UV Resonance Raman Saturation Spectroscopy of Tryptophan Derivatives: Photophysical Relaxation Measurements with Vibrational Band Resolution

Junji Teraoka,† Paul A. Harmon, and Sanford A. Asher*

Contribution from the Department of Chemistry, University of Pittsburgh, Pittsburgh, Pennsylvania 15260. Received June 5, 1989

Abstract: We have developed a new technique to monitor photophysical relaxation processes with the resolution inherent in vibrational Raman scattering. We examine the ground-state ultraviolet resonance Raman saturation behavior of tryptophan derivatives excited in their S0, S1, and S2 excited states in a well-defined, low laser pulse energy flux regime where multiphoton absorption is negligible and no Raman scattering is observed from photon-induced species. We derive a model for Raman saturation in this pulse energy flux regime which quantitatively relates the Raman saturation behavior to the absorption cross section and to the excited state relaxation rates. We detect novel differences in internal conversion efficiencies from the higher excited states of several tryptophan derivatives. We discuss specific applications of this new technique for the study of aromatic amino acid residue environments in proteins.

The sensitivity of molecular excited state relaxation rates to molecular microenvironment has motivated the development of numerous fluorescence methodologies.1 The fact that the intrinsic fluorescence from many biological molecules is generally dominated by tryptophan emission has led to the extensive use of both time-resolved and steady-state tryptophan fluorescence as a probe of protein and enzyme structure and conformational dynamics.2,3 Conformational and environmental changes are often accompanied by significant tryptophan fluorescence intensity changes associated with specific quenching interactions. There are, however, a few drawbacks in applying fluorescence techniques to large biological molecules. In the case of a system with a single tryptophan residue, the data analysis is, in principle, straightforward, but for multiple tryptophans, the residue fluorescence overlaps to prevent the acquisition of individual residue specific information. Additionally, fluorescence methodologies are generally not amenable to study the decay characteristics of the other aromatic amino acids, tyrosine (tyr) and phenylalanine (phe), which show low fluorescence quantum yields when incorporated into polypeptides and proteins.2,4 The prospect of examining tyr and phe fluorescence with deeper UV excitation, where these chromophores strongly absorb, appears unlikely due to the breadth and resulting overlap of tyr (maximum ca. 300 nm) and phe (maximum ca. 280 nm), as well as tryptophan (maximum ca. 340 nm) emission bands. The inability of fluorescence methodologies to study tyr and phe emission is unfortunate as these residues could provide two additional probes of protein structure.

The recent development of ultraviolet resonance Raman spectroscopy (UVRR) has been partially motivated by its potential utility for probing aromatic amino acid environments in proteins.5,6 Previous UVRR studies have demonstrated the ability to selectively excite and resolve individual Raman bands of all three of the aromatic amino acids in proteins.7-10 It appears possible to even distinguish between environmentally different amino acids by stepwise deuteration difference spectral techniques.11 Typically, UVRR measurements probe changes in aromatic amino acid environment by monitoring vibrational frequency shifts and/or changes in Raman cross sections. The frequency shifts would derive from alterations in interresidue interactions such as hydrogen bonding, while cross-section changes would derive from alterations in the resonant and preresonant electronic transitions, hypochromic interactions, and changes in the chromophore local refractive index. It would be expected that UVRR band frequencies and cross sections might also be sensitive to excited-state relaxation rates, but unfortunately this is not generally realized because the resonant electronic transitions are often dominated by inhomogeneous broadening in condensed-phase systems. Indeed, in most UVRR applications, excitation occurs in upper singlet excited states in order to avoid fluorescence interference. The relaxation rates from these levels are dominated by intramolecular (intraresidue) processes and are thus insensitive to the molecular microenvironment.

We demonstrate here the use of a new Raman spectroscopic technique which probes excited-state relaxation rates with the vibrational resolution inherent in Raman measurements. We carefully examine the nonlinear dependence of UVRR intensities upon the laser pulse energy flux. This nonlinear behavior, or "saturation", reflects ground-state depletion of the analyte species during the laser pulse. The degree of ground-state depletion is a function of the rates of both excited-state population (via optical absorption) and relaxation during the laser pulse. Although a few previous studies have also examined nonlinearities in UVRR measurements,12-14 these studies only qualitatively modeled ground-state saturation and did not relate the saturation to excited-state population and relaxation. In addition, two of these previous studies12,13 examined these phenomena at photon fluxes ca. 30-fold larger than reported here, in a regime where photon-driven relaxations (vide infra) and excited-state absorption mask the first-order excited-state relaxation processes.

In this work we examine the saturation of ground-state resonance Raman vibrational intensities over a low pulse energy flux regime. We demonstrate that in this flux regime the saturation behavior can be quantitatively related to excited-state population relaxation rates occurring during nanosecond laser pulse excitation.

(6) Harada, I.; Takeuchi, H. Spectroscopy of Biological Systems; Clark, R. J., Hestor, R. E., Eds.; John Wiley and Sons: New York, 1986; Chapter 3.
(9) Rava, R. P.; Spiro, T. G. Biochemistry 1985, 24, 1861.
This provides a methodology to monitor photophysical relaxation processes with the vibrational resolution inherent in Raman measurements. We call this new spectroscopic technique UV resonance Raman saturation spectroscopy.15 We examine the Raman saturation behavior of a series of tryptophan derivatives with essentially identical electronic transitions, but which have widely varying nonradiative relaxation rates from their lowest excited singlet states. These compounds were selected because their radiative and nonradiative rates have previously been well characterized by time-resolved fluorescence techniques.16-22 Thus, these compounds provide quantitative tests for our UV Raman saturation model. Further, the photophysics of tryptophan remains of considerable interest, and certain aspects of its photophysics, particularly the mechanism for photoionization from higher singlet excited states,23-24 remains unclear. The major motivation for the present study is that the study of tryptophan derivative saturation is a logical first step for studying tyrosine, phenylalanine, and tryptophan residue excited-state relaxation rates in proteins, as probes of amino acid residue environments and protein conformation.

Our UV saturation results are analyzed in the context of the known tryptophan-derivative excited-state relaxation rates. We present a simple model which quantitatively describes the saturation behavior in terms of absorption cross sections, laser pulse energy fluxes, and excited-state lifetimes. Our saturation data also reveal novel differences in internal conversion efficiencies for several derivatives, which involves the presence of a protonated amino group near to the indole ring. In a subsequent report we will describe the utilization of UV resonance Raman saturation spectroscopy to study aromatic amino acids in proteins.

Experimental Section

L-Tryptophan (TRP), N-acetyl-L-tryptophanamide (NATA), L-tryptophanamide (TRPAM), and N-acetyl-L-tryptophan ethyl ester (NATE) were obtained from Sigma Chemical Co. and used without further purification. The instrumentation used to obtain the UVRR spectra has been described in detail elsewhere.29 The Nd-Yag based system generates 20 Hz UV pulses with pulse widths of ca. 4 ns. The Raman scattered light was collected in a backscattering geometry. The ca. 20 mL samples were pumped rapidly through a dye laser jet nozzle and recirculated during the 5-10 min Raman measurement. The flow rate was sufficient to supply fresh sample to the illuminated sample volume between laser pulses. The excitation beam was focused 2 cm beyond the sample with a 25 cm focal length lens. The beam was positioned at a point where the sample thickness was estimated to be 150-200 μm at ca. 1 mm from the jet nozzle tip. The spot size and intensity distribution of the excitation beam at the front face of the sample were measured by translating a razor blade mounted on a micrometer stage across the beam and monitoring the transmitted power. Typical laser beam spot sizes at the samples were ca. 1 mm. The pulse energy was measured by using a Scientech Model 361 power meter. Pulse energies were attenuated by placing Suprasil neutral density filters (Melles Griot) in the beampath. The TRP derivative concentrations were ca. 3 mM for the 273 nm excitation studies and 0.5-1 mM for the 225 nm excitation studies. This gave absorbances of ca. 0.3 through the jet sample thickness. NaClO4 (1.0 M) was used as a non-absorbing internal Raman intensity standard. Small adjustments of the analyte concentrations were made so that the absorbance at the excitation frequency was kept identical in all cases. This ensures identical exponential decreases in the laser intensity between samples as the beam transmits through the imaged sample volume. This

---

Figure 2. Resonance Raman spectra of tryptophanamide (TRPAM, pH 9) excited at 273 nm obtained with increasing pulse energy flux. Pulse energy fluxes are listed on the right in units of mJ/cm². The internal standard perchlorate band at 932 cm⁻¹ is shaded. Note the decreases in the relative intensity ratios of the TRPAM bands with higher energy fluxes.

was verified by monitoring the absolute perchlorate Raman intensities as a function of laser energy flux for each of the TRP derivative saturation studies. We observed identical ClO₄⁻ intensities for each TRP derivative with identical incident pulse energy fluxes. The ClO₄⁻ intensities increased linearly with increasing laser energy flux, indicating no significant bleaching of the TRP derivative absorption along the laser beam path.

The relative intensity ratios used for our calculations derived from peak height measurements. No changes in band shapes were observed over the flux regimes studied here. No permanent loss of analyte was observed during the 5–10 min scan times. Further, we only illuminate 5–10% of the total sample volume in this time and replace the sample between scans.

Results

S₁/S₂ Excitation. Figure 1 shows the absorption spectra of L-tryptophanamide (TRPAM) (pH 5) in aqueous solution. The absorption spectra of all of the tryptophan derivatives are very similar and show the same absorption bands with similar relative intensities (±20%) and band maxima. The molecular structure of TRPAM (pH 5) is also shown as are the electronic transitions and the excitation wavelengths used. Table I shows the substitution pattern for the other tryptophan derivatives studied here. Figure 2 shows four representative resonance Raman spectra of L-tryptophanamide (TRPAM) at pH 9 obtained with increasing pulse energy flux. The spectra were excited at 273 nm in resonance with the 1Lₑ, 1L₃ + A σ−σ* singlet electronic transition of the indole moiety, which we will refer to as S₁/S₂. The 1622 and 1578 cm⁻¹ νₓ₇ and νₓ₇ benzene-like indole vibrations are selectively enhanced because they vibronically mix with strong higher energy indole electronic transitions. The spectra in Figure 2 demonstrate that the Raman intensities of the TRPAM bands decrease relative to the internal standard band (shaded) as the energy flux increases. The open circles in Figure 3 show the energy flux dependence of the ratio of the TRPAM (pH 9) 1578 cm⁻¹ Raman band intensity relative to that of the CICO₂⁻ internal intensity standard band. The decreasing relative intensity ratio simply reflects the decreasing number of TRPAM ground-state species (integrated over the laser pulse) in the imaged sample volume that are available for Raman scattering as the pulse energy flux increases. The initial rate of decrease of the TRPAM (pH 9) relative intensity ratio begins to level off at ca. 30 mJ/cm². The solid line in Figure 3 was calculated from a photophysical model (vide infra) which was fit to the 0–30 mJ/cm² data which utilizes only the known radiative and nonradiative relaxation rates from S₁/S₂ states. Although the model easily fits the 0–30 mJ/cm² pulse energy flux data, the model underestimates the relative intensity ratio at higher energy fluxes. Apparently, recovery of the ground state is augmented by some photon driven process which begins to turn on at ca. 30 mJ/cm² under our experimental conditions. This additional photon driven process dominates the saturation behavior between 30 and 250 mJ/cm² and causes the 1578 cm⁻¹ band to ClO₄⁻ band intensity ratio to become only weakly dependent upon the pulse energy flux. In fact, the behavior is rather insidious in the high energy flux regions, where the analyte and the internal standard Raman intensity increase together almost linearly with laser power. However, the intensity ratio is artificially low and any Raman cross section determined from this observed relative ratio will be more than 2-fold smaller than its true value.

The solid circles in Figure 3 show a similar saturation curve for a TRPAM (pH 9) solution containing 0.5 M sodium iodide. Iodide is a commonly used collisional fluorescence quencher that increases the S₁/S₂ → S₀ nonradiative rate. These data initially show a less steep energy flux saturation slope than was observed for unquenched TRPAM. The dashed curve is a fit for the 0–30 mJ/cm² data using the same model used for the unquenched TRPAM. In this case, the model successfully describes the observed data until ca. 85 mJ/cm² where the calculated relative ratios are again less than those observed, which again indicates an enhanced ground-state recovery rate. We find that all the TRP analogues excited in the S₁/S₂ transition exhibit a behavior where an additional ground-state recovery process becomes active at high pulse energies. The energy flux threshold for this additional recovery process increases as the S₁/S₂ lifetime decreases. Although interesting in its own right, this photon-induced relaxation limits the energy flux range over which we can acquire data dominated by the normal well-known radiative and nonradiative processes from S₁/S₂.

Figure 4 shows the saturation behavior for S₁/S₂ excitation for several TRP derivatives at energy fluxes below where this photon driven relaxation is observed. The data have been corrected for concentration differences arising from our requirement that the absorbance of each sample be identical at the excitation frequency. Shown in Figure 4 is N-acetyl-L-tryptophanamide (NATA) quenched with 0.5 M NaI, TRPAM (pH 5), L-tryptophan (TRP), and TRPAM (pH 9) data from Figure 3. NATA was quenched to give a wider range of S₁/S₂ lifetimes for our study. The S₁/S₂ lifetime of NATA as a function of added sodium iodide is known. The relative S₁/S₂ lifetimes of these compounds are the following: NATA quenched with 0.5 M NaI < TRPAM (pH 5) < TRP < TRPAM (pH 9) (vide infra). We expect that a longer lived S₁/S₂
Figure 4. Saturation of the 1578-cm\(^{-1}\) band of TRP (solid squares), TRPAM (pH 5) (open circles), NATA quenched with 0.5 M NaI (solid circles), and TRPAM (pH 9) (open circles). Note the lower flux energy regime compared to Figure 3. TRP derivative concentration is 3 mM. 1 M perchlorate, 273-nm excitation.

Figure 5. Resonance Raman spectra of TRP (pH 6.5) excited at 225 nm with pulse energy fluxes below 15 mJ/cm\(^2\). Pulse energy fluxes listed in units of mJ/cm\(^2\). Internal standard band shaded.

Figure 6. Raman saturation of the 1555-cm\(^{-1}\) band of TRPAM (pH 9) (open circles) and TRPAM (pH 5) (open squares) at 225-nm excitation. 0.5 mM analyte, 1 M perchlorate. Solid curves derive from a quantitative model for the saturation. See text for details.

Figure 7. Raman saturation for NATA (circles) and NATE (triangles). 0.5 mM NATA and NATE, 1 M perchlorate.

Excited state will result in more extensive ground-state depopulation and a more rapid decrease in the analyte to internal standard intensity ratio with increasing pulse energy flux. This is clearly observed in Figure 4. It should be noted that the different relative intensity ratios at zero energy flux reflect the intrinsically different Raman cross sections at 273-nm excitation for the 1578-cm\(^{-1}\) band for these TRP analogues.

\( S_1 \) Excitation. Excitation at 225 nm is in resonance with the allowed \( ^1B_0 \leftarrow ^1A \) electronic transition\(^{30}\) of the indole ring which we denote as \( S_1 \). At 225 nm the molar absorptivities are ca. 20,000–25,000 M\(^{-1}\)cm\(^{-1}\), which are 4- to 5-fold larger than at 273 nm. The 225-nm excited resonance Raman spectra of the TRP derivatives are dominated by the totally symmetric indole modes at ca. 760, 1015, and 1555 cm\(^{-1}\) (Figure 5). As observed for \( S_1/S_2 \), we see decreases in the intensity ratio of the TRP derivative to the internal standard bands as the energy flux increases, and we also observe the onset of photon-driven relaxation at higher fluxes. Additionally, photochemical transient Raman bands which overlap the 760- and 1015-cm\(^{-1}\) ground-state bands begin to contribute to the spectra at high-energy fluxes. At high-energy fluxes the analyte to internal standard intensity ratio again becomes relatively independent of energy flux. Significant ground-state depopulation and Raman saturation occurs at pulse energies below ca. 15 mJ/cm\(^2\), well before any transient Raman bands are observed. This is evident in Figure 5 which shows four 225-nm excited TRP (pH 6.5) resonance Raman spectra obtained with pulse energy fluxes below 15 mJ/cm\(^2\). The TRP to internal standard relative intensity ratios change by about a factor of 2 over this energy flux region, which corresponds to a maximum average laser power of ca. 2 mW at a 20 Hz repetition rate focused to a 1 mm\(^2\) spot size as described in the Experimental Section. Figures 6–8 show the 225-nm saturation curves for the 1555-cm\(^{-1}\) band for all the compounds studied in a low energy flux region.

---

Figure 8. Raman saturation for the 1555-cm⁻¹ band of TRP. Conditions as described in Figures 6 and 7.

where photochemical transient and photon-induced relaxation contributions are absent. Other vibrational modes show similar behavior. The data in Figures 6–8 have been corrected for concentration differences. The different relative rates at zero energy flux reflect the differing Raman cross sections for the 1555-cm⁻¹ vibration for the different TRP derivatives. Since the normal mode character of the 1555-cm⁻¹ band and the nature of the S₁ transition in all these derivatives is identical, Raman theory predicts that the Raman cross sections should be roughly proportional to the square of the molar absorbivities near 225 nm. Our results quantitatively agree with this expectation. In Figure 6, for example, the ratio of the TRPAM (pH 9)/TRPAM (pH 5) Raman cross sections is 0.76. The calculated relative molar absorbitivity ratio at 225 of 0.87 is close to the 0.84 measured ratio.

Discussion

TRP S₁/S₂ Relaxation Mechanisms. Table 1 displays the structures and S₁/S₂ lifetimes of the TRP derivatives studied here as determined by Petrich et al. using time-resolved fluorescence techniques. The NATA quenched with 0.5 M NaI fluorescence lifetime value derives from Wijnaendts Van Resandt. The variation in the S₁/S₂ lifetimes has been successfully explained by Petrich et al. and refined later by Engh et al. and involves a modulation of the nonradiative S₁/S₂ → S₀ rate by the R and R' groups. The explanation utilizes three assumptions: (1) the R and R' groups may charge transfer quench the excited indole ring to give ground-state indole; (2) the quenching efficiency depends on the electrostaticity of the R and R' groups and their proximity to the indole ring, and (3) various possible rotational isomers about the α-β and β-γ C-C bonds (see note indicated in Table 1) define the proximity of R and R' groups to the indole ring. Rotomers about the α-β bond may interconvert on the fluorescence time scale while rotomers about the β-γ bond cannot. The observed S₁/S₂ fluorescence lifetimes and decay (i.e., single or double exponential) can then be rationalized on the basis of the relative rotational isomer populations and group electrostatics in the order CO₂H > CO₂CH₂CH₂ > NH⁺ > CONH₂ ≅ NHCOCH₃ > CO₂⁻ > NH₂. Compounds where R and R' have similar electrostatics (NATA), or where R and R' combined are weakly electrophobic (TRPAM, pH 9), will exhibit single exponential decays. All other compounds will exhibit double exponential decays as seen in Table 1.

The reciprocal of the fluorescence lifetimes displayed in Table 1 gives the sum of the radiative and nonradiative decay rates out of the thermalized S₁/S₂ fluorescing levels. A quantitative correlation between Raman saturation behavior and fluorescence lifetimes will only be possible if the rate of decay of molecules

from S₁/S₂ corresponds to the rate of repopulation of S₀ during the laser pulse. Decay from S₁/S₂ to states that are long lived with respect to the laser pulse width must be minimal; this is the case for each of the compounds in Table 1. The three processes that contribute to the S₁/S₂ decay rates are (1) charge-transfer quenching, (2) radiative decay, and (3) intersystem crossing. Photoionization from S₁/S₂ fluorescing levels is quite small (less than 1%). Only intersystem crossing leads to the formation of long-lived states while charge-transfer quenching directly yields ground-state indole. Fluorescence emission yields ground electronic state species in higher vibrational levels of S₀ which thermalize on the picosecond time scale to a Boltzmann vibrational ground-state population. The radiative and intersystem crossing rates for the compounds in Table 1 are relatively independent of R and R' and are ca. 6 × 10⁷ and 3 × 10⁷ s⁻¹, respectively.

The ca. 10-fold smaller intersystem crossing rate compared to the S₁/S₂ relaxation rate gives 5–10% triplet quantum yields for TRP, NATA, NATE, and TRPAM (pH 5) and shows that S₁/S₂ relaxation largely corresponds to repopulation of S₀.

Raman Saturation Behavior in S₁/S₂. For S₁/S₂ excitation of TRP derivatives at 273 nm, the simplest scheme for the depletion of the ground state during the laser pulse is absorption followed by rapid picosecond order collision induced solvent relaxation to thermalized S₁/S₂ levels. In the absence of other processes (such as the photon-induced relaxation discussed above), the radiative and nonradiative S₁/S₂ → S₀ relaxation rates should determine the population of ground-state molecules available for Raman scattering during the entire laser pulse. Thus, a simple model describing the saturation behavior is illustrated in Figure 9. P is the S₀ ground-state population at time t and σᵦ is the absorption cross section (cm²/µ火锅) measured at 273 nm. L(t) = I₀L(t) and describes the laser pulse intensity at time t; I₀ is the energy flux (mJ/cm²) per pulse in the imaged sample volume. L(t) is a normalized temporal pulse shape function. The state designated as P* in Figure 9 represents all of the S₁/S₂ fluorescence levels. K represents the sum of the rates of all radiative and nonradiative processes from S₁/S₂ to S₀. The curved arrow depicts the rapid collisional deactivation of the initially excited S₁/S₂ vibronic levels to thermalized S₁/S₂ levels at a rate K'. Under the photon fluxes considered here, K' is fast enough that absorption by the initially excited S₁/S₂ vibronic levels is completely negligible; we show below that absorption from thermalized S₁/S₂ levels is also negligible. The rate equation describing the ground-state population during the laser pulse is then

\[ \frac{dP}{dt} = \sigma_A I(t)P - KP^* \]  

The total number of analyte molecules in the imaged sample volume is P₀. Since the excited-state lifetime associated with K' is ps, whereas the lifetime for excited states relaxing with the rate K is ns, P₀ is given at any time during the pulse by

\[ P_0 = P + P^* \]  

Substituting eq 2 into eq 1 and solving the rate equation for P(t) yields

\[ \frac{d}{dt}P = \sigma_A I(t)P - KP^* \]  

---


The Raman intensity of the non-absorbing internal intensity standard, \( I_0 \), will linearly increase with pulse energy flux because no depopulation of its ground state occurs during the laser pulse

\[
I_S = \int_0^\phi \sigma_l C(t) \, dt = \sigma_0 C_0 f_0
\]  

where \( C_0 \) is the initial concentration of the internal standard and \( \sigma_0 \) is the internal standard Raman cross section. The relative ratio of the analyte Raman intensity to the internal standard Raman intensity expected for one laser pulse is then just eq 6 divided by eq 7.

\[
I_{RR} = \frac{\sigma_R P_0}{\sigma_0 C_0 (\sigma_l f_0 + K_{l0})^2 + \sigma_l f_0 (1 + K_{l0} - \exp[-(\sigma_l f_0 + K_{l0})])}
\]  

Since the sample volume is regenerated between laser pulses, the integrated intensity observed is an average of many single pulse ratios described by eq 8. Also implicit in eq 8 is that \( \sigma_R \) does not change with increasing \( f_0 \). \( \sigma_R \) can be a function of \( f_0 \) if field strengths and dipole moments are large enough to produce Rabi frequencies high enough where vibrational and electronic population relaxation rates and \( T_2 \) dephasing relaxation rates begin to mix.\(^{33-35}\) In this case the resonance Raman cross sections and line shapes begin to change. This phenomena has been invoked to explain the broadening of resonance Raman line shapes in \( \beta \)-carotene\(^{36}\) and deoxy hemoglobin\(^{37}\) at a pulse power flux of ca. 10\(^6\) and 10\(^7\) W/cm\(^2\), respectively, in regions where molar absorbptivities are ca. 10\(^5\). We observe no spectral broadening in any data reported here. The highest pulse power flux data analyzed with eq 8 utilized power fluxes of ca. 7 \times 10\(^7\) W/cm\(^2\) with molar absorbptivities of 5 \times 10\(^3\).

The saturation scheme leading to eq 8 requires that each vibrational mode of a species will exhibit the same nonlinear behavior. Equation 8 predicts a smoothly decreasing intensity ratio with increasing \( f_0 \). Indeed, the observed \( S_1/S_2 \) saturation behaviors for the 1578 cm\(^{-1}\) TRP analogue bands in Figure 4 are well-described by eq 8. We can extract both \( \sigma_R \) and \( K \) by nonlinear least-squares fitting of eq 8 to the measured Raman saturation data. We experimentally determine the absorption cross sections (\( \sigma_a \)) at 273 nm. The values of \( 1/(K) \) obtained in this manner are given in column VI in Table I and are an average of the results of fitting both the 1578 and 1622 cm\(^{-1}\) band saturation behaviors over the flux range shown in Figure 4. Quoted errors are estimates of sample standard deviations. The values of \( 1/(K) \) derived for quenched NATA and TRPAM (pH 9) of 0.75 \pm 0.07 and 7.2 \pm 1.5 ns, respectively, can be directly compared to the fluorescence lifetimes in column IV since the decays are single exponential. For the double exponential decays of TRPAM (pH 5) and TRP, we assume identical Raman cross sections for each decay component. In the case of TRP, for example, we then expect to obtain values of \( 1/(K) \) from the TRP Raman data corresponding to the best fit of eq 8 to a saturation response given by 22% of the molecules relaxing with a 0.62-ns lifetime and 78% with a 3.2-ns lifetime. These calculated values are given in column V of Table I. It is not possible, over the low flux ranges we utilize and with the current signal-to-noise ratios, for us to distinguish between two independent relaxation rates and the “single” lifetimes given in column V. The good agreement between columns IV and V and column VI in Table I indicates the following: (1) within the experimental error associated with measuring and defining \( f_0 \) our simple rectangular model for \( L(t) \) provides adequate absolute agreements with fluorescence decay rates as well as relative agreements between different TRP derivatives, (2) the radiative and nonradiative \( S_1 \rightarrow S_0 \) relaxation rates dominate the relative...

saturation behaviors, and (3) the triplet quantum yields must be relatively small and do not vary greatly between TRP derivatives. It should also be noted that least-squares fitting with eq 8 to give $I_{RR}/I_0$ at zero laser flux provides a logical, quantitative method for determining Raman cross sections free from saturation artifacts. This will be discussed further below.

The photon-driven relaxation apparent at high-energy fluxes in Figure 3 must be avoided for eq 8 to be useful for deriving $S_1/S_2$ lifetimes. This photon-driven relaxation process derives from macroscopic properties; it is not a result of the field and the "isolated molecule" as was the power broadening in $\beta$-carotene and deoxyhemoglobin discussed above. This relaxation appears only at pulse energy fluxes where a $S_1/S_2/S_0$ population inversion occurs. Even at these higher fluxes, the incident photon flux is far too small to stimulate emission from the levels initially populated near 273 nm which survive only for ps. This suggests that the new photon-driven ground-state repopulation is amplified spontaneous emission (ASE) from the $S_1/S_2$ fluorescing levels. The spontaneous fluorescence could be radially amplified along the gain plane provided by the overlap of the ca. 1 mm excited beam spot and the 2.0 x 2.0 x 0.15 mm jet sample volume (the beampath is normal to the 2 x 2 mm jet plane). Our observation that shorter lifetime derivatives show a higher energy flux threshold for the onset of the photon-driven process is consistent with an ASE process that requires a population inversion. In fact, comparing TRPAM (pH 9) with $K = (7 \text{ ns})^{-1}$ to that quenched with NaI with $K = (2.4 \text{ ns})^{-1}$ we see that the threshold increases from ca. 30 to ca. 85 $\text{mJ/cm}^2$. The ratio of these pulse energy fluxes (ca. 3) is almost equal to the ratio of relaxation rates (7/2.4).

We also measured the relaxed fluorescence intensity dependence upon energy flux and find that it drops off faster than does the Raman intensity at high energy fluxes; this is consistent with a directional ASE process.

$S_1$ Raman Saturation. In condensed phases internal conversion from higher excited electronic states to $S_1$ is generally considered to be fast (ps) and essentially 100% efficient. Normally, $S_1$ fluorescence quantum yields are found to be independent of excitation wavelength. Some notable exceptions to this general trend are phenol, tyrosine, indole derivatives, and tryptophan. In the case of tryptophan, for example, although the fluorescence quantum yield throughout $S_1/S_2$ is constant, the quantum yield decreases by 30-50% with $S_0$ excitation. The fluorescence quantum yield decrease has been shown to be accompanied by an increase of photoinization, which occurs rapidly enough to compete with internal conversion to the $S_1/S_2$ states (vide infra).

If we initially assume complete internal conversion from $S_0$ to $S_1/S_2$ for all the TRP analogues studied here, the model for saturation behavior in $S_1$ is identical with that depicted in Figure 9 except that the initial excitation is into $S_0$ which rapidly relaxes to $P^*$ ($S_1/S_2$). Equation 8 then describes the expected saturation behavior and values of $(1/K)$ derived in $S_1$ should be similar to those derived in $S_0/S_2$. We use the measured absorption cross sections at 225 nm and utilize the rectangular pulse shape described previously. The least-squares best fits of eq 8 to the 1555-cm$^{-1}$ data below ca. 15 $\text{mJ/cm}^2$ are shown in Figures 6-8 by the solid curves. We obtain good relative and absolute agreements to the fluorescence decay components for NATA, NATE, and TRPAM (pH 9) (see column VII, Table I). The values of $(1/K)$ listed in column VII are averages of the values obtained by fitting the 760-, 1015-, and 1555-cm$^{-1}$ bands of each compound separately. Errors quoted are estimated standard deviations. The values of $(1/K)$ of 2.9 $\pm$ 0.5, 1.3 $\pm$ 0.2, and 7.1 $\pm$ 1.5 ns for NATA, NATE, and TRPAM (pH 9) are essentially identical with their $S_1/S_2$ fluorescence lifetimes, which indicates similar internal conversion efficiencies for these three compounds. Thus, the $S_1$ saturation is determined by the radiative and nonradiative $S_1/S_2 \rightarrow S_0$ relaxation rates as observed with $S_1/S_2$ excitation at 273 nm. In contrast, the solid curves modeled by eq 8 shown in Figures 6 and 8 for TRPAM (pH 5) and TRP result in significantly longer $(1/K)$ lifetimes than those $S_1/S_2$ lifetimes (column VII). This suggests that for TRP and TRPAM (pH 5) for excitation in $S_0$ an additional process competes effectively with internal conversion to $S_1/S_2$ and leads to a state or states that are long lived compared in our laser pulse length. For comparison, the expected saturation behavior for TRP based on the TRP $S_1/S_2$ lifetime is shown by the dashed curve in Figure 8.

To estimate the fractional number of species that do not convert from $S_0$ to $S_1/S_2$ for TRP and TRPAM (pH 5), we utilize a saturation model shown in Figure 10 where all parameters are as defined in Figure 9. $P_{out}$ represents states derived directly from $S_1$ excitation, which are long lived relative to our 4-ns laser pulse. $P_{out}$ in Figure 10 is populated prior to internal conversion to $S_1/S_2$ with a quantum yield of $\phi_{out}$. This phenomenological models, for example, monophotonic photoionization from $S_P$, which could compete with internal conversion to give long-lived radical species. The rate equations for the model in Figure 10 are given by eq 1 and eq 9.

$$\frac{d}{dt}P_{out} = \phi_{out} \sigma_A f(t)P$$

(9)

The total number of molecules in the imaged sample volume is $P_0$ and is given at any time during the laser pulse as

$$P_0 = P + P^* + P_{out}$$

(10)

It is shown in the Appendix that the pulse energy flux dependence of the analyte to internal standard Raman intensity ratio for the saturation model of Figure 10 is

$$\frac{I_{RR}}{I_0} = \frac{\sigma_R P_0}{\sigma_C C_0} \left[ \frac{1 + (2\phi_{out}\sigma_A I_0 - K_{0} - \sigma_A I_0 - F^{1/2}) \exp[(\gamma_0 -(K_{0} + \sigma_A I_0 ) + F^{1/2}]}{2\phi_{out}\sigma_A F^{1/2} \left(2\phi_{out}\sigma_A I_0 - K_{0} - \sigma_A I_0 + F^{1/2}) \exp[(\gamma_0 -(K_{0} + \sigma_A I_0 ) - F^{1/2})] \right)}{2\phi_{out}\sigma_A F^{1/2}} \right]$$

(11)

where $F = (K_0 + \sigma_A I_0)^2 - 4\phi_{out}K_0\sigma_A I_0$. We can determine $\phi_{out}$ for TRP and TRPAM (pH 5) by using eq 11 with the values of $(1/K)$ that we obtained with $S_1/S_2$ excitation for these compounds (or we can use the measured values of $(1/K)$ based on the $S_1/S_2$ fluorescence lifetime data). The least-squares fit extracts $\sigma_R$ and $\phi_{out}$. For TRP, we find that $\phi_{out} \approx 80\%$, while for TRPAM (pH 5) $\phi_{out} \approx 55\%$; these fits give essentially identical solid curves as those in Figures 6 and 8.

Equations 11 and 8 can be used to derive accurate resonance Raman cross sections for $S_2$ excitation where the saturation is particularly severe. For example, the fit of the relative intensity ratio $I_{RR}/I_0$ below 15 $\text{mJ/cm}^2$ extrapolated to zero pulse energy flux, gives at 225-nm excitation Raman cross sections of 2.2, 1.4, and 1.8 barns/(molecule-steradian) for the 1555-, 1015-, and 760-cm$^{-1}$ bands of TRP. These values are 2 to 3 times larger than those recently reported by Fodor et al. 40 for TRP at 225 nm. Fodor
et al. claimed that the ratio of the TRP bands to the nonabsorbing sulfite internal standard bands was constant over the pulse energy range utilized. However, only one data point below ca. 90 mJ/cm² was obtained. Figure 8 demonstrates a 2-fold change in the TRP/internal standard intensity ratios occurs between 0 and 15 mJ/cm² pulse energy flux. This clearly shows that the data of Fodor et al. were measured in an energy flux regime where ASE relaxation processes discussed above lead to a very weak dependence of TRP/internal standard intensity ratios upon I₀. This also explains why their relative intensity ratios are 2- to 3-fold smaller than our values.

**Origin of φout Differences between TRP Derivatives in S₁.** Our ability to quantitatively determine φout is limited both by the signal-to-noise ratios of the data and by the error associated with specifying I₀ and J₁(t). Thus, we estimate that although we find φout values of ca. "zero" for S₃ excitation of NATA, NATE, and TRPAM (pH 9), our uncertainties permit φout values as large as ca. 20%. The central point is, however, that we obtain a φout value considerably larger for TRP and TRPAM (pH 5).

To ensure that these variations in φout did not derive from sequential two-photon absorption where photoionization occurs by S₁/S₂ excited-state absorption, we studied the saturation behavior of TRP at pH 1.3 (where the carboxylate group is protonated). Protonation of the carboxylate group leads to a large decrease in the S₁/S₂ lifetime due to enhanced charge-transfer quenching. We measure an 8-fold fluorescence intensity decrease. The shorter S₁/S₂ lifetime must result in a decreased S₁/S₂ absorption probability. Equation 11 was fit to our data, which were obtained between 0 and 15 mJ/cm² energy fluxes. Utilizing the appropriate value of (1/K) for S₁/S₂ relaxation, we obtain a similar φout = 70% value for TRP (pH 1.3) excited at 225 nm.

Our S₃ resonance Raman saturation results, which indicate large φout values for TRP and TRPAM (pH 5), are qualitatively consistent with the few other relevant observations of S₃ photochemistry of TRP derivatives. As discussed above, the S₃/S₄ fluorescence quantum yields of a few indole derivatives as well as TRP decrease with excitation in S₃. Steen et al. measured a decrease of 30% in the S₃/S₄ quantum yield of TRP between 260 and 230 nm while Tatschewi and Klein measured a 50% decrease over the same region. It was demonstrated that this decrease does not derive from a change in the intersystem crossing rate. In this same spectral region, several workers observed dramatic increases in electron ejection (photoionization) quantum yields (φₚ), although the absolute values are not all in agreement. For TRP, Steen et al. found at 220 nm φₚ = 15-20%, while Amouyal et al. found φₚ larger than 50% even at 240 nm, the short wavelength limit of their experiment. Considerable data support the idea that a "charge transfer to solvent state" occurs sufficiently rapidly from initial excited state to intramolecular charge transfer to solvent state. Electron release to solvent from the "charge transfer to solvent state" is thermally activated and may compete with direct back-reaction relaxation to the ground state of the indole. The radical species formed after electron release have microsecond lifetimes and are likely to dominate our φout values. The lack of appearance of new Raman bands due to the photoionized or even S₃/S₄ excited-state species is primarily due to the low-energy fluxes. At higher laser powers with 235-nm excitation, for example, we observe new bands.

Our observation of much larger φout values for TRP and TRPAM (pH 5) than for NATA, NATE, and TRPAM (pH 9) is novel. To our knowledge fluorescence quantum yields and electron ejection quantum yields in S₁ are unknown for any of the compounds studied here except TRPAM. In common with S₁/S₄ excitation studies, we find that the S₁ excited-state photochemistry is influenced by the identity of the R and R' groups. The fact that TRP and TRPAM (pH 5) have large φout values suggests that the protonated amino group promotes electron ejection. The NH₃⁺ group must either assist the initial formation of the charge transfer to solvent state or influence the yield of electron release. An electrostatic field effect of the protonated amino group on the indole nucleus, facilitating initial electron ejection, was postulated previously for S₁/S₂ excitation. Although the work here does not conclusively determine the identity of the long-lived transient species, it demonstrates its existence through the observed saturation phenomenon. In the future we will attempt to resolve these transient species to uniquely identify them, in a manner similar to our previous study which identified the formation of tyrosyl radical with excitation at 220 nm in tyrosine and at 245 nm in tyrosinate. UV Resonance Raman Saturation Spectroscopy. The quantitative relationships demonstrated here between the UV Raman saturation behavior and S₁ → S₃/S₄ internal conversion efficiencies and S₁/S₂ lifetimes indicate that the UV resonance Raman saturation spectroscopy may be useful as a methodology for monitoring photophysical relaxation processes in complex systems and environments. Although time-resolved fluorescence techniques also give essentially identical information, the UV Raman saturation methodology has the advantage of vibrational resolution. The wavelength dependence of the resonance Raman effect and the vibrational resolution of the Raman spectra make it possible to resolve saturation of a single Raman band of a particular chromophore. It is obviously difficult to resolve between the broad overlapping fluorescence bands of condensed phase aromatic species. Another advantage is that, unlike time-resolved fluorescence techniques, where the detector response time determines the possible temporal resolution (in the picosecond time scales), the time scales that can be probed by the UV saturation method are, in principle, limited only by the energy flux of the laser pulse. In principle, any photon counting device will serve as a detector. It should be noted, however, that the optimal laser pulse length for ps and fs relaxation studies probably would be in the ps and fs time regime. Study of these fast relaxations with ns pulse lengths would require high pulse powers, which could lead to higher order nonlinear processes that would complicate the measurements.

Raman saturation spectroscopy at present suffers from a lower sensitivity than time-resolved fluorescence due to the signal-to-noise ratios presently available. Thus, time-resolved fluorescence techniques will be superior for determining multieponential decays, or if highly accurate decay rates are required. However, the two techniques are highly complimentary. Fluorescence techniques monitor excited-state dephasing while the UV Raman saturation technique reports directly on ground-state recovery. Fluorescence techniques are dominated by those chromophores with the largest resonance quantum yields while the UV Raman saturation method is equally sensitive to species with large or zero fluorescence quantum yields.

We envision a number of applications of UV Raman saturation spectroscopy. Of particular interest to our laboratory is utilizing this saturation methodology to determine tyrosine, phenylalanine, as well as tryptophan residue excited state relaxation rates in order to monitor protein structure and conformational changes. The difficulties in applying fluorescence techniques, such as the low fluorescence quantum yields of the and tryr in proteins, have already been discussed. Other ground-state recovery techniques, such as measuring the laser flux dependence of the optical density, would also be extremely difficult due to the overlap of aromatic amino acid and amide group absorption spectra in typical proteins. The UV Raman excitation method has promise since at least one vibrational mode of each aromatic amino acid can be completely resolved without interference from other aromatic residues or amide vibrations with excitation in the 240-195 nm spectral region. From the work reported here, we anticipate that the Raman saturation methodology will be sensitive enough to detect...
interactions with nearby residues that alter the major decay components or which influence photochemistry. We will show in a subsequent report that Förster energy transfer strongly influences the UV resonance Raman saturation behavior of aromatic amino acids in a number of peptides and proteins.

Conclusions

We have derived expressions to extract ground-state recovery rates from resonance Raman saturation behaviors of a number of tryptophan derivatives. These recovery rates agree well with excited-state decay rates as determined by fluorescence techniques. Decreases in the relative $S_1 \to S_2/S_1$ internal conversion efficiencies for several tryptophan derivatives have been observed that appear to be related to the presence of a protonated amino group near the indole ring. We suggest these differences derive from a greater degree of monophotonic photoionization upon $S_2$ excitation. The quantitative success of these Raman saturation studies suggests that UV resonance Raman saturation spectroscopy is a new methodology that will be useful for studying photophysical relaxation processes. This technique is complementary to time-resolved fluorescence techniques since it reports directly on ground-state recovery rates. The Raman saturation technique has the advantage of vibrational resolution, as chromophore-specific relaxation rates can be probed by vibrational markers. The UV Raman saturation methodology will be particularly useful in monitoring phenylalanine and tyrosine, as well as tryptophan residue relaxations in proteins and other biological molecules.

Acknowledgment. We gratefully acknowledge support of this work from NIH Grant 1R01GM37041-08. Sanford A. Asher is an Established Investigator of the American Heart Association. This work was done during the tenure of an Established Investigatorship of the American Heart Association, Pennsylvania affiliate.

Appendix

We show here the derivation of eq 11. Substitution of eq 1 into eq 10 gives

$$-\frac{dP}{dt} = (\sigma_A(t) + K)P - KP_0 + KP_{out}$$

(12)

Equation 9 is rearranged to

$$P = \frac{1}{\sigma_A(t)\phi_{out}} \frac{d}{dt} P_{out}$$

(13)

Equation 13 is substituted for $P$ into eq 12 yielding

$$\frac{d^2}{dt^2} P_{out} + (\sigma_A(t) + K) \frac{d}{dt} P_{out} + (K\phi_{out}\sigma_A(t))P_{out} = K\phi_{out}\sigma_A(t)P_0$$

(14)

Solving this differential equation for $P_{out}$

$$P_{out} = C_1 \exp(\alpha t) + C_2 \exp(\beta t) + C_3$$

where $C_1$, $C_2$, and $C_3$ are to be determined from the boundary conditions and $\alpha$ and $\beta$ are

$$\alpha = \frac{1}{2} \sqrt{A + A^2 - 4B}$$

$$\beta = \frac{1}{2} \sqrt{-A - A^2 - 4B}$$

where

$$A = \sigma_A(t) + K$$

$$B = K\phi_{out}\sigma_A(t)$$

Using the initial condition for $P_{out}(t = 0) = 0$ and eq 13 and with the initial condition for $P(t = 0) = P_0$ yields

$$P(t) = \frac{P_0}{\beta - \alpha} [(K + \beta) \exp(\beta t) - (K + \alpha) \exp(\alpha t)]$$

(15)

which, for $\phi_{out} = 0$, is identical with eq 4 since $\sigma_A(t) = (\sigma_A t_0)/t_0$. The expression for $P(t)$ of eq 15 is substituted into eq 5 and integrated from 0 to $t_0$ to give

$$I_{RR} = \frac{\sigma_R P_0 \phi_0}{t_0} \left[ \frac{K + \beta}{\beta(\beta - \alpha)} \exp(\beta t_0) - \frac{K + \alpha}{\alpha(\beta - \alpha)} \exp(\alpha t_0) + \frac{K}{\alpha\beta} \right]$$

(16)

Equation 16 divided by eq 7 gives $I_{RR}/I_S$, yielding eq 11.

Registry No. TRP, 73-22-3; NATA, 2382-79-8; TRPAM, 20696-57-5; NATE, 2382-80-1.