, exciting the upper electronic state. The rapid rise of the transient anti-Stokes signal indicates that excited vibrations in the intermediate levels of the ground electronic state are populated very rapidly following photoexcitation. Figure 2.13 illustrates that the rise of the $\nu_2$ anti-Stokes signal is coincident with the experimental rise time of the apparatus. Anti-Stokes scattering from the $\nu_7$ mode also appears promptly. Such rapid appearance ($<2$ ps) of population in the lower vibrational levels is consistent with rapid cooling following an electronic relaxation known to occur with 300 fs time constant.$^{46}$

The recovery of the depleted $\nu_2$ Stokes Raman signal indicates that the vibrationless level in this mode completely refills with a 2 to 5 ps time constant. The recovery of the depleted $\nu_2$ Stokes transient signal and the simultaneous decay of
the positive $\nu_2$ anti-Stokes transient signal is the spectral signature of VER. Figure 2.5 shows the dynamics of the anti-Stokes transient signal at three Raman band positions. Figure 2.5A shows the rise and fall of the transient anti-Stokes signal at the position of $\nu_7$; figure 2.5B shows the same dynamics at the position of a symmetric scissoring mode of the terminal $=\text{CH}_2$ on the two vinyl substituent groups; figure 2.5C plots the dynamics of $\nu_2$ as previously discussed. Within the uncertainty of the data all three spectra are identical. Therefore the cooling dynamics of the $\nu_2$ skeletal mode, the in-plane $\nu_7$ ring deformation mode, and the $\delta_s$ side chain mode are identical within the time resolution of this experiment.

### 2.5.4 Temperature Estimate

In non-resonant Raman scattering, temperature can be directly computed from the Stokes/anti-Stokes ratio assuming Boltzmann statistics. For resonance Raman scattering this is not the case because the resonance enhancement can be different for Stokes and anti-Stokes bands. However, it is possible to factor out the mode-dependent resonance enhancement by the following method which utilizes only transient anti-Stokes data. We can extract a quantitative estimate of heme temperature by comparing the anti-Stokes transient intensity of a band in the low frequency region to the anti-Stokes transient intensity for a band in the high frequency region. The purpose of this temperature estimate is not to confirm the conclusion that the heme is hot on our 8 ps time scale. This conclusion is firm from the complementary Stokes and anti-Stokes dynamics. Rather, we estimate the temperature in order to gain insight into how much the heme has cooled on an 8 ps time
Figure 2.13: **DeoxyHb anti-Stokes rise times.** The circles are individual data points for the experimental rise time of the apparatus. Stimulated emission in coumarin 500A laser dye was used for this purpose. The solid line is the experimental curve for the rise of the deoxyHb anti-Stokes transient signal of $\nu_2$ at -1563 cm$^{-1}$. The dashed line is the experimental curve for the rise and decay of the deoxyHb vinyl group anti-Stokes transient signal at -1424 cm$^{-1}$. These data show the rise times of several spectra overlapped to illustrate that they rise together. All rise times are 0 to 2 ps when the laser pulse is deconvolved out of the curve.
scale. The question of whether or not we are justified in assigning a temperature to the heme is treated under the DISCUSSION section.

Anti-Stokes Raman scattering can only occur for vibrational levels \( v \geq 1 \). In this temperature estimate, we assume that the anti-Stokes signal provides a window to look at population moving through the \( v = 1 \) level of a particular mode, neglecting possible anti-Stokes scattering from \( v > 1 \). The height \( H_n \) of an anti-Stokes band for mode \( n \) is given by \( H_n \propto P_n \times \sigma_n \). Here \( P_n \) represents the population in \( v = 1 \) of mode \( n \), while \( \sigma_n \) stands for the resonant Raman enhancement of mode \( n \) at the probe wavelength. For the unexcited anti-Stokes intensity \( H_n \) of given Raman band, \( P_n \) is given by the Boltzmann population in \( v = 1 \) of mode \( n \) at room temperature.

The temperature calculation is based on the observation that the unknown \( \sigma_n \) for mode \( n \) is factored out by taking the ratio of excited anti-Stokes band height to unexcited anti-Stokes band height. The quantity \( H_n^*/H_n \) is therefore independent of resonant Raman enhancement and is equal to the \( v = 1 \) population ratio in mode \( n \) for the two temperatures.

The \( \nu_4 \) mode of deoxyHb lies 1359 cm\(^{-1} \) in energy above the zero point, while \( \nu_7 \) lies at 671 cm\(^{-1} \). The time-zero transient anti-Stokes \( \nu_4 \) band at 1359 cm\(^{-1} \) is 43\% of the unexcited anti-Stokes \( \nu_4 \) band height. For the \( \nu_7 \) anti-Stokes band at 671 cm\(^{-1} \), the time-zero transient is 15\% of the unexcited band height. In the notation used above, \( H_4^*/H_4 = 1.43 \) and \( H_7^*/H_7 = 1.15 \).
We assume that the excess heat is distributed statistically among all modes on the time scale of our experiment. This assumption is justified in the DISCUSSION section of this chapter. Application of the Boltzmann distribution for the \( v = 1 \) level of two vibrations of different energy yields the equation

\[
\frac{H_4^*/H_4}{H_7^*/H_7} = \exp \left[ \frac{E_4 - E_7}{k_b} \left( \frac{1}{T_{\text{room}}} - \frac{1}{T_{\text{hot}}} \right) \right]
\]

Let it be emphasized again that the band height ratios in this equation are not an attempt to relate Stokes to anti-Stokes intensities. Rather \( H_4^*/H_4 \) and \( H_7^*/H_7 \) represent the ratio of a (532 nm-pump + 355 nm-probe) anti-Stokes band height to a 355 nm-probe only anti-Stokes band height at the same Raman shift. In this way, the resonant Raman enhancement is factored out.

An accurate temperature estimate requires that the data be corrected for the day-to-day variations in laser power and alignment of pump and probe beams. Time-zero transient spectra in the 1260-1640 cm\(^{-1}\) Stokes region were taken in conjunction with each experiment. The ground state and transient Raman band intensities in these test spectra allow us to factor experimental conditions out of the data before calculating the temperature. We also need to correct for the fact that we photoexcite only 20 to 25% of the hemes in the sample volume. This is accomplished by scaling the transient signal upward by a factor of four for the purposes of the temperature estimate. This method provides an approximate but useful estimate of the (Boltzmann) temperature of the photoexcited heme averaged over the duration of the convolution of our 8 ps pump and 8 ps probe pulses.
Assuming a Boltzmann distribution of excess vibrational energy yields a vibrational temperature best estimate of 36 K above room temperature averaged over our 8 ps pulse at time zero. The initial temperature before energy exchange with the protein is \( \approx 460 \text{ K} \) above room temperature.\(^{33}\) If we consider the worst-case error due to the noise level of the data, the estimated temperature jump we observe could be as low as 12 K or as high as 63 K. In any case, we conclude that we are seeing the latter stages of cooling in our 8 ps time resolution.

2.5.5 Band Shifts

Raman band shifts to lower frequency upon photoexcitation have been reported by Petrich et al.\(^{12}\) for photolysis of carbonylhemoglobin. We observe shifting of some deoxyHb Raman bands at time zero.

2.5.5.1 Modified Subtraction Procedure

It is difficult to see small shifts in band position directly in figures 2.6 and 2.7. A modification to the normal subtraction procedure makes such shifts clearly visible. If the influence of the 532 nm pump pulse in these experiments is simply to make ground state bands grow or shrink, then the transient features in the subtracted spectra will have the same shape as the unexcited Raman bands. But the effect of the 532 nm excite pulse may also be to broaden or shift a band, relative to its shape and position in the unexcited spectrum. All transient spectra previously shown are subtracted directly without any other data manipulation. However, we can multiply the unexcited spectra by various constant factors before subtraction. If the bands
simply grow or shrink upon excitation, then a factor should be possible which will force transients to the baseline within the noise. If this is not possible, then the 532 nm pump pulse also induced a shift or broadening in the Raman bands shapes, in addition to the primary effect of changing the band intensities. This procedure emphasizes small frequency shifts.

The transient spectrum of figure 2.7 is produced by directly subtracting the probe-only spectrum from the pump-probe spectrum. The spectrum of figure 2.14 is produced from the same data as figure 2.7, except that the probe-only spectrum is multiplied by 0.94 prior to subtraction. These data are interpreted below for the porphyrin skeletal modes and the vinyl modes.

2.5.5.2 Interpretation of High Frequency Band Shifts

All deoxyHb Raman bands in the high frequency region of the spectrum of Figure 2.14 shift to lower frequency upon photoexcitation. The shifting of individual bands in the congested region from 1500 to 1640 cm\(^{-1}\) is not discernible due to overlap of several bands. Such shifting in photoexcited carbonylhemoglobin (carbonylHb) has been interpreted\(^ {12}\) in terms of anharmonic coupling to vibrationally excited lower frequency modes in the femtosecond Stokes Raman experiment of Petrich et al. However, Czernuszewicz et al.\(^ {47}\) have shown that heme Raman bands are not expected to be so temperature sensitive. Anharmonic coupling to lower frequency hot vibrations should account for part of the shifting, but the magnitudes of the observed band shifts indicate that additional mechanisms are likely involved. We have not attempted to obtain the necessary S/N to study these
Figure 2.14: Stokes frequency spectrum showing shifting. A) 355 nm probe-only spectrum of the high frequency region for deoxyHb. B) Transient spectrum obtained by subtracting with a correction factor: transient = (pump-probe) − factor × (probe-only). This illustrates that the transient features of figure 2.7 are not simply due to depletion, but also to a shifting to slightly lower frequency. The positive and negative features of frame B) are not noise. Note that each Raman band in frame A) has a corresponding feature in frame B) which goes positive on the left side of the frame A) band and negative near the center of the corresponding frame A) band position. The shifting in the congested region between 1500 and 1650 cm\(^{-1}\) is not as simple due to the overlapping bands in this region. The shifting is assigned to anharmonic coupling of these high frequency modes to hot lower-frequency vibrations. The transient spectrum has been smoothed with a three-point Fourier transform filter. Frequency units are cm\(^{-1}\).
Figure 2.14: Stokes frequency spectrum showing shifting.
band shifts. We do note that the shifting behavior is readily discernible on all high frequency bands in Figure 2.14 with apparently similar magnitude of shift. The \( \nu_7 \) mode at \( 672 \text{ cm}^{-1} \) does not shift within the limit of our S/N. The key point to notice is that whatever mechanisms control the band shifting seem to couple to the vinyl scissoring mode as well as to the porphyrin skeletal modes.

2.6 Summary of Transient Raman Data

The following items summarize the transient spectra obtained by subtracting the one color probe-only spectra from the two color pump-probe Raman spectra. The features compiled below are due to photoexcited hemes only. Each feature is discussed fully in the previous sections.

1. Every deoxyHb Stokes Raman band studied shows a negative transient band at time zero. This indicates removal of vibrational population from the vibrationless level of the ground electronic state.

2. We detect positive anti-Stokes bands in the time zero transient spectrum at the positions of three ground state Raman bands between 650 and 800 cm\(^{-1}\) and at the positions of the high-frequency modes between 1260 and 1640 cm\(^{-1}\). These positive anti-Stokes transient bands indicate hot vibrations in the low-lying levels of the ground electronic state.

3. No transient is observed at the position of the 982 cm\(^{-1}\) sulfate band at time zero. Considering the noise level, any transient on the sulfate band must be less than \( \pm 0.7\% \) of the band height in the probe-only spectrum. Heme bands in the same spectrum show negative transients which are more than 6\% of
the band height in the probe-only spectrum. This indicates that the dynamics observed in the transient Raman spectrum are not due to transient absorption, but rather to pure vibrational dynamics.

4. Time-delay scans which track the dynamics on a specific Raman band were taken at several Stokes and anti-Stokes Raman shift positions. After deconvolution of the laser pulsewidths, we find that all transient features appear promptly and decay with a time constant of 2 to 5 ps. These data show that hot vibrations detected in the $v_7$ mode, the vinyl $\delta_s$ mode, and the $v_2$ mode of the heme cool with 2 to 5 ps time constant. The dynamics of these modes are identical within our time resolution.

5. We observe shifting to lower frequency for Stokes Raman bands between 1260 and 1640 cm$^{-1}$. No such shift is observed for bands between 650 and 800 cm$^{-1}$ within our S/N. S/N on anti-Stokes transient bands is inadequate to comment on possible shifting. Shifted Stokes transient bands are believed to arise from anharmonic coupling to excited low-frequency vibrations.

2.7 Discussion

2.7.1 Evidence for Boltzmann Distribution

The picosecond Raman data reported in this chapter provide evidence that we observe hemes with Boltzmann-distributed internal energies on the time scale of our 8 ps laser pulses. The primary internal conversion event back to the electronic ground state occurs with a time constant of 300 fs.$^{(46)}$ One case in which
the heme vibrational modes might not be in thermal equilibrium with themselves is if intramolecular vibrational redistribution (IVR) is not rapid enough to statistically redistribute the internal energy on an 8 ps time scale. Experiments have suggested that IVR times in condensed phase media are less than 1 ps for several large molecules which are smaller in size than isolated hemes. Theoretical discussions of heme protein cooling suggest that heme IVR following photoexcitation should be complete in 1 ps or a few picoseconds. It is reasonable to believe that dissipative IVR produces a thermal distribution over heme internal modes on a much shorter time scale than our 8 ps laser pulse.

The other case where Boltzmann statistics would not be applicable is if there are some uncoupled modes where vibrational energy can hang up long after most modes have cooled to room temperature. However, the picosecond Raman data show positive anti-Stokes transient bands at the position of $\nu_7$, $\nu_{16}$, $\nu_{32}$, $\nu_4$, a vinyl mode, $\nu_3$, $\nu_2$, and $\nu_{37}$. Actually, every unexcited heme Raman band in our spectrum has a positive feature in the anti-Stokes transient spectrum. It seems quite unreasonable to suppose that all of the bands that are active when probed at 355 nm coincidentally happen to be bottleneck modes which trap mechanical energy. Furthermore, we have already observed from Figure 2.5 that $\nu_7$, the $\delta_s$ vinyl mode, and $\nu_2$ in the congested region all undergo VER at the same rate. Clearly these hot modes are not relaxing independently with individual rates. Rather they seem to be strongly coupled to a common heat bath. Finally, previous Raman experiments have suggested that many high-frequency porphyrin skeletal modes are an-
harmonically coupled to low frequency modes. Anharmonic coupling of $\nu_4$ to hot low-frequency modes is responsible for at least part of the time-dependent shifts observed by Petrich et al.$^{(12)}$ Asher and Murtaugh$^{(48)}$ have demonstrated that several of the very bands shown in our spectra are anharmonically coupled to low-frequency porphyrin modes in Ni-octaethylporphyrin (NiOEP). Thus the modes we are studying not expected to be places for vibrational energy to hang up.

Ultimately the question of whether the internal energy in the heme is Boltzmann distributed on the time scale we observe is difficult to answer without measuring population data for all of the modes in the molecule. Clearly this is beyond the ability of any one spectroscopic technique. An objection to assuming Boltzmann statistics could be raised on the grounds that the vibrational modes observed in resonant Raman spectra tend to be the same ones which are involved in optical transitions. This is due to the fact that both electronic absorption and resonant Raman scattering require good Franck-Condon overlap between upper and lower vibrational wave functions. Thus our argument that IVR is complete within a few picoseconds might not be true if the Franck-Condon modes are very loosely coupled to the non-Franck-Condon modes. That possibility, however, does not change the conclusion that the modes which we can see are in equilibrium with each other, based on similar decay dynamics on our time scale. In any case, if the IVR rate is faster than the heme-to-pocket cooling rate (which is faster than our pulse width), then our assumption of thermal equilibrium is within reason. If the IVR rate were actually slower than the cooling rate, then those non-Franck-Condon modes would
play only a minor role in the dynamics. We conclude that an assumption of Boltzmann statistics is the most reasonable basis for interpreting the data, and we are justified in assigning a temperature to the heme.

2.7.2 Theory and Simulation of Heme Cooling

Heme protein cooling has been qualitatively compared to the cooling of the S1 state of photoexcited pentacene (PTC) in a naphthalene molecular crystal host.\(^{22}\) Picosecond coherent stimulated Raman scattering data for vibrational cooling of the PTC/naphthalene molecular crystal\(^{49}\) are adequately explained by theories of VER in which the guest molecule is coupled to the phonon spectrum of the crystal host.\(^{22,50-51}\) Picosecond coherent anti-Stokes Raman scattering measurements by Dlott and coworkers have shown that the anharmonic coupling and densities of states are similar for crystalline proteins and amino acids as compared to naphthalene.\(^{22}\) Dlott estimates a cooling rate for the protein-bound porphyrin that is 25% faster than that observed in PTC/naphthalene, based on the lower threshold for rapid IVR in the larger porphyrin chromophore. The threshold for fast IVR in porphyrin can be estimated at \(\approx 700\ \text{cm}^{-1}\), based on supersonic free jet measurements of phthalocyanine by Levy and coworkers.\(^{52}\) Dlott estimates a time constant for VER in hemoglobin of \(\approx 20\ \text{ps}\), following an initial few picoseconds of fast IVR.\(^{22}\)

Henry et al.\(^{33}\) have simulated the cooling process in laser excited heme proteins using classical molecular dynamics. The mechanical relaxation of both deoxymyoglobin (deoxyMb) and cytochrome c (cyt c) were studied following the sim-
ulated absorption of both 530 and 350 nm photons. In the simulated systems, the kinetic temperature of the heme drops with two exponential time constants. The faster relaxation time in both proteins occurs with time constant 1 to 4 ps, while the slower relaxation time constant is 20 ps in cyt c and 40 ps in deoxyMb. The conclusion drawn from these simulations is that the pathway for mechanical energy transfer out of the heme consists of the many van der Waals contacts with the hydrophobic interior of the pocket. The fast component is attributed to continuous channeling of vibrational energy to highly efficient relaxation modes during the early time when rapid IVR is operative. The slow component is thought to be associated more nearly with the individual mode relaxation time constants after the vibrational energy has thermalized.

2.8 Experimental Results Show Faster Cooling

The above predictions can be tested by the picosecond Raman experiments on deoxyHb presented in this chapter. The Dlott work\(^{(22)}\) directly references cooling in hemoglobin, while the MD calculation\(^{(33)}\) addresses cooling in deoxyMb. This poses no problem for comparison, though, because the cooling times in deoxyHb and deoxyMb should not differ significantly. The chromophore in hemoglobin and myoglobin is identical, specifically protoporphyrin IX.\(^{(23)}\) Furthermore, myoglobin and the $\alpha$ and $\beta$ chains of hemoglobin have strikingly similar three dimensional structures, even though the amino acid sequences for the three polypeptide chains vary significantly.\(^{(23)}\) The only covalent link between the heme and the protein
in both cases is the proximal histidine located at the central iron atom's fifth ligand position. Thus the myoglobin simulation is directly applicable to the case of hemoglobin. The data presented in this chapter warrant comments on three theoretical expectations.

1. Within our 8 ps pulsewidth, we observe only one time scale of cooling. The cooling is best fit by a (deconvolved) 2 to 5 ps decay constant. A time constant of 2 to 5 ps is approximately that of the fast component predicted by Henry et al. Approximately 50% of the chromophore mechanical energy in the myoglobin simulation decays with the slower time constant of 40 ps. In the simulation that 40 ps component represents a kinetic temperature drop from \( \approx 425 \text{ K} \) back down to room temperature at 300 K. According to our temperature estimate, the heme vibrational temperature averaged over our 8 ps pulse is \( \approx 36 \text{ K} \) above room temperature. With the current S/N we can say that after 20 ps the heme internal temperature is within 5 K of room temperature. Thus we do not believe it is likely that we see Henry et al.'s fast component while missing the slow one due to lack of sensitivity. We assert that the deconvolved 2 to 5 ps time constant represents the major fraction of the cooling process. We conclude that vibrational energy transfer from the chromophore to the protein matrix is faster than predicted by the simulation or by comparison to the PTC/naphthalene system.

2. It is reasonable to expect that the long time component of vibrational cooling should closely reflect the relaxation times of individual modes. The decay times
of the low-lying excited vibrations in a particular mode are given by the time-
dependence of the anti-Stokes Raman signal for that mode. Figure 2.5 shows
that the cooling dynamics for heme vibrations of 671, 1424, and 1563 cm$^{-1}$
are identical within our time resolution. We do not have experimental access
to the very low frequency vibrations (< 500 cm$^{-1}$) where bottleneck vibra-
tions may hold internal energy for longer periods of time. However, we have
argued above that the 2 to 5 ps component of cooling represents the removal
of most of the excess vibrational energy. This implies that most of the internal
energy is able to escape through the efficient pathways, leaving little energy
to hang up in the bottleneck vibrations. The tight heme-pocket coupling (that
leads to fast heme-to-protein energy transfer) probably lowers the threshold
for rapid IVR. This allows mechanical energy to exit through the most efficient
relaxation channels even when the heme internal temperature is relatively low.
The 700 cm$^{-1}$ collision-free IVR threshold value estimated$^{(22)}$ for porphyrin
will be lowered by heme-pocket collisions. An additional possibility is that
the static heme-protein coupling may effectively increase the size of the por-
phyrin; this would result in an increase in the number of low frequency modes
contributing to the heme vibrational density of states.

3. Henry et al.$^{(33)}$ see no difference in the rise or decay of heating for the por-
phyrin skeletal modes compared to the side chains. We do find, within our
uncertainty, that the vinyl mode anti-Stokes transient rises and decays concur-
tently with the skeletal mode anti-Stokes transients. However, it is not clear
how to compare this to the simulation results. The vinyl modes should be con-
sidered separately from the other side chain modes in terms of coupling to the
electronic transition. The C=C double bond of the vinyl substituent group
conjugates into the porphyrin ring \( \pi \) electron system. Because the Q absorp-
tion band which we pump at 532 nm is a \( \pi \rightarrow \pi^* \) transition, we expect the vinyl
mode to experience the electronic transition just as those atoms in the skeletal
\( \pi \) system. We cannot comment experimentally on the time scale of transfer of
energy to the other side chain modes.

The simulation of Henry et al. ignores all hydrogen atoms in the system except
those directly involved in hydrogen bonds between a nitrogen and an oxygen atom.
The heme-pocket van der Waals contacts will be altered by leaving out the non
H-bonding hydrogen atoms. This clearly changes the nature of the heme-pocket
collisions by reducing the size of the heme and increasing the effective radius of
the pocket. Omitting these hydrogen atoms may be responsible for some of the
discrepancy between the simulation and the experimental results. The other obvi-
ous reason that the simulation might give a slow cooling rate is the neglect of water
solvent molecules.\(^{(33)}\) The simulation has been repeated with the inclusion of sol-
vent molecules. Preliminary analysis indicates that the inclusion of solvent has no
more than a factor of 2 effect on the simulation results.\(^{(53)}\) Thus the inclusion of
heme-solvent coupling is probably insufficient to explain the discrepancy between
the simulation and experiment.
2.8.1 Role of the Side Chain Modes

The van der Waals contacts between the heme side chains and the pocket are expected to deexcite the heme vibrations collisionally.\(^{(32-33)}\) The two vinyl and two propionate side chains of the heme expose both low- and high-frequency vibrational modes directly to collisions with the pocket. Both groups have a number of van der Waals contacts with the protein pocket and may constitute significant pathways for vibrational energy flow out of the pocket. In contrast, the single Fe-histidine covalent bond linking the heme to the globin is perpendicular to the plane of the ring. Vibrational motion along this bond is expected to be weakly coupled to the ring vibrational modes.\(^{(32)}\)

The vinyl side chains are the one set of porphyrin side chains amenable to resonant Raman study.\(^{(25)}\) We noted in figure 2.5 above that the vibrational dynamics for the vinyl \(\delta_\text{a} (=\text{CH}_2)\) mode are identical to the dynamics for both low and high frequency skeletal modes. Previous work has demonstrated the coupling of a high-frequency vinyl mode to high-frequency porphyrin skeletal modes. Spiro and coworkers\(^{(25-26)}\) performed a series of IR and resonant Raman experiments in which they deuterated the \(\text{C}_\alpha\) and \(\text{C}_\beta\) carbons of the vinyl side chains in order to study the vibrational coupling between the vinyl local modes and the porphyrin skeletal modes. Several low \((<500 \text{ cm}^{-1}\)\) and high \((>1400 \text{ cm}^{-1}\)\) frequency vinyl modes were found to be strongly coupled to skeletal modes. Figures 2.13 and 2.5 demonstrate that this coupling results in very efficient vibrational communication between the skeletal and the vinyl modes.
To the degree that the frequency shifts of Figure 2.14 can be ascribed to anharmonic coupling to hot low frequency modes, the data of Figure 2.14 illustrate that the $\delta_s$ vinyl mode is coupled to low frequency vibrations just like the porphyrin skeletal modes. Whatever the cause of shifting, it is clear that the same mechanism which shifts the porphyrin skeletal modes also operates on the vinyl side chain modes. Together these experimental observations show the coupling of the vinyl modes to low-frequency vibrations and to both medium- and high-frequency skeletal modes. This suggests that the heme can very quickly transfer vibrational energy to the side chain modes and then out of the protein, even from a mode which is seemingly isolated on the porphyrin skeleton.

### 2.9 Comparison to Previous Experimental Work

Petrich et al.\(^{(46)}\) interpret absorption transients of photoexcited heme proteins in terms of electronic relaxation from two different intermediate states. If the branching ratios for deoxyHb are similar to deoxyMb, then two-thirds of the deoxyHb excited electronic population returns to the ground electronic surface with 300 fs time constant, and the remaining one-third returns with a 3.2 ps time constant. The 2 to 5 ps VER time constant we measure represents the sum of electronic relaxation and the vibrational dynamics on the ground electronic surface. According to the interpretation of Petrich et al., what we observe must be the vibrational dynamics of the final one-third which relaxes with 3.2 ps time constant. In that case, the VER time constant we measure is dominated by electronic relaxation, and the pure vibrational relaxation time constant might actually be 1 to 2 ps.
The possibility also exists that the slower electronic relaxation of Petrich et al. is actually VER. Vibrational excitation within an electronic state is well known to produce transients lying to the red of the static, room temperature absorption band.\(^{(33)}\) In that experiment, the transient absorption ascribed to the slower intermediate electronic state indeed lies just to the red of the Soret band.\(^{(46)}\) Femtosecond absorption studies of nickel porphyrins lend credence to this suggestion.\(^{(54)}\)

Several ultrafast experimenters have approached the question of VER in photoexcited hemes. Genberg et al.\(^{(32)}\) studied the picosecond change in the temperature of the water surrounding heme proteins with a transient grating technique. That study concluded that the water interface heated up in $<20$ ps following photoexcitation of the heme chromophore. Thus they concluded that heme-to-protein vibrational energy transfer occurs on a time scale faster than 20 ps (which is the time resolution of that technique). That experiment points toward our direct Raman determination that VER occurs with a 2 to 5 ps time constant.

Petrich et al.\(^{(12)}\) have indirectly studied VER with femtosecond Stokes Raman spectroscopy. Central to their interpretation is a theory of vibrational dephasing of a high frequency mode by energy exchange with a low frequency mode.\(^{(55)}\) That theory has been calibrated for the NiOEP $\nu_{10}$ band ($\approx 1650$ cm\(^{-1}\)) in the static limit by Asher and Murtaugh.\(^{(48)}\) Petrich et al.\(^{(12)}\) applied the result of Asher and Murtaugh to interpret picosecond band shifts of the $\nu_{4}$ band following photolysis of carbonylHb. The first 10 ps of $\nu_{4}$ shifting were interpreted as cooling, while further shifting up to 95 ps was ascribed to relaxation of the strain energy of cooperativity.
However, Czernuszewicz et al.\textsuperscript{(47)} have since demonstrated that the strong temperature-dependence of the NiOEP $\nu_{10}$ band studied by Asher and Murtaugh is anomalously high. Dynamical effects which are specific to nickel porphyrin make the NiOEP $\nu_{10}$ band a poor predictor of the temperature dependence of iron porphyrin vibrational frequencies.\textsuperscript{(47)} A resonance Raman study of the deoxyHb $\nu_4$ band position at temperatures ranging from 4.2 K to 300 K by Rousseau and Friedman\textsuperscript{(56)} has shown that the position of $\nu_4$ is weakly dependent on temperature. This suggests that the 11 cm$^{-1}$ shift observed by Petrich et al. is too large to be explained by heating effects alone. Schomacker and Champion\textsuperscript{(34)} have also challenged the validity of applying the dephasing theory\textsuperscript{(55)} to the case of porphyrin VER. We cannot comment on the subpicosecond dynamics or protein relaxation (there is no deligation in our deoxyHb to trigger protein relaxation). However, we can say that the Petrich et al. conclusion of substantial cooling of the heme in 10 ps seems to be correct. The analysis and interpretation leading to this conclusion are nevertheless inconclusive.

Alden et al.\textsuperscript{(13,35)} have also questioned the work of Petrich et al.\textsuperscript{(12)} They studied the ratio of Stokes intensity to anti-Stokes intensity for the $\nu_7$ mode of deoxyHb and concluded that there is no evidence for hot ground state vibrations on the time scale of their 30 ps laser pulses. Alden et al.\textsuperscript{(13)} use a method due to Schomacker and Champion to factor out the resonance enhancement arising from the complicated electronic structure of an iron porphyrin in an attempt to extract the pure temperature effect on the Stokes/anti-Stokes ratio.
2.10 Advantages of Transient Anti-Stokes Spectroscopy

Using transient Stokes and anti-Stokes Raman data in tandem, we can unambiguously determine the time scale of vibrational cooling in the chromophore. The spectral features which characterize VER in transient Raman spectroscopy can be easily distinguished from structural changes and electronic relaxation. Unlike previous Raman studies, this experiment has determined the time constant for VER with no dependence on the accuracy of our temperature estimate. The sensitivity to hot vibrations of Alden et al.\(^{13}\) is critically dependent on the ability of the Kramers-Kronig transform to yield accurate temperature information. The same can be said of the femtosecond Stokes Raman experiment of Petrich et al.\(^{12}\) In that case, one also must be concerned that shifts due to structural dynamics might interfere with shifts due to heating. On the other hand, Stokes and anti-Stokes data together allow vibrational heating to be readily distinguished from conformational rearrangement. For a given absolute value of Raman shift, negative Stokes transient in tandem with positive anti-Stokes transient can only be due to transient heating.

The ability to measure the dynamics of specific vibrations has yielded information on modes with energy from 600 cm\(^{-1}\) to 1600 cm\(^{-1}\). Furthermore we see that, within our time resolution, these modes have identical VER dynamics. The implication that coupling between these modes is strong follows by inspection from
figure 2.5. This illustrates another distinction between detection of VER in deoxyHb in this experiment and the indirect methods employed in previous experiments. The femtosecond data of Petrich et al.\(^{(12)}\) is interpreted in terms of anharmonic coupling of a high frequency mode (\(\nu_4\) at 1358 cm\(^{-1}\)) to a low frequency mode (528±150 cm\(^{-1}\)). Spectroscopy of the high frequency mode is used in that work to infer information about VER for the low frequency mode. We have directly measured VER of both the lower (671 cm\(^{-1}\)) and higher (1563 cm\(^{-1}\)) frequency modes.

A distinct advantage of doing a transient Raman experiment at kHz repetition rates is illustrated by an examination of the peak intensities used in the present work and the 10 Hz experiment of Petrich et al.\(^{(12)}\) The peak powers in the Petrich et al. 10 Hz experiment approach \(5 \times 10^{12}\) W/cm\(^2\) for the 575 nm pump beam and \(2 \times 10^{11}\) W/cm\(^2\) for the 435 nm probe beam. Because we operate at 2 kHz, we are able to deliver one order of magnitude higher average power to the sample while maintaining a probe peak power almost two orders of magnitude lower than Petrich et al.\(^{(12)}\) Clearly, kiloHertz repetition rates present a significant advantage in avoiding nonlinear effects such as multiphoton transitions and the type of nonlinear broadening of Raman bands noted by Alden et al.\(^{(35)}\)

### 2.11 Summary

We have detected the unambiguous spectral signature of VER in photoexcited deoxyHb using picosecond Raman spectroscopy by comparing complementary Stokes and anti-Stokes dynamics. The time constant for mechanical energy
transfer from the chromophore to the protein is between 2 and 5 ps. In addition, we have estimated the vibrational temperature of the porphyrin chromophore in deoxyHb within 8 ps after photoexcitation. Analysis of the transient anti-Stokes resonant Raman data indicates a temperature jump of 36 K above room temperature. This represents an average over our 8 ps pump and probe laser pulses. The conclusion is drawn that we are observing the latter stages of cooling. A summary of major conclusions follows.

1. VER in deoxyHb has been measured directly and unambiguously for the first time. Time-dependent Stokes and anti-Stokes data directly probe the movement of vibrational population through excited levels in the ground electronic state.

2. The temperature at time zero, averaged over the convolution of our pump and probe laser pulses, is estimated to be $\approx 36$ K above room temperature.

3. VER occurs with a 2 to 5 ps time constant. The rate of VER is found to be identical within our time resolution for several modes over a wide range of frequencies.

4. Fast cooling suggests that there are very efficient conduits for vibrational energy flow out of the heme into the protein. Previous estimates and MD simulation have not adequately described this.

5. Rapid cooling also implies that vibrational energy within the chromophore can be rapidly redistributed to the conduit modes, even when the internal energy is
relatively low. The threshold for dissipative IVR may be lowered by the tight coupling to the protein.

6. The role of the side chains in providing efficient pathways for vibrational energy flow is emphasized by the picosecond spectroscopy of the vinyl Raman bands.

7. The time scale for VER measured here can be ascertained by visual inspection of the data. Previous attempts to measure the time scale for VER relied on indirect analyses. Important contributions have been made by past studies, but in no way could they be considered conclusive.

The power of utilizing complementary time-dependent Stokes and anti-Stokes spectroscopy to study large molecules of biological significance is demonstrated here. One advantage of this technique is direct interpretation of vibrational population transients. We also demonstrate sensitivity of the technique to vibrational modes over a wide range of frequencies.
3. STUDY OF REACTION COORDINATE AND VER

3.1 Significance of This Experiment

Ultrafast spectroscopic studies have provided valuable information on the photophysics and photochemistry of hemoglobin.\(^{46,57-70}\) Pico- and femtosecond spectroscopy has been extensively used to investigate the ligand binding reaction of hemoglobin by photodissociating the ligand. The most studied topics include the photodeligation of the heme, subsequent ligand (CO, O\(_2\) and NO) rebinding with the heme, and the structural changes in the coupled heme/protein system. Knowledge of the time dependence of the dissociation and recombination processes provides insight into the potential surface along the heme-ligand reaction coordinate. However the absorption of a photon by a protein-bound chromophore can, in general, trigger a very complicated series of events involving electronic, vibrational, conformational, and photochemical dynamics. All of these types of relaxation phenomena will cause transients in the visible and ultraviolet absorption spectra of a complex biomolecule such as hemoglobin. Therefore, it is often difficult to confidently link a ultrafast absorption transient with a specific relaxation mechanism. This difficulty obscures the available information about the reaction coordinate for ligand rebinding.

In this paper, we will show that the mode-specific dynamics provided by picosecond pump-probe Raman spectroscopy allow us to sort out the complex dynamics in a large biomolecule, specifically hemoglobin. Raman spectroscopy is an ideal tool for the study of photoinitiated dynamics in a large molecule, since
the vibrational spectrum is very sensitive to small changes in the structure of the molecule. In addition, we have recently shown\(^{71-72}\) that transient Raman spectroscopy can quantitatively characterize excess vibrational energy in a photoexcited molecule. The spectral signature of vibrational energy relaxation (VER) in the Stokes and anti-Stokes transient Raman spectra is quite distinct from the spectral features which result from chemical or conformational changes in the molecule. It will also be shown that ligand binding has specific effects on the vibrational spectrum which allow us to further separate ligand dynamics from other structural changes (such as those in the protein).

The photodeligation/recombination reactions for myoglobin and hemoglobin have been studied on various time scales.\(^{46,59,61-62,66,73-77}\) It has been shown that photodeligation from the heme occurs in less than 50 fs.\(^{78}\) On the same time scale a new species appears, which then decays in approximately 300 fs to a deoxy-like photoproduct.\(^{64}\) Structural information about the motion of the iron atom out of the heme plane has also been obtained. Raman scattering and other optical studies show that doming of the heme occurs within 350 fs and perhaps as rapidly as 50 fs following photodeligation.\(^{12,68,79}\) This motion of the iron and its associated tilting of Fe-proximal histidine bond have been discussed in detail.\(^{67,80}\)

Results from both absorption and Raman spectroscopic investigations show that there is a slow ligand recombination occurring on the nanosecond time scale for both oxyhemoglobin (oxyHb) and carbonylhemoglobin (carbonylHb) following photodeligation.\(^{46,59,75,77}\) However, studies which have attempted to observe
a picosecond geminate recombination following photodlingation of oxyHb have obtained several different time constants. There have also been recent conflicting results for fast geminate recombination following photodlingation of carbonylHb. Obviously our understanding of subnanosecond geminate recombination processes in hemeprteins remains incomplete.

A further question concerns what effect, if any, the excess energy left in the heme by the photon might have on the subsequent reactivity of the heme. Henry et al. have estimated that the energy of a 530 nm photon, equipartitioned among the vibrational modes of the heme ground state, will produce a temperature jump in the heme of 500-700 K. Breaking the Fe-ligand bond will take \( \approx 40\% \) of that energy in the case of photolysis of oxyHb or carbonylHb; nevertheless the heme internal temperature jump should still be substantial in those cases. A time constant of 2-5 ps has recently been measured for the dissipation of this energy from the heme into the protein in photoexcited deoxyhemoglobin (deoxyHb). It is possible that the local heating in the heme resulting from photoexcitation can alter the early stages of a picosecond geminate reaction between heme and ligand. It is also possible that the vibrational cooling dynamics could hinder identification of fast geminate recombination in the transient spectrum if the two dynamics occur on similar time scales. The influence of the VER dynamics in hemoglobin on the reaction coordinate of ligand rebinding remains unknown. Therefore, it is of fundamental significance to study and differentiate VER from dynamics of chemical change in the hemeprteins.
This study contributes to the investigation of ligand rebinding and VER occurring in oxyHb and carbonylHb by recording both Stokes and anti-Stokes transient Raman spectra of the photoexcited molecules. For a given vibrational mode, Stokes Raman data provide population information on the vibrationless level while anti-Stokes data yield population information for the low-lying excited vibrations. A vibrationally hot heme should have Stokes Raman bands which are weaker than those of an unexcited heme. Similarly, the anti-Stokes bands of a vibrationally hot heme will be more intense than those of an unexcited heme. In the transient (hot - cold) spectrum, the population changes result in a negative Stokes transient band and a corresponding positive anti-Stokes transient band. For a given mode it has been shown that this is the unambiguous spectral signature of VER. However, a structural change in the heme resulting from the photochemistry of ligand release or rebinding will alter the frequencies and/or resonant Raman enhancements for each vibration of the heme in a well-known and mode-specific fashion. The Stokes and anti-Stokes transients for that band will be negative or positive as determined by the (known) resonant Raman enhancements for the precursor and photoprod- uct. Thus, the spectral evidence for vibrational cooling and conformational changes are entirely different and can therefore be unambiguously distinguished. These results provide direct information on the reaction coordinate for ligand binding to the protein-bound heme.
3.2 Experimental

3.2.1 Apparatus

The experimental apparatus has been previously described in section 1.5 in detail. Briefly the laser system\(^{(11)}\) consists of a high repetition rate (2 kHz) chirped-pulse regenerative amplifier which provides 1 mJ pulses with an 8 ps pulse width at 1.064 \(\mu\)m. After frequency upconversion the width of the 532nm pump pulse is expected to be 6ps and the width of the 354.7nm probe pulse 5ps. A mode-locked cw Nd:YAG laser in combination with a single-mode fiber optic is used to produce the seed pulse to the regenerative amplifier. Laser excitation occurs in an o-ring sealed spinning cell. The spinning cell prevents multiple probing of the same sample spot. Collinear pump and probe laser pulses enter through a suprasil window, and the Raman scattering is collected through the same window at \(\approx 135^\circ\) with respect to the laser pulse direction. Raman scattering is dispersed by an Instruments SA U1000 double monochromator. Large (2 mm) monochromator slits were used for increased throughput of light. The signal is detected by a photomultiplier tube and processed by a gated integrator. The typical experimental time for dynamics data at a single Raman frequency is three hours for Stokes and 12 hours for anti-Stokes. In all cases, these data are recorded by taking repetitive scans of 5 to 10 minute duration and adding the results using a computer. Dynamics are obtained by varying the optical delay between the pump and probe pulses using a retroreflector and 1m long delay stage. The timing between pulses was measured experimentally
using stimulated emission from the laser dye coumarin 500A in methanol. The difference spectrum technique is used for both frequency-resolved and time-resolved data. Our difference spectrum transient Raman technique will be described below.

Dynamics data at a single Raman frequency were obtained by changing the optical delay using a Velmex translation stage outfitted with a retroreflector. Dynamics data above were fit with the equation

\[
C_1 \left[ - \exp(-k_{\text{rise}}(1)t) + \exp(-k_{\text{decay}}(1)t) \right] + \\
C_2 + C_3 \left[ - \exp(-k_{\text{rise}}(2)t) + \exp(-k_{\text{decay}}(2)t) \right].
\]

Within our uncertainty, both the negative Stokes transient and the positive anti-Stokes transient appear with <2 ps time constant.

### 3.2.2 Difference Spectrum Transient Raman Technique

We have studied oxyHb by exciting at 532 nm and probing at 355 nm. A pure transient Raman spectrum is obtained by using a difference spectrum technique. This is a crucial aspect of the experiment because the transient spectrum can be isolated from the large signal resulting from unexcited molecules. Therefore it is necessary to describe this important technique in some detail. In our experiment a single scan of the monochromator collects two spectra: the spectrum produced by the pump-probe sequence and the spectrum produced by the probe color only. This is accomplished by rapidly chopping (mechanically) the pump laser at \( \approx 100 \text{ Hz} \) and recording the total two-color signal as well as the one-color probe-only background spectrum. The pure transient Raman spectrum is produced by subtracting the background probe-only components from the spectrum obtained with the pump-probe
sequence. We do not record the pump-only spectrum, because the monochromator is scanning wavelengths which are $\approx 9000$ cm$^{-1}$ to the blue of the 532 nm pump pulses. No Raman scattering of the green pump pulses is possible at these UV observation wavelengths. In fact, no background signal is observed from 532 nm pump pulses.

The (pump-probe minus probe-only) spectrum is referred to as the subtracted or transient spectrum. Any feature appearing beyond the noise level in this spectrum is due to a transient species created by the pump photons and is characteristic of the time delay between pump and probe pulses. If photoexcitation bleaches a ground state Raman band, then the subtraction procedure will yield a negative transient band. If the pump photon creates a new Raman band or increases the intensity of an existing band, then subtraction will yield a positive transient band. It is often useful to describe a transient band by its magnitude relative to the magnitude of the corresponding probe-only band. This subtraction procedure removes all contribution of Stokes and anti-Stokes scattering from vibrational levels which are thermally populated at room temperature.

We excite oxyHb and carbonylHb with the YAG second harmonic (532 nm) and probe the Raman spectrum of the excited sample at the third harmonic wavelength (355 nm). This pump photon energy falls in the oxyHb and carbonylHb Q absorption band and has a millimolar extinction coefficient of $\approx 11.5$ for oxy-heme and $\approx 12.5$ for carbonyl-heme at 532 nm.$^{(82)}$ Using a 532 nm pump beam of an
average power of 50 mW, we deliver 25 μJ in an 8 ps pulse over a spot of radius
≈250 μm for a peak intensity of less than 5×10^9 W/cm^2 at the pump wavelength.

The probe wavelength of this experiment is 355 nm. This wavelength is near the
maximum of the N absorption band which has its origin in the π → π* transitions
of the porphyrin ring (ε_{m,M} ≈ 25.0 for oxy-heme and ε_{m,M} ≈ 20.0 for carbonyl-
heme.\(^{82}\)) The both absorption coefficients are smaller than that of deoxy-Heme
which is ε_{m,M} ≈ 32.5. Our probe intensity is somewhat smaller than our pump
intensity at approximately 3×10^9 W/cm^2. Hochstrasser and Johnson assert that the
peak power density should be less than ≈ 3×10^{10} W/cm^2 to avoid photodamage.\(^{10}\)
Both the pump and probe peak power densities used in this experiment are below
that limit.

3.2.3 Sample Preparation

The preparation of hemeproteins has been described in section 2.3.2. OxyHb
was stored in liquid nitrogen until needed. A UV-vis absorption spectrum of oxyHb
is shown in Figure 3.1A. We used a 200 mM sodium phosphate buffer solution at pH
7.5. The carbonylHb were prepared from oxyHb by directly flushing CO gas into
oxyHb for one hour. A UV-vis absorption spectrum of fresh carbonylHb is shown in
Figure 3.1B. The absorption spectra of fresh oxyHb and deoxyHb are also displayed
in Figure 3.1B for comparison. The final hemoglobin sample was prepared by di-
luting the heme concentration to about 0.8 mM with the buffer. Na_2SO_4 was used
in one experiment to calibrate transient absorbance in the sample. Due to the high
absorbance of the heme solution, it is necessary to keep the heme concentration
lower in order to see the sulfate non-resonant Raman band. The concentrations necessary to bring out the sulfate band were 100 \( \mu \)M heme, 200 mM sodium phosphate buffer, and 150 mM Na_2SO_4.

The sample was typically used for 24 hours. Cumulative effects due to long exposure of the sample to laser radiation were ruled out by recording the visible absorption spectra prior to and after prolonged photoexcitation. Figure 3.1A shows two absorption spectra of oxyHb. The solid curve is a spectrum of fresh oxyHb. The dotted curve is the absorption spectrum of the same sample taken after 24 hours of laser irradiation. The spectra are virtually identical, indicating that the sample can be used for one day without degradation.

### 3.3 Results and Interpretation

In order to interpret the dynamics of photoexcited oxyHb it is necessary to first understand how various processes affect the transient vibrational spectrum. The primary factors we consider are (1) vibrational energy relaxation or vibrational cooling (VER), (2) changes in the optical density of solution due to the absorbance of transient species, (3) resonant Raman enhancement, and (4) chemical change (structural changes which affect the vibrational frequencies).

We first demonstrate how VER can be unambiguously identified in deoxyHb. For deoxyHb there is no known photochemistry for 532nm excitation. Absorption of a photon leads to a hot ground state molecule on a subpicosecond time scale. Data are presented which show that VER can be characterized by using the Stokes and anti-Stokes spectra. Then the one-color ground state resonant Raman spectra
Figure 3.1: **Static absorption spectra of hemoglobins.** A) Static absorption spectra of oxyHb before (solid curve) and after (dotted curve) 24 hours of laser excitation. B) Static absorption spectrum of oxyHb (solid curve) overlaid with the spectra of carbonylHb (dotted curve) and deoxyHb (dash curve).
Figure 3.1: Static absorption spectra of hemoglobins.
are briefly discussed. For oxyHb, these spectra are crucial in determining the relative intensities of bands originating from the oxyHb precursor and the deoxyHb photoproduct. We also demonstrate that optical density changes do not affect the transient Raman spectra. Using the insight gained from these calibration experiments, we then interpret the transient vibrational spectra of photoexcited oxyHb and carbonylHb in terms of both chemical changes and VER.

3.3.1 Observation of Vibrational Cooling in DeoxyHb

In order to understand the experimental results for oxyHb and carbonylHb, it is necessary to look back briefly at the results of our previous study of deoxyHb.\(^{(72)}\) Figure 3.2 shows the resonant Raman spectra of deoxyHb at time zero. A pure transient resonant Raman spectrum is produced by subtracting the background probe-only components from the spectrum obtained with the pump-probe sequence. The Stokes spectrum of Figure 3.2B shows a depletion for each of the unexcited Raman bands seen in Figure 3.2A. The corresponding anti-Stokes spectrum of Figure 3.2C shows positive transient features. The observation of negative transient Stokes bands accompanied by positive anti-Stokes transient bands cannot be explained either by shifts in the frequencies of Raman bands resulting from structural changes in the heme or by picosecond transient absorption effects near the probe wavelength of 355 nm. The negative/positive pattern in the Stokes/anti-Stokes transient spectrum can only be assigned to transient heating in the ground electronic state of the heme. The negative Stokes transient signals indicate the removal of
population from the vibrationless level in the heme. Similarly, the positive anti-Stokes transients indicate an increase in the population of levels within the ground electronic state following photoexcitation. This negative-positive symmetry of the transient Raman spectra is the unambiguous spectral signature of a vibrationally hot heme.

The data of Figure 2.4 illustrate the complementary nature of transient Stokes and anti-Stokes dynamics data as a probe of VER. The bleaching of the high frequency $\nu_2$ Stokes Raman band represents the instantaneous removal of population from the vibrationless level of the ground electronic state by the absorption of a green laser photon. The prompt rise of the transient anti-Stokes signal indicates that the excited vibrations in the intermediate levels of the ground electronic state are populated very rapidly upon photoexcitation. The prompt bleaching of the Stokes Raman signal and the instantaneous rise of the anti-Stokes Raman signal show the rapid formation of a hot heme following photoexcitation. Deconvolution of the probe and pump pulses indicates that the transients appear in < 2 ps. This is consistent with rapid cooling following an electronic relaxation known to occur with a 300 fs time constant.\(^{(46)}\) Clearly the recovery of the depleted $\nu_2$ Stokes transient signal and the rapid decay of the positive $\nu_2$ anti-Stokes transient signal are the dynamical spectral signature of VER. The deconvolved time constant of VER for deoxyHb is about 2-5 ps.
Figure 3.2: **Time zero Raman spectra of deoxyHb.** This figure demonstrates the spectral signature of a vibrationally hot deoxyHb transient. Figure 3.2A shows the ground state Stokes probe-only Raman spectrum. Figures 3.2B and C show pure transient picosecond Raman spectra obtained by subtracting the 355nm probe-only spectrum from the two-color spectrum. Figure 3.2B shows depleted Stokes Raman scattering. Positive anti-Stokes transient bands at the position of \( \nu_7, \nu_{16}, \nu_4 \), the vinyl mode, \( \nu_3, \nu_2 \), and \( \nu_{37} \) argue for Boltzmann distribution of heme internal modes, since all observed modes appear to be hot. Each of the six individual spectra are scaled independently to fill the height of the plot. The marked bands are assigned\(^{25-26,35}\) as \( \nu_7 \) at 672 cm\(^{-1}\), \( \nu_{16} \) at 755 cm\(^{-1}\) (shoulder on quartz band), broad quartz window bands centered at 790 cm\(^{-1}\) (clearly labeled), weak \( \nu_{32} \) on top of quartz bands at 790 cm\(^{-1}\), \( \nu_4 \) at 1359 cm\(^{-1}\), a vinyl mode at 1430 cm\(^{-1}\), \( \nu_3 \) at 1475 cm\(^{-1}\), and a broad group of overlapping bands including \( \nu_2 \) plus \( \nu_{37} \) plus others centered at 1580 cm\(^{-1}\). The Stokes negative transients are 6 to 8\% of the corresponding unexcited band heights. Figure 3.2C shows anti-Stokes Raman scattering at time zero. The positive anti-Stokes transient band height for \( \nu_7 \) is \( \approx \) 12\% of the corresponding unexcited anti-Stokes band height (not shown). The positive anti-Stokes transient band height for \( \nu_4 \) is \( \approx \) 40\% of the corresponding unexcited anti-Stokes band height (not shown). Frequency units are in cm\(^{-1}\). Unexcited spectra are not smoothed. Transient data have been minimally smoothed with Fourier transform filters ranging from three to six points.
Figure 3.2: Time zero Raman spectra of deoxyHb.
3.3.2 Effects of Transient Absorption

It is possible that the transient Raman spectrum can be affected by changes in the optical density of the solution. For example, Raman bands of the ground electronic state could change intensity due to a simple transient absorbance change in the sample. Changes in the optical density of the solution alter the effective path length of the Raman probe pulse in the solution. Transient absorption might arise from an electronically excited minority species such as those seen in model porphyrins \(^{44-45}\), from vibrational heating, or from the photochemistry of ligand dissociation. The importance of optical density changes is evaluated by adding sulfate ion to the hemoglobin solution and then looking for a transient feature associated with the sulfate ion Raman band. Presumably, the sulfate band will exhibit no dynamics unless there is a significant change in the optical density of the solution.

The data of Figure 3.5 show that picosecond transient absorption effects are not responsible for the transient heme Raman bands of Figures 3.6 and 3.7. Figure 3.5 is a time zero Stokes Raman spectrum of oxyHb with sodium sulfate. Figure 3.5A shows the ground state Raman bands observed by probing at 355nm, and the corresponding transient Raman spectra are shown in Figure 3.5B. In Figure 3.5A we can obviously see the C\(_6\)-vinyl stretching band \(\nu_{44}\) at 1129 cm\(^{-1}\), the oxidation state marker band \(\nu_4\) at 1377 cm\(^{-1}\), the sulfate band at 982 cm\(^{-1}\), and other small Raman bands. Figure 3.5B shows significant bleaching of the transient Raman signal at 1377 cm\(^{-1}\), a little bleaching at 1129 cm\(^{-1}\) and almost no transient Raman signals at any other band. Within the signal-to-noise of the scan, any
**Figure 3.3:** Stokes Raman spectra of oxyHb with Na$_2$SO$_4$. A) The unexcited spectrum. The second most prominent feature is the 982 cm$^{-1}$ Raman band of the sulfate ion. All features to higher frequency are due to Raman scattering from oxyHb: $\nu_{44}$ at 1129 cm$^{-1}$, $\nu_{30}$ at 1172 cm$^{-1}$, possibly $\nu_{13}$ at 1229 cm$^{-1}$, $\nu_4$ at 1377 cm$^{-1}$, the vinyl side chain $\delta_s (=\text{CH}_2)$ at 1426 cm$^{-1}$. The vertical dashed lines are provided to mark corresponding features in frames A and B. B) The transient spectrum at time zero. The most obvious depletion is at the $\nu_4$ band, while there is a little bleaching of the $\nu_{44}$ band. There is no apparent depletion for the other heme bands because of the lower concentration of oxyHb necessary to bring out the sulfate band. The most important feature is that sulfate ion Raman band shows no measurable transient feature, whereas the $\nu_4$ transient is strong and obvious. This indicates that heme Raman band transients are due to pure vibrational dynamics, not transient absorption. The transient spectrum has been smoothed with a three-point Fourier transform filter. Frequency units are cm$^{-1}$. 
Figure 3.3: Stokes Raman spectra of oxyHb with Na$_2$SO$_4$. 
transient feature at the sulfate band position in Figure 3.5B is limited to ± 1% of the probe only unexcited sulfate band in Figure 3.5A. In contrast, the negative transient for the \( \nu_4 \) band in Figure 3.5B is 15% of the unexcited \( \nu_4 \) band intensity in Figure 3.5A. This is a factor of 15 greater than the limit set for the sulfate band. The positive oxyHb transient Raman bands in the high frequency anti-Stokes region in Figure 3.7B are \( \approx 22\% \) of the unexcited anti-Stokes band heights. Any picosecond absorbance change in the sample is too small to account for the prominent transient bands shown in Figure 3.6 and 3.7.

3.3.3 Dynamics of Photoexcited OxyHb and CarbonylHb

3.3.3.1 Procedure for Interpreting Vibrational Dynamics

For oxyHb and carbonylHb, the transient vibrational spectrum is expected to be quite different from the deoxyHb spectra previously discussed. For oxyHb and carbonylHb, photoexcitation produces a new species—the deoxy-like photoprod-uct which we will write as deoxyHb'. The vibrational spectrum of this new species has bands which occur at or very near the band frequencies of the precursor. In ad-
dition, photoexcitation can also produce a vibrationally hot oxyHb or carbonylHb. Ideally, it would be convenient to tune the Raman probe frequency to enhance se-
lectively the precursor or deoxyHb' photoprod-uct. However, this is not possible with the current apparatus. In the present experiment each vibrational feature in the transient spectrum must therefore be interpreted as signal resulting from the combination of hct precursor and deoxyHb'. Note that precursor bands which are
not affected by the pump pulse simply subtract out of the transient spectrum. However, the interpretation is not hopeless because we know the relative intensities and frequencies of bands corresponding to the precursor and deoxyHb photoprocess.

Figures 3.3 and 3.4 are, respectively, resonant Raman spectra of hemoglobin in the Stokes and anti-Stokes regions using the 355 nm probe pulse only. Both spectra are scaled on the basis of the intensity of the sulfate Raman band. The assignments of bands are similar to those in our previous work.(71) A large quartz band is prominent on the low frequency edge of all the spectra. There is also a broad silica band centered at 790 cm$^{-1}$ which overlaps with the $\nu_{32}$ heme Raman band peaked at 793 cm$^{-1}$.

The band positions and ratios of intensity for oxyHb, carbonylHb and deoxyHb at $\nu_4$, $\nu_7$ and $\nu_{16}$ band are listed in Table 3.1. The resonant Raman enhancements for the three hemoglobin molecules are completely different. In fact, the resonant Raman enhancements for the Stokes and anti-Stokes band of the same vibrational mode vary independently of one another. In addition, band shifts for different species are also apparent. For example, the band position of the $\nu_4$ mode for oxyHb and deoxyHb is 1377 cm$^{-1}$ and 1355 cm$^{-1}$ respectively. However, for the $\nu_{16}$ mode, the band position is 755 cm$^{-1}$ for both oxyHb and deoxyHb.

These relative resonant Raman enhancements along with the frequency shifts for different species indicate what species will dominate the transient vibrational spectrum at a specific frequency. Note that this method assumes that the vibrational spectrum of deoxyHb' is very similar to deoxyHb. Table 3.1 contains the
Figure 3.4: **Stokes resonant Raman spectra of hemoglobins.** The band assignments have been previously documented\(^{(25,26,41)}\) A) CarbonylHb spectrum B) OxyHb spectrum C) DeoxyHb spectrum All three spectra are scaled to give the same intensity of sulfate band at 982 cm\(^{-1}\). These spectra show the different resonant Raman enhancements and band shifts of the three hemoglobins in the Stokes region. It is obvious for the \(\nu_4\) band that the resonant Raman enhancement is much larger for oxyHb (at 1377 cm\(^{-1}\)) and carbonylHb (at 1373 cm\(^{-1}\)) than for deoxyHb (at 1355 cm\(^{-1}\)). However the \(\nu_{16}\) band resonant Raman enhancement is bigger for deoxyHb than for oxyHb. Table 3.1 contains quantitative details.
Figure 3.4: Stokes resonant Raman spectra of hemoglobins
Figure 3.5: **Anti-Stokes resonant Raman spectra of hemoglobins.** A) CarbonylHb spectrum B) OxyHb spectrum C) DeoxyHb spectrum All three spectra are scaled to give the same intensity of sulfate band at 982 cm$^{-1}$. These spectra show the different resonant Raman enhancements and Raman shifts of the three hemoglobins in the anti-Stokes region. For example, at $\nu_{16}$ band the RRE is much larger for deoxyHb than for oxyHb or carbonylHb. AT the $\nu_4$ and $\nu_7$ band positions, the resonant Raman enhancements is bigger for deoxyHb than for oxyHb or carbonylHb.
Figure 3.5: Anti-Stokes resonant Raman spectra of hemoglobins.
**Table 3.1. Intensities of Raman bands of hemeproteins.** Relative intensities for ground state Raman bands measured with the 355nm probe laser in the absence of the pump laser. All bands are measured relative to the sulfate ion Raman band. The concentration of hemoglobin and sulfate ion was constant for each species so that the true relative intensities for all species is reflected in the values listed in the table. See text for details.

<table>
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<th>Rel. Int.</th>
<th>$\nu_{16}$ (cm$^{-1}$)</th>
<th>Rel. Int.</th>
<th>$\nu_4$ (cm$^{-1}$)</th>
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<table>
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<th>$\nu_{16}$ (cm$^{-1}$)</th>
<th>Rel. Int.</th>
<th>$\nu_4$ (cm$^{-1}$)</th>
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</table>
vibrational frequencies and relative intensities of the Stokes and anti-Stokes bands for the primary bands used to interpret the heme dynamics below. The information in this table is used to determine what will happen in the picosecond transient spectrum.

3.3.3.2 Dynamics of OxyHb $\nu_4$ Band

The $\nu_4$ band is the well studied oxidation-state marker band. The position of this band is sensitive to the $\pi$ electron density on the tetrapyrrole ring which is determined by the oxidation state of iron atom and shifts with removal of the ligand. Table 3.1 shows that the Stokes $\nu_4$ in oxyHb is shifted 22 cm$^{-1}$ higher from the corresponding deoxyHb band and is 3.6 times as intense. Probing $\nu_4$ at 1377 cm$^{-1}$ in the Stokes spectrum provides a clean measure of the oxyHb dynamics. In the anti-Stokes spectrum the resonant enhancements for the deoxyHb band and oxyHb band are similar. However, the 22 cm$^{-1}$ frequency difference between these two bands should make the oxyHb band dominate the signal at 1377 cm$^{-1}$ in the anti-Stokes spectrum.

Figures 3.6 and 3.7 show the frequency spectra at time zero for oxyHb in the Stokes and anti-Stokes regions, respectively. Consider the oxyHb $\nu_4$ band at 1377 cm$^{-1}$. In the Stokes transient spectrum $\nu_4$ depletes whereas in the anti-Stokes transient spectrum it is a positive band. We have shown that the 1377 cm$^{-1}$ frequency is dominated by oxyHb dynamics in both the Stokes and anti-Stokes spectrum. We can then interpret the dynamics of this band in the same way as the deoxyHb vibrational dynamics previously published$^{(71)}$. The oxyHb $\nu_4$ band has the
negative-positive symmetry in the Stokes/anti-Stokes spectra which is characteristic of a vibrationally hot heme.

The dynamics of the oxyHb $\nu_4$ are shown in Figure 3.8 in the Stokes (9A) and anti-Stokes (9B) regions. The dynamics of the Stokes spectrum show a prompt depletion followed by two recovery times of 5 ps and 1000 ps ± 500 ps. The initial depletion of the Stokes spectrum is a total result of the photochemical conversion of oxyHb to deoxyHb and vibrational heating.

The rise of the anti-Stokes spectrum indicates that transient oxyHb is formed with an excess internal energy. The initial loss of oxyHb molecules is not observed at time zero in the anti-Stokes spectrum. Apparently, photoexcitation results in the formation of a substantial fraction of hot oxyHb. The increased internal temperature results in a positive transient which offsets the population bleaching at early times in the anti-Stokes spectrum. Ultimately the anti-Stokes signal decays below the baseline at times longer than 20 ps. This depletion reflects the population bleaching resulting from the photochemistry. It is important to note that the population bleach at long times in both the Stokes and anti-Stokes spectrum verifies that the vibrational spectrum at $1377 \text{ cm}^{-1}$ originates from oxyHb.

The Stokes spectrum in Figure 3.8 has a signal which rises from zero to 40 ps. The kinetic rate for this process is the same as that occurring with opposite sign in the anti-Stokes spectrum. The process responsible for these dynamics is assigned to vibrational cooling because of the negative-positive symmetry of the Stokes and
**Figure 3.6: Picosecond Stokes Raman Spectra of OxyHb.**

A) The unexcited Stokes Raman spectrum using only the 355 nm probe. 
B) Transient Raman spectrum at time zero. All the bands in this spectrum are due to the effect of the 532 nm pump pulse. OxyHb transient bands are negative due to removal of population from the vibrationless level of the ground electronic state by the optical excitation. There is a positive transient signal at 755 cm\(^{-1}\) because the photodeligation of oxyHb produces a photoproduct of deoxy-like hemoglobin which has stronger resonant Raman scattering than oxyHb. The negative/positive pattern of the other Stokes/anti-Stokes transient bands is due to VER. Dashed lines show the baselines for the transient spectra. Note that all spectra are independently scaled, so the transient spectra have different baselines. As in the assignment of bands in Figure 3.2, the marked bands are assigned as \(\nu_7\) at 676 cm\(^{-1}\), \(\nu_{16}\) at 755 cm\(^{-1}\) (shoulder on quartz band), broad quartz window bands centered at 790 cm\(^{-1}\) (clearly labeled), weak \(\nu_{32}\) on top of quartz bands at 793 cm\(^{-1}\), \(\nu_4\) at 1377 cm\(^{-1}\), a vinyl mode at 1426 cm\(^{-1}\), \(\nu_3\) at 1503 cm\(^{-1}\), \(\nu_2\) at 1591 cm\(^{-1}\), and \(\nu_{37}\) at 1637 cm\(^{-1}\).\(^{25-26,41}\) The quartz bands are absent in the subtracted spectrum. The transient spectrum has been smoothed with a five-point Fourier transform filter. Frequency units are cm\(^{-1}\).
Figure 3.6: Picosecond Stokes Raman Spectra of OxyHb.
Figure 3.7: **Picosecond anti-Stokes Raman Spectra of OxyHb.** A) The unexcited anti-Stokes Raman spectrum using only the 355 nm probe. B) Time-zero transient spectrum of oxyHb following absorption of a 532 nm. All the oxyHb Raman modes observed in the unexcited spectrum show positive transients here. The positive transients are interpreted as vibrational heating on the ground electronic surface due to photoexcitation at 532 nm in comparison with Figure 3.2C. Dashed lines show the baselines for the transient spectra. Note that all spectra are independently scaled, so the transient spectra have different baselines. The quartz band is absent in the transient spectrum. The assignment of bands is identical with those for the Stokes spectrum in Figure 3.6A. Because of the much weaker Raman signals in anti-Stokes region, not every Raman band can be well-resolved at our S/N level. For example, we cannot resolve the \( \nu_2 \) and \( \nu_{37} \) bands. However, we can obviously see \( \nu_7 \) at 676 cm\(^{-1} \), \( \nu_{16} \) at 755 cm\(^{-1} \), a broad quartz band centered at 790 cm\(^{-1} \), \( \nu_4 \) at 1377 cm\(^{-1} \) and a broad oxyHb Raman band centered at 1565 cm\(^{-1} \). The transient feature at 793 cm\(^{-1} \) corresponds to \( \nu_{32} \) which is obscured in the probe-only spectrum by the quartz band. The transient spectrum has been smoothed with a five-point Fourier transform filter. Frequency units are cm\(^{-1} \).
Figure 3.7: Picosecond anti-Stokes Raman Spectra of OxyHb.
Figure 3.8: **Dynamics of VER and GR at $\nu_4$ of oxyHb.** The Stokes and anti-Stokes transient Raman intensity at $\nu_4$ (1377 cm$^{-1}$) is plotted as a function of delay time between pump and probe pulses. At this band oxyHb dominates the observed dynamics because the resonant Raman enhancement is stronger for oxyHb than for deoxyHb and because the oxyHb $\nu_4$ band is shifted 22 cm$^{-1}$ higher than the corresponding deoxyHb band. A) The Stokes dynamics of the $\nu_4$ band. The prompt depletion is assigned to population removal from the lowest vibrational levels of the $\nu_4$ mode on the ground electronic surface, the fast recovery to vibrational cooling of hot oxyHb molecules and the slow recovery to geminate recombination of photoproduct deoxy-like hemoglobin with the O$_2$ ligand. B) The anti-Stokes dynamics of the $\nu_4$ band. The fast rise of the transient Raman signal is assigned to vibrational heating following photoexcitation, the fast decay to vibrational cooling, and the slow recovery to geminate recombination. The fits show that the deconvolved time constant of VER is about 2-5 ps, while the slow geminate recombination occurs with $\approx$ 1000 ps $\pm$500 ps time constant. No fast geminate recombination is observed on 2-100 ps time scale. The baselines are shown by the dashed lines. The circles are the experimental data points. The solid line is the fit to the dynamics. The spectra of frames A and B are independently scaled.
Figure 3.8: Dynamics of VER and GR at $\nu_4$ of oxyHb.
anti-Stokes spectra. These dynamics cannot be the result of geminate recombination. If geminate recombination of $O_2$ with the heme was responsible for the rise of the Stokes spectrum, a transient signal with a slope of the same sign should be observed in the anti-Stokes spectrum. In fact, this is not observed.

For times much greater than 100 ps (not shown but see reference (83)) the signals in both the Stokes and anti-Stokes spectra return toward the baseline. This results from the $\approx 1000$ ps geminate recombination reported here, in addition to slower recombination previously reported.(77)

It might at first seem curious that a positive $\nu_4$ band at 1355 cm$^{-1}$ corresponding to deoxyHb' is not observed in the transient frequency spectrum of Figure 3.6. There are two reasons why this is not observed. First the oxyHb band is 3.6 times larger than the corresponding deoxyHb' band. Secondly, there is a small band in the oxyHb spectrum at approximately 1355 cm$^{-1}$ which offsets the chances of observing the deoxyHb' band at the same frequency. This oxyHb band at 1355 cm$^{-1}$ has been previously documented.(41)

3.3.3.3 Dynamics of DeoxyHb' $\nu_{16}$ Band

Table 3.1 indicates that the Stokes $\nu_{16}$ band is 2.7 times as intense for deoxyHb compared to oxyHb. In Figure 3.6 the positive transient of the $\nu_{16}$ band at 755 cm$^{-1}$ is observed. This is clearly a band associated with the deoxyHb' photoproduct. There is no way to account for a positive band attributed to oxyHb in the Stokes spectrum. In the anti-Stokes spectrum the $\nu_{16}$ band is even more prominent for deoxyHb as compared to the ligated hemes. Table 3.1 indicates that $\nu_{16}$ is at least
three times more intense for deoxyHb compared to oxyHb. The dynamics of the vibrational spectrum at 755 cm\textsuperscript{-1} is therefore expected to be largely that of deoxyHb.

Figure 3.9 shows the dynamics of the vibrational spectrum at 756 cm\textsuperscript{-1}. The anti-Stokes spectrum rises promptly and decays to a steady state value with a (deconvolved) time constant of 5 ps. The Stokes spectrum rises with a (deconvolved) time constant of 5 ps. Both Stokes and anti-Stokes spectra are expected to rise because of the newly formed deoxyHb' photoproduct. The fact that the anti-Stokes spectrum decays with nearly the same time constant as the rise in the Stokes spectrum indicates that the deoxyHb photoproduct is undergoing vibrational cooling. Again, this is the same negative-positive symmetry previously observed for vibrational cooling. For the deoxyHb' $\nu_{16}$ band, the negative-positive symmetry of vibrational cooling is superimposed on the deoxyHb' formation kinetics. At long times (>40 ps) a positive offset is observed in both spectra in Figure 3.9 which verifies that the $\nu_{16}$ band is largely the result of deoxyHb'.

### 3.3.3.4 Dynamics of carbonylHb $\nu_4$ Band

Table 3.1 shows that the carbonylHb Stokes band at 1373 cm\textsuperscript{-1} is 5.8 times the intensity of the deoxyHb' $\nu_4$ band at 1355 cm\textsuperscript{-1}. Vibrational dynamics at 1373 cm\textsuperscript{-1} should be entirely that of the carbonylHb precursor. For the anti-Stokes region Table 3.1 indicates that it is hard to distinguish the band from the carbonylHb precursor from that of deoxyHb'. This is particularly true when the high quantum yield for photodeligation is taken into account.
Figure 3.9: **Dynamics of VER and GR at $\nu_{16}$ of deoxyHb'.** The Stokes and anti-Stokes transient Raman intensity at $\nu_{16}$ (755 cm$^{-1}$) is plotted as a function of delay time between pump and probe pulses. Because the resonant Raman enhancement is much larger for deoxyHb than for oxyHb at $\nu_{16}$ in both the Stokes and anti-Stokes regions, the observed dynamics are dominated by the photoproduction deoxyHb'.

A) The Stokes dynamics of the $\nu_{16}$ band. The fast rise is assigned to vibrational cooling of photoproduction deoxy-like hemoglobin, that is, the appearance of cold deoxyHb. The slow decay is attributed to geminate recombination.

B) The anti-Stokes dynamics of the $\nu_{16}$ band. The prompt rise of the transient Raman signal is assigned to the appearance of vibrationally hot deoxyHb', the fast decay to the vibrational cooling of deoxyHb', and the slow recovery to geminate recombination. The circles are the experimental data points. The solid line is the fit to the dynamics. The fit show that the deconvolved time constant of VER is about 2-5 ps, while the geminate recombination time constant is $\approx$ 1000 ps $\pm$500ps. No fast geminate recombination is observed on 2-100 ps time scale. The spectra of frames A and B are independently scaled. The baselines are shown by the dashed lines.
Figure 3.9: **Dynamics of VER and GR at $\nu_{16}$ of deoxyHb'.**
Figure 3.10: **Dynamics of VER and GR for CarbonylHb**. The Stokes and anti-Stokes transient Raman intensity at $\nu_4$ (1373 cm$^{-1}$) is plotted as a function of delay time between pump and probe pulses. Because the resonant Raman enhancement is 5.8 times larger for carbonylHb than for deoxyHb at $\nu_4$ in the Stokes region, the observed Stokes dynamics are dominated by carbonylHb. A) The Stokes dynamics of the $\nu_4$ band of carbonylHb. The instantaneous bleaching is assigned to population removal from the vibrationless level in the ground electronic state of carbonylHb. No fast recovery of the bleach (due to VER of hot carbonylHb) is observed in this spectrum, because most of the photoexcited carbonylHb undergoes deligation. The slow decay is ascribed to geminate recombination of photoproduct deoxyHb$'$ with CO ligand. B) The anti-Stokes dynamics of the $\nu_4$ band of carbonylHb. Signal is a mixture of carbonylHb and deoxyHb$'$ but is assigned as largely deoxyHb$'$ as a result of the large quantum yield for photodeligation. The fast rise of the anti-Stokes transient is assigned to the appearance of vibrationally hot deoxyHb$'$, while the fast decay is due to cooling of deoxyHb$'$. The fit shows that the deconvolved time constant of VER is about 2-5 ps and slow geminate recombination with $\approx 1000$ ps ±500 ps time constant. No fast geminate recombination is observed on 2-100 ps time scale. The circles are the experimental data points. The solid line is the fit to the dynamics. The spectra of frames A and B are independently scaled. The spectra of frames A and B are independently scaled.
Figure 3.10: Dynamics of VER and GR for CarbonylHb.
The dynamics of $\nu_4$ at 1373 cm$^{-1}$ for carbonylHb is shown in Figure 3.10. The Stokes spectrum shows an immediate depletion with no recovery on the 100 ps time scale. The short time recovery due to vibrational cooling observed in the $\nu_4$ band of oxyHb is absent for carbonylHb. We believe that this is a consequence of the very high quantum yield of dissociation for carbonylHb compared to oxyHb(77). There are simply not enough photoexcited carbonylHb molecules remaining in the ligated form to see the vibrational cooling process.

The Stokes dynamics indicate that there is no geminate recombination observed on the 100 ps time scale. If geminate recombination occurred, we would expect to observe a prompt decay accompanied by a rise with time constant equal to that of geminate recombination. Even a geminate recombination time constant of 2 ps could easily be observed with the 5 ps probe pulse. In fact, the VER dynamics prominently shown in Figures 2.4, 3.8, and 3.9 are an example of a 2-5 ps dynamics convolved with the experimental time resolution. Therefore, the absence of a recovery in Figure 3.10 indicates that geminate recombination takes less than 2 ps or more than 100 ps.

The anti-Stokes spectrum in Figure 3.10 shows the prompt rise and decay of vibrationally hot molecules. Again, we cannot distinguish between carbonylHb and deoxyHb in the anti-Stokes region for $\nu_4$. However it is reasonable to assign these dynamics to deoxyHb since the quantum yield of photodissociation is about 0.65 to 0.8(77,84) measured on the nanosecond timescale. The cooling time is similar to that discussed above for oxyHb and deoxyHb.
3.3.3.5 Evidence for No Fast Geminate Recombination

As described above, the rapid decay dynamics immediately following the instantaneous rise of signal in the anti-Stokes spectrum is complemented by fast recovery in the Stokes spectrum. This is the dynamical spectral signature of VER.

In contrast, geminate recombination must appear in the same way (same sign and decay rate) in both the Stokes and anti-Stokes spectra. Similarly, any structural change at the heme should give rise to the same signal in both the Stokes and anti-Stokes spectra. However, we have not seen such dynamics on a 2 to 100 ps time scale. This is evidence that geminate recombination does not occur on a 2 - 100 ps time scale. Figure 3.8 and 3.10 show the dynamics of ligated heme. If geminate recombination had occurred on a 2 to 100 ps time scale, there should have appeared a positive-going transient signal in both Stokes and anti-Stokes dynamics in Figure 3.8 and 11. Similarly, there should have appeared a negative-going dynamics in Figure 3.9A and 3.9B, where the photodeligated deoxy-like hemoglobin dominates the measured dynamics. The experimental results confirm the non-existence of fast geminate recombination on 2 to 100 ps time scale.

3.3.3.6 Existence of Slow Ligand Rebinding

As stated above, we have not seen a fast geminate recombination occurring on 2 to 100 ps time scale. However, we do observe the existence of slower ligand rebinding for both oxyHb and carbonylHb. There is a slow component in Figure 3.8, 3.9 and 3.10A, which has a long time constant. We have also observed the dynamics out to 800ps for oxyHb (not shown but see reference (83)) and in this case the
slow recovery is much more obvious. Clearly the same dynamical behavior in both Stokes and anti-Stokes (both show the same slow recovery) indicates the recovery of population of oxyHb (or carbonylHb) following photodeligation. It is well-known\(^{46,73,75,81,85}\) that slow ligand rebinding with the heme on a nanosecond time scale occurs for both oxyHb and carbonylHb. The deconvolved geminate rebinding time constant for oxyHb\(^{(83)}\) is 1000 ± 500 ps.

3.3.3.7 Summary of Experimental Results

1. For deoxyHb a negative/positive pattern is observed for the Stokes/anti-Stokes transient spectra which is the unambiguous spectral signature of vibrational cooling. These spectral features occur in both the transient frequency spectra and dynamics spectra. The cooling time is 2-5 ps.

2. Relative intensities and frequencies for bands in the Stokes and anti-Stokes spectra were recorded for deoxy, oxy, and carbonylHb. These spectra account for different resonant Raman enhancements and allow the spectral changes responsible for structural change at the heme to be separated from the dynamics of vibrational cooling.

3. Starting with oxyHb, the transient \(\nu_4\) spectra show the appearance of vibrationally hot oxyHb following photoexcitation of oxyHb with a 532 nm pulse. The cooling time is 2-5 ps.

4. Starting with oxyHb, the transient \(\nu_{16}\) band probes deoxyHb'. The dynamics exhibit the negative-positive symmetry characteristic of vibrational cooling.
This signal is superimposed on the growth of deoxyHb' in the Stokes and anti-Stokes region. This indicates the appearance of vibrationally hot deoxyHb' upon 532 nm photoexcitation of oxyHb. The cooling time is 2-5 ps.

5. Starting with oxyHb, the transient $\nu_4$ and $\nu_{16}$ bands show that there is no geminate recombination occurring on a 2 to 100 ps time scale. Only vibrational cooling is observed. Once deoxyHb is formed, there are no frequency shifts associated with the $\nu_4$ and $\nu_{16}$ bands which would indicate fast structural rearrangement in the heme. Apparently, the excess internal energy in the heme does not affect the ligand rebinding reaction.

6. For oxyHb, a slow (1000 ps ± 500 ps) recovery is observed which is assigned to slow geminate recombination.

7. For carbonylHb, the $\nu_4$ dynamics indicate a pulse width limited photodissociation. Geminate recombination does not occur on a 2 to 100 ps time scale.

8. For carbonylHb, the $\nu_4$ anti-Stokes dynamics indicate vibrational cooling of the deoxyHb' photoproduct with a 2-5 ps time constant.

### 3.4 Discussion

#### 3.4.1 Reaction Coordinate for Ligand Rebinding

Geminate recombination dynamics in hemoglobins$^{(46,62,73-76,81,85)}$ have been extensively studied. Previous investigations agree that there are nanosecond ligand rebinding processes following photolysis of oxyHb and carbonylHb.$^{(46,66,73,75,85)}$ Our results show this recombination process clearly in Figures 3.8, 3.9 and 3.10.
A number of authors have interpreted picosecond absorption transients as geminate recombination processes. Chernoff et al.(59) observed 200 ± 70 ps transient in oxyHb (but not in carbonylHb or oxymyoglobin) which was assigned to geminate recombination. Friedman et al.(75) also reported geminate recombination results for oxyHb on the 100 to 200 ps time scale using transient absorption spectroscopy. All previous studies agree that there is no geminate recombination for photolyzed carbonylHb on a 10 to 1000 ps time scale. Recently, Petrich et al.(46) have reported absorption transients of 2.5 - 3.2 ps which exist in the femtosecond dynamics of all photoexcited heme proteins. They interpret this result as geminate recombination from a deligated but highly reactive excited electronic state that relaxes back to the ligated form.(46)

Our data indicate that there are no detectable geminate recombination events for either oxyHb or carbonylHb which have a 2 to 200 ps time constant under the pH and temperature conditions used in this experiment. However, we do observe a \( \tau = 1000 \text{ ps} \pm 500 \text{ ps} \) time constant of geminate recombination for oxyHb in the dynamics of the oxy precursor. This appears to conflict with the 200 ps result of Friedman et al.(75) However, that reference mentions time scales for the biexponential decay dynamics without specifying whether or not the data were actually fit to a biexponential function. We have digitized the data from Friedman et al.(75) and fit that data to a biexponential. Their data are very well fit by a biexponential decay with 1/e time constants of 667 ps and 6250 ps. Therefore the faster time constant
(≈ 700 ps) of Friedman et al. actually agrees with our result within experimental error. We also observe a recombination time constant in the nanosecond range in the dynamics for the carbonyl precursor. We identify geminate recombination in our transient spectra based on the known, mode-specific changes that ligand binding causes in the Raman spectra of the heme.

We can also comment on the 2.5 ps geminate recombination for all ligands inferred from transient absorption by Petrich et al. In our transient Raman experiment we have studied the vibrational dynamics of the heme and have found that no geminate recombination occurs on a 2 to 100 ps time scale for either oxyHb or carbonylHb. We set the lower limit at 2 ps because that is the shortest time constant that we obtain by deconvolution for the very fast VER observed in deoxyHb.\(^{72}\) In fact the only transients we see in the transient Raman spectra of photoexcited oxy and carbonylHb are 2 - 5 ps transients which must be assigned to VER, as in the case of deoxyHb. If geminate recombination occurred, it should appear in the dynamics of Figure 3.9 as a loss of deoxyHb in both the Stokes and anti-Stokes spectra as previously noted. Our results indicate that the 2.5 ps transient absorption dynamics observed by Petrich et al.\(^{46}\) cannot be due to geminate recombination.

Recent femtosecond IR experiments on carbonylHb photolysis\(^{81}\) also contradict the existence of the 2 - 3 ps geminate recombination claimed by Petrich et al. In that experiment Hochstrasser and co-workers have directly probed the CO dynamics and observed heme-CO recombination with a time constant in the range
of nanoseconds. Their results indicate that no geminate recombination occurs on the time scale of 10 ps.

Geminate recombination is affected by many factors. In an oversimplified picture geminate recombination is largely affected by energetic and diffusional factors. The lack of fast (∼2 - 100 ps) geminate recombination suggests that the barrier for ligand rebinding forms promptly in oxyHb and carbonylHb. This barrier can be divided into a structural, electronic, and heating barrier.

The structural change of the heme has been discussed by Friedman and co-workers. Upon photodigitation, the low spin Fe quickly changes to high spin, thus the central Fe becomes too large to be in the original position. The iron rapidly moves out of the heme plane within several hundreds of femtoseconds in order to reduce the repulsive potential between the imidazole and pyrrole nitrogen. This doming effect dramatically affects the dynamics of geminate recombination. For geminate recombination to occur, the iron in the heme has to move back onto the heme plane. The repulsive energy between the histidine and the heme must increase, and this raises the barrier height for ligand rebinding. This fast structural change of the heme will obviously influence the dynamics of fast geminate recombination.

There are other potential contributions to the reaction barrier, such as the so-called steric barrier and the electronic barrier. The steric barrier arises when neighboring atoms rearrange to fill the site vacated due to the leaving of ligand. The electronic barrier is due to the fact that heme-ligand recombination appears
to be singly spin-forbidden for $O_2$ and NO ligands and doubly spin-forbidden for CO.\cite{65} It should be pointed out that the analysis of spin states is based on the most likely electronic pathways for recombination, but is not experimentally certain.

In addition there is a structural barrier which arises from the different structures of various ligands and their bonding properties with the center iron atom. A final contribution to the structural barrier comes from the bond angle between ligand-Fe and the heme plane. A comparison of the heme-ligand bonding geometry for various ligands in solution versus in the protein suggest that the barrier to recombination is higher for CO than $O_2$.\cite{86}

Although the barrier to recombination is complicated we find experimentally that a sufficient barrier to ligand rebinding forms immediately ($<2$ ps) and thereby inhibits geminate recombination.

3.4.2 Photoexcitation and Photodeligation of Hemoglobins

One of the puzzling aspects of hemoglobin photochemistry is the quantum yield of photodissociation. Using nanosecond flash photolysis the dissociation yield for oxyHb\cite{77} is about 24 %, while for carbonylHb\cite{77,84} it is about 65 to 80 %. Under steady illumination, the quantum yields are $\approx 5$ % for oxyHb and $\approx 50$ % for carbonylHb.\cite{87} The ligand bond strength, however, is higher for CO than for $O_2$. Several mechanisms have been proposed to account for these facts. One is that the barrier height to recombination affects the dissociation yields. Many dynamics studies are consistent with this barrier mechanism.\cite{46,65,75,81} If, as is suggested, the
barrier to recombination were higher for CO than O₂, the yield of picosecond geminate recombination with O₂ would be expected to be higher than for CO. However, we find no significant geminate recombination for either ligand, on a picosecond time scale. This result suggests that the barrier height to recombination may not be the crucial factor in determining dissociation yields.

It has also been suggested that the dissociation yield is determined by electronic relaxation. Different electronic states will have different probabilities of transition at the crossing point where the electronic relaxation to the dissociative state occurs. Let us assume that the photoexcitation of 532 nm photon creates two different electronic excited states for oxyHb and carbonylHb (charge transfer state for oxyHb and 3T₁ state for carbonylHb according to Petrich et al.). A cursory examination of the energy level diagram of oxyHb, carbonylHb, and their dissociation products quickly demonstrates the possibility that the two electronic excited states have different probabilities of photodeligation.

Our results support the importance of electronic relaxation processes in determining quantum yields of photodeligation. In oxyHb (Figure 3.8) we see the transient oxyHb molecule undergoing vibrational cooling with a time constant of 2 - 5 ps. The same data indicates that no geminate recombination occurs. Taken together, these two results suggest one of the following two conclusions. (1) Photodeligation occurs on a subpicosecond time scale from an electronic excited state. However, significant non-radiative electronic relaxation occurs producing a hot oxyHb molecule. In this mechanism different non-radiative relaxation rates for CO and
O$_2$ will greatly influence the photodeligation yields. (2) Photodeligation occurs from the excited electronic state with unit quantum efficiency. In this event, the hot oxyHb we observe comes from subpicosecond geminate recombination. In this mechanism, photodeligation yields for CO and O$_2$ are controlled by the barrier to recombination. We believe that this mechanism is unlikely. For this mechanism to be operative, the barrier would have to be nonexistent initially in order to allow a significant fraction of oxyHb to form by geminate recombination. Next the barrier would have to appear suddenly so that geminate recombination essentially stopped by the end of the first picosecond. (This must occur to match our observation that no significant geminate recombination occurs on a 2-100 ps time scale.) With this in mind it is hard to see how a barrierless reaction could exist in the first picosecond following photodeligation.

Figure 3.10 shows that relatively little hot carbonylHb is formed upon photolysis. In addition we have already found that a large transient due to hot oxyHb is obvious in Figure 3.8. These results are consistent with the long-established fact that the quantum yield for photodeligation is much higher for carbonylHb than oxyHb on the long time scale.$^{(77,87)}$ Thus the picosecond dynamics data for the two different ligands show the same qualitative trend for deligation yield as the long time and steady state measurements.$^{(77,87)}$
3.4.3 Vibrational Cooling

As in our previous study on deoxyHb\(^{(71-72)}\) we believe that dissipative intramolecular vibrational redistribution (IVR) produces a thermal distribution over the heme internal modes on a much shorter time scale than our laser pulse. In fact, experiments have suggested that the IVR times in condensed phase media are less than 1 ps for several large molecules which are smaller in size than isolated hemes.\(^{(29)}\) Theoretical discussions of heme protein cooling also suggest that IVR in hemes following photoexcitation should be complete in 1 ps\(^{(33)}\) or a few picoseconds.\(^{(22)}\) In our experiment we cannot determine if the internal energy in the heme is a Boltzmann distribution on our observation time scale since we are not able to measure population data for all of the modes in the heme. However, we think that an assumption of Boltzmann statistics is the most reasonable basis on which to interpret our data.

Experimentally, the time constant for the cooling time of every heme mode we have measured is 2-5 ps. This is the same observation as in our previous work.\(^{(71-72)}\) Apparently, this excess internal energy does not affect the geminate recombination chemistry.

3.5 Summary

We have studied the photoexcitation and photodeligation dynamics of oxyHb and carbonylHb using two color transient Raman spectroscopy. The vibrational heating and cooling of the heme following photoexcitation have been directly observed by comparing the complementary Stokes and anti-Stokes transient Raman
spectra. The measured time constant of vibrational cooling is the same for oxyHb, carbonylHb and deoxyHb, which is 2 - 5 ps by deconvolving our laser pulse.

In order to investigate ultrafast structural changes in the heme, the relative intensities of the oxyHb, carbonylHb, and deoxyHb photoproduct bands were separately measured in both the Stokes and anti-Stokes regions. This information was used together with the vibrational cooling dynamics characterized by the complementary negative/positive symmetry of the Stokes/anti-Stokes spectra. In this analysis we are able to sort out the structural changes in the heme from the vibrational cooling dynamics.

The vibrational dynamics indicate that no fast geminate recombination of O₂ or CO with the original heme occurs on a 2 to 100 ps time scale. The slow ligand rebinding occurring on a nanosecond time scale is observed for both oxyHb and carbonylHb.

The reaction coordinate of ligand rebinding is discussed. The lack of fast (2 to 100 ps) geminate recombination indicates that the barrier height for recombination forms immediately following photodeligation. This barrier inhibits fast ligand rebinding.

A hot oxyHb molecule was observed despite the lack of geminate recombination. This was interpreted as evidence for the importance of non-radiative processes which quench the electronically excited state. This observation supports the previous hypothesis that non-radiative processes rather than chemical barrier heights control the quantum yield of photodeligation.
4. DYNAMICS OF PHOTOEXCITED CH₃I

4.1 Rationale of This Experiment

There is great interest in investigation of the photolytic dynamics of small polyatomic molecules in liquid solvents. Methyl iodide is one of the smallest organic molecules. It is due to the existence of I atom that CH₃I absorbs near UV light. Thus, CH₃I has been considered as a model for the photolysis of organic compounds in condensed phase for ultrafast spectroscopists.

Previous investigations of photodissociation of CH₃I in gas phase have provided a large amount of information about the photophysics and photochemistry of CH₃I. The lifetime of methyl iodide has been reported. The photoproducts, CH₃, I*, and I and the distribution of the available energy in these photoproducts have been quantitatively measured. Especially, the distribution of vibrational energy of CH₃ radical has been observed. Theoretical techniques for treating dynamics of small polyatomics have also advanced to the point where fairly accurate predictions can be made\(^{(88-91)}\). All of these previous results have given us a beautiful picture about the photolysis of methyl iodide in gas phase.

As a result of what is known in the gas phase, CH₃I becomes an attractive molecule for investigation of liquid dynamics. The well-known gas phase photophysics of CH₃I are expected to be largely unaffected by the presence of the solvent. This is a consequence of the modest potential of mean force exerted by the solvent on CH₃I in comparison to the rather large intermolecular force of CH₃I. However, in solution the frequent intermolecular collisions make the subsequent reactions...
of the radicals much more complicated. Many ultrafast radical reactions are possible because of the solvent cage effect. One of the most interesting questions is the geminate recombination of the those radicals:

\[ CH_3 + I^* (or I) \rightarrow CH_3I \] (4.1)

The geminate recombination reaction has been indirectly recognized in previous experiments of flash light photolysis in solution\(^{92-94}\). However, how fast do these two primary reactions of geminate recombination occur? How much recomines on the ultrafast time scale? What are the pathways for these two reactions of geminate recombination? What do these measurements tell us about liquid dynamics? These questions have not been answered yet.

The CH\(_3\) radical is one of the most important species in combustion reactions and is of interest both as product and reactant in photodissociation studies. The vibrationally hot CH\(_3\) will be produced with the photodissociation of CH\(_3\)I in gas phase. Once again, if the same reaction occurs in condensed phase, the interesting questions are how fast the vibrational cooling of the hot CH\(_3\) radical occurs following the photodissociation of CH\(_3\)I and how the vibrationally hot CH\(_3\) transfers its excess energy to the solvent molecules. The investigations of these questions is of fundamental significance.
An ideal technique to study the photodissociation of CH₃I in solution is transient Raman spectroscopy. The biggest advantage of using transient Raman technique is that the vibrational cooling and geminate recombination dynamics can be directly distinguished in the Raman spectrum\(^{(7,24,95)}\). The resonance Raman spectrum of CH₃I and CH₃ in gas phase has been obtained by Zhang et al.\(^{(96-98)}\). The CH₃ radical has a weak and strongly peaked gas phase absorption centered at 215.8 nm\(^{(97,99)}\). However, we have found that the transient species, CH₃ cannot give a strong enough Raman signal in solution when probed by the 213 nm laser line. Therefore, we cannot obtain the transient Raman spectrum upon the photodissociation of CH₃I in solution.

We have overcome the disadvantage of transient Raman spectroscopy using transient absorption spectroscopy because the latter has much higher sensitivity than the former. In fact, many ultrafast spectroscopists have been very anxious to study the photodissociation of CH₃I in condensed phase for many years. However, it has not been possible to generate ultrafast pulses at 213 nm until very recently. Our powerful high repetition picosecond laser system can generate enough power at both 266 nm and 213 nm for a pump-probe experiment. This work is the first successful attempt to study the photodissociation of CH₃I in liquid solvents using ultrafast transient absorption spectroscopy.
4.2 Transient Absorption Spectroscopy

Since its development by Porter and coworkers\(^\text{(100)}\), the technique of flash photolysis has served as a powerful experimental tool for the spectroscopic study of molecular excited states. In its conventional form a powerful broadband excitation flash is directed into the sample and followed, after a delay of many microseconds, by an interrogating light flash used for detecting induced electronic absorption bands at coarse levels of spectral resolution. Increasing interest in the properties and dynamics of excited molecules has led over the years to steady refinements in flash photolytic methods. Today, ultrafast lasers have been applied to flash photolysis resulting in picosecond and even femtosecond time resolution\(^\text{(101)}\). This forms the so-called ultrafast transient absorption spectroscopy.

The transient absorption study is based upon the Lambert-Beer law. The photolysis of parent molecules produces a new transient species, which has a different absorption coefficient at the probing wavelength. For samples that are optically thin at the excitation wavelength, the density of excited molecules that may be generated is

\[
\rho_{\text{exc}} = N_{\text{exc}}(\sigma/a)\rho
\]

Here, \(N_{\text{exc}}\) is the number of excitation photons, \(\sigma\) is the ground-state absorption cross section at the excitation wavelength, \(\rho\) is the sample particle density, and \(a\) is the area of the laser beam. If we let \(\sigma'/\) denote the difference between excited
(transient species) and ground state (parent molecule) absorption cross sections at the probing wavelength, then the collinear induced absorbance, $\Delta A$, in a sample of length $l$ is given by

$$\Delta A = N_{exc}(\rho\sigma'\lambda/2.303)a$$

(4.3)

It is clear that transient signals will be dependent on the intensity of excitation laser, concentration of the sample, absorption cross section, and the optical path-length of the cell. However, the key point is the following: on one hand, the larger the absorption cross section of the sample molecule at the excitation wavelength, the more excited the sample molecules. Therefore, the more transient species are generated and the transient absorption signal becomes stronger. On the other hand, the bigger the difference between excited and ground state absorption cross section of the sample molecule at the probing wavelength, the stronger the transient absorption signal. Of course, the transient signal will be bigger when the optical path length is longer. But the long optical path length will reduce the time-resolution of the experiment. Clearly, it is not a good way to lengthen the optical path length in order to improve the transient absorption signal. The best way to increase the transient signal is apparently to increase the laser power and tightly focus the laser beam. However, multiphoton absorption can result from the very tightly focused beam. So some compromise has to be made experimentally. In the
following sections I will describe the development and application of the ultrafast transient absorption spectrometer in our laboratory.

4.3 Photodissociation of CH$_3$I in Gas Phase

The photodissociation of molecular beams of aryl halides$^{(89,103)}$ has shown that methyl iodide directly dissociates but that the aryl iodides predissociate. The excited state lifetime of methyl iodide is about 70 femtoseconds$^{(89,103)}$.

Many spectroscopic experiments$^{(104-110)}$ of CH$_3$I have shown the photolytic dissociation of CH$_3$I at wavelengths shorter than 290 nm produces energy-rich methyl radicals CH$_3$, ground state I($^2$P$_{3/2}$), and electronically excited iodine atoms I$^*$(2P$_{1/2}$). Iodine-atom laser emission following the photolysis of alkyl iodide$^{(111)}$ at 7603 cm$^{-1}$ due to I$^*$($^2$P$_{1/2}$) $\rightarrow$ I($^2$P$_{3/2}$) transition is evidence for the appearance of (I$^2$P$_{1/2}$) upon the photodissociation of CH$_3$I. However, it is not until Wilson and Wiesenfeld's experiments in 1972 that there was a quantitative result for the ratio of I$^*$ ($^2$P$_{1/2}$) and I($^2$P$_{3/2}$) produced in the methyl iodide photolysis$^{(107,112)}$. Their experimental results show that 80% to 92% of the iodine atoms are formed in I$^*$ following the photodissociation of CH$_3$I. The exact value of the yield of I$^*$ depends on the photolysis wavelength.

The UV absorption spectrum of CH$_3$I from 200 to 360 nm is shown in Figure 4.4. This absorption band has been studied by Goodeve and Kosower$^{(113-114)}$. They interpreted the spectrum in terms of two potentials, one dissociating to CH$_3$ + I($^2$P$_{3/2}$) and the other dissociating to CH$_3$ + I$^*$(2P$_{1/2}$). Figure 4.1 shows the potential curves of CH$_3$I, which is drawn from CI calculations$^{(102)}$. Rowe and
Figure 4.1: The potential curves of CH$_3$I. Ground state ($^1$A$_1$) and excited state $^3$Q$_0$(A) and $^1$Q(E) surfaces of methyl iodide, drawn from CI calculations(102).
Mulliken\textsuperscript{(115-116)} reported that at 266 nm nearly 96\% of the absorption is to the $^3Q_0$ state, which is of $A_1$ symmetry. Furthermore, Mulliken has assigned the $^3Q_0$ band as correlating to $I^*(^2P_{1/2})$, while the $^1Q$ correlates to the ground $I(^2P_{3/2})$ state. Thus, $I^*(^2P_{1/2})$ is the dominant photodissociation pathway, while $I(^2P_{3/2})$ is possibly generated by surface crossing from the $^3Q_0$ to $^1Q$ state.

The energy distribution of photofragments of methyl iodide has also been studied by Riley et al.\textsuperscript{(112,117)}. The fraction of the energy available after breaking the I-C bond which goes into internal excitation of the alkyl fragments increases from $\sim 12\%$ for methyl iodide to $\sim 50\%$ for the propyl iodides\textsuperscript{(112)}. Bass et al. also show that following the photodissociation of methyl iodide, the methyl is formed with up to 9.5 kcal mol\textsuperscript{-1} vibrational energy and about 23 kcal mol\textsuperscript{-1} translational energy\textsuperscript{(118)}. This shows that the "hot" methyl radicals formed in methyl iodide photolysis are predominantly translationally rather than internally excited, but that the internal excitation of the alkyl fragment increases sharply with the size of the radicals\textsuperscript{(112)}.

The hot CH\textsubscript{3} radical has been studied by using many methods\textsuperscript{(117,119-121)}. These results have provided clear evidence that all the fragment internal energy is stored in the CH\textsubscript{3}($\nu_2$) umbrella vibration mode, and the potential energy of this vibration induces a strong negative anharmonicity up to and beyond $\nu_2 = 10$. The maximum distribution of vibrational population in $\nu_2$ mode is at $\nu_2 = 2$ level.

These results in gas phase have provided us enough information. It is on the basis of these data that our experimental results in condensed phase are interpreted.
4.4 Photolysis of CH₃I in Condensed Phase

CH₃I has been studied using flash photolysis on a minute time scale\(^{(92-94)}\). These results show that CH₃I dissociates following the absorption of a 253.7 nm photon. The observation of purple I\(_2\) unambiguously indicates the photolysis of CH₃I. The photoproducts are suggested to be "hot" CH₃, I*, and I\(^{(92)}\). Photolytic products such as, methane, ethane, hydrogen iodide, and I\(_2\) have been detected. These photoproducts are ascribed to the reaction of internal hot methyl, iodine atom, and solvent reactions. The quantum yields of many products have been measured in cyclohexane. The quantum yield is 0.19, 0.085, 0.057, 0.048, and 0.009 for methane, cyclohexyl iodide, cyclohexene, iodine, and hydrogen iodide, respectively\(^{(93)}\). Reactions of the hot CH₃ with solvents have also been observed\(^{(92,94)}\). The "effective temperature" of hot CH₃ has been reported in the range 1000-3000° K. These early experimental results clearly demonstrate that the photodissociation of CH₃I in solution must be similar to the result in gas phase as anticipated. Therefore, it is reasonable that our experimental results are interpreted according to the gas phase data.

4.5 Experimental

4.5.1 Photometry

Transient absorption spectroscopy has been developed for more than forty years\(^{(100,122)}\). However, ultrafast transient absorption spectroscopy has been widely used in the recent fifteen years\(^{(123-125)}\). The apparatus described in detail below
operates on the “excite and probe” basis, in which an intense picosecond pulse is used to excite the sample and a second weaker pulse probes the induced absorption change. The laser system used here is the same as the one described in chapter 1. Here I specify the signal detection system of our transient absorption spectrometer.

The essence of a transient absorption experiment is, of course, the measurement of absorption change for a probe beam induced by the prior passage of pump beam through the sample. We use a 266 nm laser pulse as the pump beam, which is generated by using the fourth harmonic in the output beam of the regenerative amplifier. The probe beam is the fifth harmonic (213 nm), which is generated by mixing the fourth harmonic and fundamental laser light at 1064 nm (Figure 4.2).

The 266 nm beam is optically delayed using a one meter-length delay stage. This beam is mechanically chopped before it goes into the sample cell. The 213 nm beam goes through a prism before it passes the sample cell so that the 266 nm and 532 nm beams are further separated from the 213 nm beam. In this way the possible interference from other wavelengths is eliminated. The two beams are approximately copropagating through the sample cell. A mirror or optical filter is used to block the pump beam so that only the probe beam is sampled by the photodiode. The counterpropagating collinear beam geometry cannot be chosen because it degrades the time-resolution of transient absorption experiment.

To obtain the transient absorption signal and achieve high sensitivity in a system based on pulsed light sources that fluctuate in amplitude by 80% from shot to shot, two photodetectors must be used in the system to monitor the intensity of the
Figure 4.2: **Block diagram of transient absorption experiment.** M-Mirror; B.S.-Beamsplitter; D₁, D₂-Photodiode; L₁, L₂, L₃-Lense; F-Optical Filter; GI-Gated Integrator; F.Cell-Flowing Cell.
probe beam before and after the sample cell, respectively. The photodetectors are home-made silicon photodiodes which are sensitive in the ultraviolet as well as the visible. They are operated in the photoconductive mode with a bias of 9V. The response linearity at lower laser intensity was verified by changing the beam intensity. At high laser intensity the photodiodes gradually lose their linear response.

The outputs of the photodiodes are fed to the different channels of the gated integrator, which generates voltages proportional to the laser energies acting on the detectors. Following each shot, the outputs of the integrator are digitized and reset by the computer system. The resulting digital values will be accurate representations of the laser energies only if the photodiodes are mounted behind effective optical diffusers, are well baffled against the beam cross talk, and are operated in their regions of linear response. Successful normalization at the 0.1% level requires careful attention to these points. In addition, the whole probe beams before and after the sample cell have to be sampled by the photodiodes. The laser power fluctuation from shot to shot is very big for transient absorption experiments. These fluctuations strongly affect the ratio of signal to noise of the experiment. This is a consequence of the narrow range of linearity for the photodiodes. This will be specifically discussed in the following sections.

4.5.2 Improvement of Signal to Noise

As mentioned above, the laser energy fluctuation from shot to shot results in terrible S/N ratio which seems to make this experiment impossible. However, the analysis of laser intensity of each shot gives us some clues about how to use this laser
system to do transient absorption experiment. The computer collects and stores the intensity of each shot. Computer software plots the probability versus the shot intensity (Figure 4.3).

As soon as the probability of shot energy is known, a window can be set in the software. This means that a lower and a higher threshold for the shot intensity (Figure 4.3) can be set in the computer software. In this case, the computer will only collect the photodiode signals resulting from the laser shot energy in this window. It is obvious that this method will discard a large number of laser shots which have energy below the lower threshold or above the higher threshold. Thus, the S/N ratio is greatly improved, but the experiment is slowed down. However, our high repetition laser system (2 kHz) can still finish the experiment in a short time (less than 10 minutes for each scan).

4.5.3 Extraction of Transient Absorption Signals

The computer records the data from each diode in channel one and two at the same time. These signals correspond to the signal from the photodetectors before and after the sample cell, respectively. The photometric quantity which is proportional to the concentration of a transient species is ΔA, the induced absorbance. Theoretically the subtraction or ratio of channel one and channel two data will give the net transient absorption signal. However, in this experiment the slow baseline drift resulting from the absorption of ground state molecules, photodetector response and/or integrator capacitance gives a huge background. It is impossible
Figure 4.3: The probability of different shot energy
to observe the transient absorption dynamics by only collecting the ratio or subtraction of the two channel data. In fact, the subtraction of the two channel data only eliminates the electronic noise, but it cannot get rid of the noise resulting from the shot to shot laser pulse fluctuations. The ratio of channel one to channel two data can only remove the optical noise, but it cannot eliminate the noise from the photodetector response and/or integrator capacitance.

In order to completely subtract both the electronic and optical backgrounds, we have extracted the transient absorption signal in the following way. The pump beam (266 nm) is being mechanically chopped during the experiment. When the chopper blocks the pump beam (266 nm), the computer takes the ratio of channel one and channel two data, which is written as \( \frac{A_1}{A_2} \). This ratio corresponds to the signal from all the backgrounds (electronic and optical). When the excitation beam (266 nm) is unblocked, transient species will be produced. At this time, the computer makes a record of the ratio of channel one to channel two data, which is described as \( \frac{(A_1/A_2)'}{A_1/A_2} \). This ratio represents the total signal (background plus transient). When the background signal \( \frac{A_1}{A_2} \) is subtracted from the total signal \( \frac{(A_1/A_2)'}{A_1/A_2} \), the net transient absorption signal is obtained. This is expressed as \( Y \)

\[
Y = \frac{(A_1/A_2)'}{A_1/A_2} - \frac{A_1}{A_2}
\]  

(4.4)
This subtraction of ratio of channel one to channel two data makes our measurements effectively immune to the slow drifts due to the electronic and optical noise.

4.5.4 Some Details About This Experiment

Our beam geometry is dictated by the need for a sensitivity high enough to detect weak transient electronic absorption signals in condensed phase. The probe beam must be as collinear as possible and somewhat smaller than the excitation beam in order to monitor attenuation within the excited volume. When a limited amount of energy is available in the excitation beam, it must be focused to a smaller diameter in order to give the greatest density of molecular excited states. Although a long optical path-length is generally beneficial, it lowers the time resolution of the experiment. The optical path-length in this experiment is about 2 mm. We commonly use beam diameters of about 1 - 2 mm and both beams are aligned to be overlapped in the sample cell. This alignment is achieved by visual inspection of the spot locations using a paper card. About 5% intensity of the probe beam is transmitted through the beamsplitter and is focused into diode 2. After the sample cell, an optical filter is used to block the pump beam and transmit the probe beam which is focused into diode 1. In front of both diodes, ground quartz is used to smooth the beam and thus, the partial saturation on the photodiodes due to focused beams can be avoided.

The sample is flowing in a closed system during the experiment. A stable flowing speed for the pump is necessary, otherwise, bubbles will be generated which
strongly influence the S/N ratio of the transient absorption. Bubbles can be eliminated by adjusting the flowing speed. The dynamic data can be obtained by optically delaying the pump beam (266 nm) and keeping the optical path-length of the probe beam (213 nm) constant. The time zero can be found using a negative delay (pump pulse arrives at the cell later than probe pulse) which gives the ground state or background absorption ratio against which the positive delay value must be compared to find the induced signal. The dynamics of transient absorption are measured by scanning the transient absorption signal with the change of delay time from negative to positive. The typical experimental time for one dynamic scan is 5 to 10 minutes. Generally, the sum of five scans can give very good dynamics.

Dynamics data above were fit with curves of the form \[ c_1 \left( - \exp(-k_{\text{rise}}(1)t) + \exp(-k_{\text{decay}}(1)t) \right) + c_2 + c_3 \left( - \exp(-k_{\text{rise}}(2)t) + \exp(-k_{\text{decay}}(2)t) \right). \] The two exponential curves describe our dynamics data very well. The data will be analyzed in detail in section 4.5.2

4.5.5 Sample Preparation

The sample molecule used in this experiment is CH₃I. The solvent compounds are cyclohexane, heptane, and hexane. All of these compounds are bought from Aldrich and used without further purification. The purity for all of these compounds is > 99%. The concentration of the sample in the different solvents is about 1 mM.
4.6 Results and Interpretation

These experiments represent some of the first measurements of the fragments of photodissociation reactions using ultrafast pulses. The state-of-the-art of ultrafast lasers do not permit the investigation of the dynamics of each possible transient species. As a result, the interpretation must rely on knowledge of gas phase processes and current theory for solution dynamics. The results are not unambiguous, they are a first attempt to unravel geminate recombination dynamics.

4.6.1 Identification of Transient Species

Figure 4.4 shows the absorption spectra of CH$_3$I. The compound has a strong absorption continuum between 220 and 300 nm and the absorption maximum is at about 260 nm. This absorption band corresponds to the n $\rightarrow \sigma^*$ transition. CH$_3$I strongly absorbs 266 nm light and not 213 nm. Many experiments$^{104,106-108,112,117,126}$ in gas phase have shown that when a CH$_3$I molecule absorbs a 266 nm photon, three transient species, CH$_3$, I, and I$^*$ are generated on ultrafast time scales. Previous spectroscopic studies by Herzberg$^{127}$ and Callear et al.$^{99}$ showed that CH$_3$ strongly absorbs 213 nm light. They have assigned this absorption to the first excited electronic transition X $\rightarrow$ A$^{127}$. This indicates that the transient absorption observed in Figure 4.5 is possibly due to CH$_3$. Another experiment$^{128}$ has shown that I-solvent atom charge transfer absorption in liquid alkanes and cycloalkanes occurs in the ultraviolet region, with a poorly-defined maximum in the range 270-350 nm. No further evidence shows I-solvent absorbs light below 250 nm. I absorbs
at 183 nm and I* absorbs at 206 nm\textsuperscript{(129)}. These data indicate that the transient absorption signals in Figure 4.5 are most probably due to CH\textsubscript{3}.

### 4.6.2 Assignments of Dynamics of CH\textsubscript{3}

A CH\textsubscript{3}I molecule is photodissociated to CH\textsubscript{3}, I* or I when it absorbs a 266 nm photon. The subsequent ultrafast dynamics have been observed in this experiment. Figure 4.5 shows the dynamic curves with the photodissociation of CH\textsubscript{3}I in three different solvents, cyclohexane, heptane, and hexane. Because CH\textsubscript{3} absorbs at 213 nm\textsuperscript{(99,127)}, the abrupt increase (signal of absorption goes to negative) indicates the formation of the transient species vibrationally hot CH\textsubscript{3}, I*, and I. Following the instantaneous formation of these transient species, there are two component dynamics (Figure 4.5): a fast and a slow decay dynamics. The deconvolved time constant for the fast component is 14 ps, 25 ps, and 27 ps in cyclohexane, heptane, and hexane, respectively. The deconvolved time constant for the slow component is about 1000 ps in cyclohexane, heptane, and hexane.

In order to clearly interpret the dynamics we have observed in Figure 4.5, many processes have to be considered. All of the dissociation products, vibrationally hot CH\textsubscript{3}, I*, and I are very reactive. The possible processes for these species are: 1. The vibrationally hot CH\textsubscript{3} relaxes; 2. These radicals recombine with each other in the solvent cage; 3. These radicals escape from the solvent cage and then recombine with each other; 4. These radicals react with the solvent molecules. The following reactions are considered here

- \text{CH}_3 + \text{solvent} \rightarrow \text{CH}_3\text{(solvent)}
- \text{I} + \text{solvent} \rightarrow \text{I}\text{(solvent)}
- \text{CH}_3 + \text{I} \rightarrow \text{CH}_3\text{I}
- \text{CH}_3 + \text{I} \rightarrow \text{CH}_3\text{I}
- \text{CH}_3 + \text{I} \rightarrow \text{CH}_3\text{I}
- \text{CH}_3 + \text{I} \rightarrow \text{CH}_3\text{I
Figure 4.4: The UV absorption spectrum of methyl iodide
The primary point in the data interpretation comes from the fact that the dynamics in Figure 4.5 have two decay components of \( \approx 20 \) ps and \( \approx 1000 \) ps. This can only be explained by vibrational energy relaxation (VER) or the cage reaction 4.6.
and 4.7. The point is that the fast component appears to turn off in the dynamics as evidenced by the fact that the fast component does not decay to the baseline. This feature cannot be explained by reactions 4.8, 4.9, and 4.10 which should go to completion. Only the cage reactions 4.6 and 4.7 turn off as a result of diffusion out of the cage. VER is another possible explanation which fits the data and we adopt this interpretation for the following reasons.

### 4.6.2.1 Are the Dynamics Fast Reaction of CH\textsubscript{3} and I?

The fast decay dynamics in Figure 4.5 is unlikely due to the geminate recombination of CH\textsubscript{3} and I (4.6). According to the potential curves in Figure 4.1, there is no energy barrier for the recombination of CH\textsubscript{3} and I. Benjamin’s molecular dynamics simulation for ICN geminate recombination shows that the cage escape time of the I and CN radicals is about 500 fs\textsuperscript{(130)}. According to the theory, this geminate recombination reaction should be on a femtosecond time scale if it occurs in the solvent cage. If the radical either CH\textsubscript{3} or I moves out from the solvent cage, the geminate recombination will be diffusion controlled and occur on a nanosecond time scale.

In solution the collision between the photodissociated fragment CH\textsubscript{3} and the solvent molecules plays an important role in determining geminate recombination dynamics. The collisions result in the energy transfer from the transient species to the solvent molecules. Current thinking suggests that this energy transfer due to collisions is very effective. The transient species CH\textsubscript{3} will lose most of its translational energy in one or two collisions with the solvent molecules\textsuperscript{(1)}. Benjamin
et al. have reported results of ICN photodissociation in liquid Xe solution using molecular dynamics simulation. When an ICN molecule is photodissociated in liquid Xe solvent by 266 nm laser light, almost all of the translational energy carried by the fragments, I and CN, will be lost in 100 fs\(^{(130)}\). This indicates that the excess translational energy of CH\(_3\) will be quickly thermalized in collisions with solvent molecules, and, therefore, the photofragment CH\(_3\) is not able to travel much in the condensed solvents. Otherwise, the geminate recombination dynamics between the photodissociated fragments CH\(_3\) and I will be diffusion controlled.

Secondly, if this geminate recombination between CH\(_3\) and I were responsible for the fast decay dynamics in Figure 4.5, the total amount of recombination should not be more than 20% because photodissociation of CH\(_3\)I only generates 20% ground state I\(^{(106-107,112,121,126)}\). However, this does not fit the data because the total amount of decay in Figure 4.5 is 80%, 55%, and 45% in cyclohexane, heptane, and hexane, respectively. These results confirm that the fast decay dynamics in Figure 4.5 are not due to the fast geminate recombination of CH\(_3\) and I.

The Harris group has recently measured the geminate recombination rate of CH\(_2\)I and I in condensed phase following the photodissociation of CH\(_2\)I\(_2\). They reported that the geminate recombination reaction of CH\(_2\)I and I occurs on a femtosecond time scale\(^{(131)}\). This shows that the geminate recombination of CH\(_3\) and I occurs on a femtosecond time scale. Apparently, this geminate recombination reaction is too fast to be observed using our 8 ps laser pulse.
4.6.2.2 Are the Dynamics Slow Reaction of \( \text{CH}_3 \) and \( \text{I}^* \)?

The fast decay dynamics in Figure 4.5 cannot be attributed to the geminate recombination between \( \text{CH}_3 \) and \( \text{I}^* \) (4.7). One pathway for this reaction is that \( \text{CH}_3 \) and \( \text{I}^* \) can go back to the \( ^1Q \) potential surface by crossing from \( ^3Q_0 \) state. However, a cursory examination of the potential curves in Figure 4.1 quickly demonstrates that there is a huge barrier (\( \approx 7000 \text{ cm}^{-1} \)) for this geminate recombination. If this geminate recombination did occur by this pathway, it would take at least several hundred picoseconds. This apparently cannot fit the fast decay dynamics in Figure 4.5.

Another pathway for this reaction is that electronically excited \( \text{I}^* \) relaxes to ground state \( \text{I} \). \( \text{CH}_3 \) and \( \text{I} \) could then recombine with each other with no potential barrier. Obviously, this pathway is controlled by the electronic quenching rate of \( \text{I}^* \) by solvent molecules. The measurements of this quenching rate in gas phase\(^{106,121,132-133} \) have illustrated that this electronic relaxation of \( \text{I}^* \) in hydrocarbon compounds is very inefficient. The quenching rate\(^{106,121,132-133} \) of \( \text{I}^* \) for \( \text{CH}_3\text{I} \) is about \( 2.5 \times 10^{-13} \text{ cm}^3 \text{ molecule}^{-1} \text{ sec}^{-1} \). This quenching rate constant can be converted to relaxation time constant in solution by multiplying a typical liquid density (0.684 g/cm\(^3\)), which gives a value of about 1370 ps. Callendar and Wilson\(^{134} \) reported a value of the quenching rate of \( \text{I}^* \) for \( \text{C}_2\text{H}_6 \) of \( 2.4 \times 10^{-14} \text{ cm}^3 \text{ molecule}^{-1} \text{ sec}^{-1} \), which gives a time constant \( \approx 4000 \text{ ps} \). From these time constants we can infer that the time constants for the quenching rates of
I* by cyclohexane, heptane, and hexane are on a nanosecond time scale. Obviously, this is too slow to interpret the fast decay dynamics in Figure 4.5.

In fact, our fit of the slow decay dynamics in Figure 4.5 gives a time constant, \( \approx 1000 \) ps. Thus, the slow geminate recombination between CH\(_3\) and I* is partly responsible for this slow decay dynamics. During 1000 ps most of the radicals, CH\(_3\) and I*, will have diffused out of the solvent cage. Thus, the non-geminate recombination between CH\(_3\) and I* or CH\(_3\) (from the other CH\(_3\)I) becomes possible. These non-geminate recombination reactions partially contribute to the slow decay dynamics.

### 4.6.2.3 Possibility of Other Slow Reactions

As discussed above, reaction 4.8, 4.9, and 4.10 are ruled out because of the biexponential decay dynamics where the fast dynamics appear to turn off. There is additional supporting evidence which eliminates these reactions. The two reactions (4.8 and 4.9) are much slower because the radicals must diffuse out of the solvent cage. These non-geminate recombination reactions are diffusion controlled and must occur on at least a nanosecond time scale\(^{130}\). Reaction 4.10 occurs on hundreds of picosecond time scale because there is a big activation energy barrier (\( \approx 3500 \) cm\(^{-1}\)) for the hydrogen abstraction from hydrocarbon solvents\(^{92}\). Obviously, reaction 4.8, 4.9, and 4.10 cannot be responsible for the fast decay dynamics in Figure 4.5 and but the slow decay dynamics.

So far, the last five reactions (4.6, 4.7, 4.8, 4.9, and 4.10) have been eliminated on the basis of theoretical calculation and experimental results in gas phase. The
only process likely responsible for the fast decay dynamics in Figure 4.5 is the vibrational energy relaxation of vibrationally hot CH$_3$. In fact, VER fits our data very well.

### 4.6.2.4 Evidence for VER of CH$_3$

As stated above, upon the photodissociation of CH$_3$I using 266 nm light, although a small amount of the available energy (12%) is partitioned in the internal degrees of freedom in the transient species$^{(112)}$, CH$_3$ is vibrationally excited. Many experiments in gas phase have observed this vibrationally hot radical, CH$_3$. Many spectroscopists$^{(117,119-121,135)}$ have also observed the vibrational distribution of the CH$_3$ fragment using different techniques. Their results show that almost all of the vibrational energy is distributed in the out-of-plane bending vibration ($\nu_2$) mode of the CH$_3$ fragment. The vibrational excitation of CH$_3$ is highly up to $\nu = 10$ of the $\nu_2$ mode. The maximum distribution of vibrational population in $\nu_2$ mode is at the $\nu_2 = 2$ vibrational level. Shapiro et al.'s$^{(89)}$ theoretical calculations have also given consistent results with these experimental observations. Hermann et al.$^{(135)}$ has shown that the rotational excitation in the CH$_3$ is essentially unchanged from that in the parent. These data quantitatively illustrate that the CH$_3$ fragment is born with vibrational excitation by the photodissociation of CH$_3$I using 266 nm light.

For most molecules, the absorption becomes red-shifted with the rise of vibrational temperature. Only a few molecules show the opposite trend in absorption. The absorption of CH$_3$ and CD$_3$ shows a blue-shift with vibrational excitation because the vibrational frequency is larger in the excited electronic state compared to
the ground electronic state\textsuperscript{(99,120-121,135)}. This result has been unequivocally demonstrated by the following experiment.

Callear et al.\textsuperscript{(99)} have studied the UV absorption spectrum of CD\textsubscript{3} radical and shown that the absorption intensity of $\nu_2' - \nu_2''$ band of CD\textsubscript{3} at 613 K is three times stronger than that at 295 K for 213 nm light. This indicates that the vibrationally hot radical CD\textsubscript{3} has stronger absorption at 213 nm than the vibrationally cold CD\textsubscript{3}. Herzberg has shown that CH\textsubscript{3} has the same electronic transition as CD\textsubscript{3}\textsuperscript{(136)}. Therefore, the vibrationally hot CH\textsubscript{3} absorbs even stronger at 213 nm than the vibrationally cold CH\textsubscript{3}. The spectroscopy of CH\textsubscript{3} indicates that when the vibrationally excited CH\textsubscript{3} cools down, the transient absorption signal will drop as is observed in the Figure 4.5. These evidences show that the fast decay dynamics in Figure 4.5 are nothing else, but the vibrational cooling of vibrationally hot CH\textsubscript{3}.

4.7 Discussion

4.7.1 Vibrational Cooling of CH\textsubscript{3}

We have interpreted the fast decay dynamics in Figure 4.5 as the vibrational cooling of vibrationally hot CH\textsubscript{3}. The deconvolved time constant of this vibrational cooling of CH\textsubscript{3} is 14 ps, 25 ps, and 27 ps in cyclohexane, heptane, and hexane, respectively. This difference of vibrational cooling of CH\textsubscript{3} in the three solvents is mainly controlled by the collision frequency between the solvent and CH\textsubscript{3}. Vibrational modes and structure of the solvent molecules both possibly affect this vibrational cooling rate.
Figure 4.5: Transient absorption dynamics of CH$_3$. (A). in hexane. (B). in heptane. (C). in cyclohexane.
A crude calculation can be performed to check if VER is the right order of magnitude to fit the observed dynamics. An approximate formula\(^{137}\) for collision frequency in the condensed phase is 

\[
Z(R) = 4\pi R^2 (N/V)(k_B T/\pi \times m)^{1/2} g(R)
\]

which represents a simple density scaling of the gas phase collision frequency. The radial distribution function \(g(R)\) should be similar for cyclohexane, heptane, and hexane and is about 3. Thus, the factor, \((N/V)(1/m)^{1/2}\) is the main point to be considered, The densities of the three solvents used in this experiment are listed in Table 4.1.

The bigger the density of the solvent compound, the more frequent collisions between the CH\(_3\) and the solvent molecules and, therefore, the faster the vibrational cooling of CH\(_3\). On the other hand, the lighter molecules move faster and therefore, make more frequent collisions. The factor \((N/V)(1/m)^{1/2}\) gives 1.20, 0.95, and 1.00 for cyclohexane, heptane, and hexane, respectively. Apparently, this is quantitatively consistent with our experimental results in Figure 4.5. The fast decay components in Figure 4.5 reflect the different vibrational cooling rates of CH\(_3\) in various solvent molecules, cyclohexane, heptane, and hexane.

Baughcum et al.\(^{121}\) have observed the time behavior of the out-of-plane bending \((v_2)\) emission of CH\(_3\) in gas phase using photofragmentation infrared emission spectroscopy. They reported that the signal decays rapidly with a rate constant of about \(1.3 \times 10^{-11}\) \(\text{cm}^3\text{molecule}^{-1}\text{sec}^{-1}\) upon collisions with CH\(_3\)I. This constant is converted to a time constant of single molecular decay by scaling the gas phase data to liquid density of CH\(_3\)I. The result is about \(\tau = 8\) ps. They attributed the observed
Table 4.1. Densities of solvent molecules

<table>
<thead>
<tr>
<th>compounds</th>
<th>density (g·cm⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyclohexane</td>
<td>0.779</td>
</tr>
<tr>
<td>heptane</td>
<td>0.684</td>
</tr>
<tr>
<td>hexane</td>
<td>0.659</td>
</tr>
</tbody>
</table>
rate entirely to vibrational deactivation by $V \rightarrow V, V \rightarrow T, R$ processes. This result is also consistent with ours. Callear et al.\textsuperscript{(138)} has measured the vibrational energy relaxation of excited CH$_3$ radicals produced in the flash photolysis of Hg(CH$_3$)$_2$ in gas phase. The relaxation rate of vibrational energy of CH$_3$ is reported to be $1.1 \times 10^{-12}$ cm$^3$ molecule$^{-1}$ sec$^{-1}$ and $1.4 \times 10^{-12}$ cm$^3$ molecule$^{-1}$ sec$^{-1}$ for CH$_4$ and C$_2$H$_6$ as a collision partner, respectively. These two constants are converted to solution and give two time constants, 70 ps and 56 ps. This is the same order of magnitude as our experimental result in Figure 4.5.

Recently Harris group has studied the photoexcited dynamics of CH$_2$I$_2$ in condensed phase. They have assigned a $\approx$10 ps dynamics to the vibrational cooling of CH$_2$I radical\textsuperscript{(131)}. Our experimental result strongly supports their assignment.

4.7.2 Pathway of Vibrational Cooling of CH$_3$

How the vibrational cooling occurs in condensed phase is an important question. Our previous experimental results of I$_2$ have shown that the vibrational cooling of I$_2$ occurs in tens to hundreds picoseconds depending upon different vibrational levels and solvent molecules\textsuperscript{(7)}. Theoretically, a lower frequency vibrational mode (210 cm$^{-1}$ for $\nu=1$ level of I$_2$) should have faster vibrational cooling rate than a high frequency mode. Thus, it seems that the vibrational cooling of CH$_3$ should not be as fast as shown in Figure 4.5 because all of the vibrational modes of CH$_3$ have much higher frequencies than those of I$_2$. In fact, the pathway of vibrational cooling plays a very important role in VER.
The normal vibrations of CH$_3$ radical have been extensively studied in theory and experiment$^{(139-140)}$. There should be six normal modes of vibrations. Because two pairs of them are degenerate in energy, there are only four different vibrational frequencies in CH$_3$. They are C-H stretches ($\nu_3$), out-of-plane bend ($\nu_2$), in-plane bend ($\nu_1$), and an asymmetric vibrational mode ($\nu_4$)$^{(99)}$. The out-of-plane bend (the umbrella motion) has been measured at 611 cm$^{-1}$ in nitrogen matrix$^{(139)}$ and at 617 cm$^{-1}$ in a neon matrix$^{(140)}$. The infrared active C-H stretching vibration ($\nu_3$) has been measured at 3162 cm$^{-1}$, while the in-plane bending vibration ($\nu_1$) occurs at 1396 cm$^{-1}$$^{(140)}$. The $\nu_4$ mode has been reported to be at 1026 cm$^{-1}$$^{(99)}$.

In one of the few cases of gas phase IR spectroscopy of a free radical, the out-of-plane bending vibration ($\nu_2$) was measured at 607.0 cm$^{-1}$, with a hot band reported at 603.3 cm$^{-1}$$^{(92)}$. An analysis of isotopic effects on the out-of-plane motion showed that in a matrix the vibration is very anharmonic, with the potential energy function having both quadratic and quartic terms$^{(141)}$. More recently, the photoelectron spectrum of CH$_3$ indicates that this vibration is very anharmonic for the isolated CH$_3$ radical as well$^{(142)}$. Apparently, the $\nu_2$ is the lowest vibrational mode in energy. Thus, our interpretation is that vibrationally hot CH$_3$ cools down in the solution by directly transferring its vibrational energy from the $\nu_2$ mode to the solvent molecules by collisions instead of coupling to its other internal modes. In fact, this has been shown in the photofragment infrared emission spectroscopy experiment$^{(120-121)}$, in which no excitation of other CH$_3$ vibrations has been detected. This indicates that in spite of the considerable energy of excitation in $\nu_2$,
mode, there is little, if any, coupling to other modes. In the flash photolysis of Hg(\(\text{CH}_3\))_2, Callear et al. suggested that the vibrational relaxation of \(\text{CH}_3\) is controlled by de-excitation of \(\nu_2\) although all of the high frequency modes of \(\text{CH}_3\) are vibrationally excited in that case. These results suggest that the vibrationally excited \(\nu_2\) of \(\text{CH}_3\) transfers its excess energy to the solvent by directly coupling with the lower vibrational modes of the solvents is reasonable.

There are several ways for the vibrational energy transfer of \(\text{CH}_3\) to the solvent molecules. The possibilities are efficient \(V \rightarrow V\) to the low-lying vibrations of solvent molecules, \(V \rightarrow T\), \(V \rightarrow R\), and/or reaction. In fact, there are similar vibrational modes in the solvent molecules used in this experiment. All of these solvent molecules have many vibrational modes which are very close in energy to the \(\nu_2\) mode of \(\text{CH}_3\). The vibrationally hot \(\text{CH}_3\) can directly couple with these vibrational modes of the solvent molecules. Thus, the \(V \rightarrow V\) energy transfer becomes very efficient\(^{(143)}\). This is possibly the main pathway for the vibrational cooling of \(\text{CH}_3\) in our experiment. The other minor pathways are \(V \rightarrow T\), \(R\) processes. These \(V \rightarrow V\) is the reason why the vibrational cooling of \(\text{CH}_3\) is faster than that of \(\text{I}_2\), where the \(V \rightarrow T\) dominates the vibrational energy relaxation\(^{(7)}\).

### 4.7.3 Configuration Change of \(\text{CH}_3\)

Kinsey group\(^{(144)}\) studied the photodissociation dynamics of \(\text{CH}_3\text{I}\) using emission spectroscopy. They reported that with photodissociation of a \(\text{CH}_3\text{I}\) molecule, two major structural changes occur: The C - I bond length increases indefinitely, and the HCH bond angle increases from 108° in \(\text{CH}_3\text{I}\) to 120° in \(\text{CH}_3 + \text{I}^*\). This
shows that during the photodissociation of CH$_3$I the configuration of CH$_3$ changes from the original tetrahedral to the final planar structure. As described above, the photodissociation of CH$_3$I occurs on the order of a few tens of femtoseconds\(^{(144)}\). This indicates that this configuration change of CH$_3$ occurs in tens of femtoseconds. Thus, it is impossible to detect this process of configuration change using our 8 ps laser pulse, that is, the process of configuration change of CH$_3$ does not influence the dynamics observed in our experiment.

### 4.8 Summary

1. We have, for the first time, studied the photodissociation of CH$_3$I and the subsequent vibrational cooling dynamics of CH$_3$ in liquid solvents using transient absorption spectroscopy.

2. The signal of transient absorption is due to the absorption of CH$_3$ radical, The vibrationally excited CH$_3$ even more strongly absorbs at 213 nm than the vibrationally cold CH$_3$. We have no evidence to say that I, I*, and I-solvent gives any transient absorption signal for 213 nm laser line.

3. The dynamics of CH$_3$I upon the photodissociation consists of two components. The fast decay is attributed to the vibrational cooling of hot CH$_3$ and, the slow decay to the geminate recombination between CH$_3$ and I*, reactions of CH$_3$ and other CH$_3$, I or the solvent molecules. The reaction of geminate recombination between CH$_3$ and I is too fast to be detected using our 8 ps laser pulse.

4. The deconvolved time constant for vibrational energy relaxation of CH$_3$ is 14 ps, 25 ps, and 27 ps in cyclohexane, heptane, and hexane, respectively. The slow
reaction rate is about 1000 ps in all of the three solvents, cyclohexane, heptane, and hexane.

5. The vibrationally hot CH$_3$ radical transfers its excess energy by directly coupling with the lower-lying vibrational modes of the solvent molecules. The pathway of vibrational cooling of CH$_3$ most likely is by V $\rightarrow$ V energy transfer. The vibrational cooling rate is mainly dependent upon the pathway of energy transfer and the collision frequency between the CH$_3$ and the solvent molecules. The vibrational modes and structure of the solvent molecules also possibly affect this vibrational cooling.

6. There is an energy barrier for the reaction of geminate recombination between CH$_3$ and I$^*$ to cross to the $^1Q$ potential surface. This geminate recombination is controlled by the quenching rate of I$^*$ by the solvent molecules. Because the electronic quenching of I$^*$ by the solvents is so inefficient that the geminate recombination of CH$_3$ and I$^*$ occurs on a nanosecond time scale. This process is partly responsible for the slow decay dynamics.
5. CONCLUSIONS

This dissertation has described the applications of transient Raman spectroscopy in biomolecules. In the deoxyHb experiment we have detected the unambiguous spectral signature of VER in photoexcited deoxyHb using this technique by comparing complementary Stokes and anti-Stokes dynamics. The time constant for mechanical energy transfer from the chromophore to the protein is between 2 and 5 ps. In addition, we have estimated the vibrational temperature of the porphyrin chromophore in deoxyHb within 8 ps after photoexcitation. Analysis of the transient anti-Stokes resonant Raman data indicates a temperature jump of 36 K above room temperature. This represents an average over our 8 ps pump and probe laser pulses. This is the first direct evidence of the VER occurring following the photoexcitation of deoxyHb.

In the oxyHb and carbonylHb experiment we have studied the photoexcitation and photodeligation dynamics of oxyHb and carbonylHb using two color transient Raman spectroscopy. The complementary Stokes and anti-Stokes transient Raman spectra unambiguously show the vibrational heating and cooling of the heme. Structural changes or geminate recombination following photoexcitation are not responsible for the 2 - 5 ps dynamics. The measured time constant of vibrational cooling is the same for oxyHb, carbonylHb, and deoxyHb, which is 2 - 5 ps by deconvolving our laser pulse. The vibrational dynamics indicate that no fast geminate recombination of O₂ or CO with the original heme occurs on a 2 to 100 ps time scale. The slow ligand rebinding occurring on a nanosecond time scale is observed for both oxyHb
and carbonylHb. The reaction coordinate of ligand rebinding is discussed. The lack of fast (2 to 100 ps) geminate recombination indicates that the barrier height for recombination forms immediately following photodeligation. This barrier inhibits fast ligand rebinding. A hot oxyHb molecule was observed despite the lack of geminate recombination. This was interpreted as evidence for the importance of non-radiative processes which quench the electronically excited state. This observation supports the previous hypothesis that non-radiative processes rather than chemical barrier heights control the quantum yield of photodeligation.

This dissertation has also described the development and application of transient absorption spectroscopy. In the CH₃I experiment we have, for the first time, studied the photodissociation of CH₃I and the subsequent vibrational cooling dynamics of CH₃ in liquid solvents using this technique. The signal of transient absorption is due to the absorption of CH₃ radical, and the vibrationally excited CH₃ even more strongly absorbs at 213 nm than the vibrationally cold CH₃. We have no evidence to say that I-solvent gives any transient absorption signal for 213 nm laser line. The dynamics of CH₃I upon the photodissociation consists of two components. The fast recovery is attributed to the vibrational cooling of hot CH₃, and the slow recovery to the geminate recombination between CH₃ and I*, reactions of CH₃ and other CH₃, I or the solvent molecules. The reaction of geminate recombination between CH₃ and I is too fast to be detected using our 8 ps laser pulse. The deconvolved time constant for vibrational energy relaxation of CH₃ is 14 ps, 25 ps, and 27 ps in cyclohexane, heptane, and hexane, respectively. The slow reaction rate
is about 1000 ps in all of the three solvents, cyclohexane, heptane, and hexane. The early results in gas phase support our experimental results. The vibrationally hot $\text{CH}_3$ radical transfers its excess energy by directly coupling with the lower-lying vibrational modes of the solvent molecules. The pathway of vibrational cooling of $\text{CH}_3$ most likely is by $V \rightarrow V$ energy transfer. The vibrational cooling rate is mainly dependent upon the pathway of energy transfer and the collision frequency between the $\text{CH}_3$ radical and the solvent molecules. The vibrational modes and structure of the solvent molecules also possibly affect this vibrational cooling. There is an energy barrier for the reaction of geminate recombination between $\text{CH}_3$ and $I^*$ to cross to $^1Q$ potential surface. Therefore, this geminate recombination is possibly controlled by the quenching rate of $I^*$ by the solvent molecules. The electronic quenching of $I^*$ by the solvent is so inefficient that the geminate recombination of $\text{CH}_3$ and $I^*$ occurs on a nanosecond time scale. This process is partly responsible for the slow recovery dynamics. The configurational change of $\text{CH}_3$ occur on the same time scale as the photodissociation of $\text{CH}_3I$. It is very fast so that it does not affect the dynamics we observed in this experiment.

The future work should concentrate on the improvements of time resolution of the both techniques. This is very important for ultrafast spectroscopists because most of the photophysical process occur on a femtosecond time scale. For example, the photoexcitation of the heme occurs in tens of femtoseconds. Some chemical reactions, such as $I + \text{CH}_3$, $\text{Cr(CO)}_5 + \text{CO}$ also occur on a femtosecond time scale.
The establishment of a femtosecond laser system will provide a powerful tool for the investigation of these photophysical and photochemical processes.

We should also improve the tunability of the laser system. We have greatly contributed to the ultrafast studies using our picosecond laser system. However, some interesting experiments are limited because our picosecond laser system can only provide 1064 nm, 532 nm, 355 nm, 266 nm, and 213 nm light. For example, for oxyHb an interesting experiment is to directly observe the $O_2$ - Fe band so that the photodissociation and the subsequent geminate recombination can be directly measured. But it is very unfortunate that we could not use 418 nm laser light which greatly enhances the $O_2$ - Fe Raman band. Another example is to measure the dynamics of CH$_3$I using transient Raman spectroscopy if we can get 215 nm laser pulse. Apparently a tunable a femtosecond laser system is very urgent to establish. This is what we are doing in our laboratory at the present time.
BIBLIOGRAPHY

[1.] Private communication with professor, Hochstrasser, R. M.


[19.] Yariv, Amnon; Quantum Electronics, Wiley and Sons, New York, 1989


[38.] private communication from Dwayne Miller, Department of Chemistry, University of Rochester.


[53.] private communication from William A. Eaton, National Institutes of Health, Bethesda, MD


[109.] Mains, G. J.; Lewis, D. J. Phys. Chem. 74 1694 1970


[131.] Private Communication with Professor, Harris, C. B.


VITA

Huiping Zhu was born on September 13, 1959 in Huoxian, Shanxi, P. R. China. He grew up here and at five years of age he started primary school. After graduating from high school, he worked as a secretary for about four years and then, attended Shandong University in P. R. China in 1978 receiving his B. S. degree in 1982. He then entered the Institute of Chemistry, Academia Sinica in 1982, where he received a M. S. degree in 1985. He continued to work in this Institute on thermochemistry until 1989, at which time he resigned his job to come to LSU to study for his PH. D. degree.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Huiping Zhu

Major Field: Chemistry

Title of Dissertation: Investigations of Ultrafast Dynamics of Photoexcited Hemeproteins and CH₃I in Condensed Phase Using Transient Raman and Absorption Spectroscopies

Approved:

[Signatures]

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination:

February 1, 1993