Journal of Fluorescence, Vol. 8, No. 3, 1998

The Adsorption of L-Tryptophan to the H_2O–CCl_4 Interface, Monitored by Red-Edge Two Photon-Induced Polarized Fluorescence

D. T. Cramb[1,3] and S. C. Wallace[2]

Received April 24, 1998; revised July 16, 1998; accepted July 21, 1998

The adsorption of L-tryptophan to the H_2O–CCl_4 interface has been studied using the novel method of two photon-induced fluorescence polarization. The process of two-photon absorption allows excitation of a very small volume of molecules near an interface. In the instance where molecules maintain a preferential orientation at the interface, interface-specific phenomena are observed as a dependence of the absorption and emission processes on the polarization of the light. In this way, an isotherm for the adsorption of L-tryptophan from the bulk aqueous phase to the H_2O–CCl_4 interface was determined. Associated with this process is a change in free energy of $\Delta G^0_{ad} = -19 \pm 3$ kJ/mol. By comparing the free energy of adsorption in this study to that at the H_2O–$CH_3(CH_2)_6CH_2OH$ interface, we find that L-tryptophan binds more readily to the H_2O–CCl_4 interface. Our polarization data suggest that the plane of the indolyl chromophore of tryptophan is oriented in a more parallel than perpendicular fashion to the liquid–liquid interface. Additionally, we present a mechanism for the observed two-photon absorption, 20 nm to the red of the traditional onset of the transition into the first excited singlet state of tryptophan in aqueous solution. This mechanism involves simultaneous hot-band transitions into the ^1L_a state and direct excitation into the triplet manifold.

KEY WORDS: Liquid interfaces; fluorescence polarization; multiphoton.

INTRODUCTION

The hydrophobicity of the aromatic side chains of some amino acids plays a crucial role in many biological processes. For example, hydrophobic interactions between side chains, such as indole of the amino acid, tryptophan, and fatty acids, have been suggested as the driving force behind incorporation of proteins into lipid membranes [1–4]. Additionally, within a protein, hydrophobic side chains aggregate in order to exclude water, thus reducing their free energy. This is the principal phenomenon which leads to protein folding. Furthermore, the catalyst tryptophan synthase is known to have two active sites connected by a hydrophobic tunnel [5]. The tunnel allows indole, cleaved from indole glycerol phosphate at the α site, to be transported to the β site of the protein, where indole and serine are condensed to form tryptophan. For all these phenomena, it is important to understand hydrophobic interactions between amino acids and their environment at a microscopic level. Tryptophan is a good candidate to help-scientists in elucidating these interactions, as it has excited electronic states which are highly sensitive to the polarity of their environment.

Tanford and co-workers [6] developed a method of determining systematically the hydrophobicity of amino

[1] Department of Chemistry, 2500 University Drive N.W., University of Calgary, Calgary, Alberta T2N 1N4, Canada.
[2] Department of Chemistry, 80 St. George Street, University of Toronto, Toronto, Ontario M5S 3H6, Canada.
[3] To whom correspondence should be addressed.

1053-0509/98/0900-0243$15.00/0

acid side chains. By comparing the solubility of amino acid in mixed liquids of different polarity and mole fraction, they were able to infer the side-chain contribution to the free energy associated with this transfer. In their work, they did not account for the existence of microscopic interfaces between the liquid phases. For amphiphilic species, such as amino acids in their zwitterionic form, there will be a significant proportion of molecules adsorbed to the interface between liquids of different polarities.

It is clear that studies of amino acids at simple interfaces would be very informative in the elucidation of their hydrophobic interactions with various media. A study of surface adsorbed *t*-butyl-oxy carbonyl tryptophan (Boc-Trp) at the air–water interface has been accomplished by Smiley and Vogel [7] using the surface second harmonic generation (SHG) to probe the orientation of Boc-Trp. They found that the indole chromophore maintained a 49° tilt angle from the surface normal independent of bulk concentration. Since an artificially low pH was maintained throughout this experiment, Boc-Trp may not represent the behavior of free zwitterionic tryptophan in the neutral pH of a living organism.

The SHG signal is interface specific because the generation of the second harmonic frequency of laser light is electric dipole permitted only at the interface between two centrosymmetric media such as a gas and a liquid. If the photon energy is such that the two-photon process is resonant with an excited electronic state of the chromophore at the interface, there may be an enhancement of the SHG. Additionally, if the excited state fluoresces, the fluorescence may dominate any SHG signal. This is the case with tryptophan, where the absorption coefficient for a two-photon process is significant in the range λ = 315–260 nm [8]. Since fluorescence emission is not interface specific, the signal from the bulk emitters may overwhelm that of the chromophores at the interface. This results in the obfuscation of the interfacial SHG signal.

However, if chromophores are aligned at an interface, it may be possible to extract interface specific fluorescence emission using a suitable choice of excitation polarization and by selecting a single polarization component of the emitted fluorescence. Furthermore, by employing two-photon excitation one can reduce the volume of interaction to those chromophores near the interfacial region. This results from the fact that a two-photon process depends quadratically on the intensity of the incident laser field, which will be greatest at the beam's focus. Thus, a gaussian laser beam of 5-mm radius can be focused by a 55-mm singlet lens to a spot radius of 3.0 μm parallel to the surface maintained ~ ± 13 μm perpendicular to the surface [9]. It is in this volume that two-photon excitation will be most efficient.

Previously, we reported the adsorption of L-tryptophan to the interface of water–octanol [10]. In the present study, we observe the adsorption of L-tryptophan to the H_2O–CCl_4 interface and compare the two. Using two photon-induced fluorescence and fluorescence polarization, we have been able to measure an adsorption isotherm for Trp at the above-mentioned interface.

In this study, we have chosen to excite tryptophan with two photons at a longer wavelength than is traditionally used. The onset of the electronic transition into the first excited singlet state is thought to be around 315 nm [8]. In an attempt to perform an SHG experiment on the liquid–liquid interface, we originally chose to use an excitation wavelength of 660 nm (two photons of which equals 330 nm) to avoid fluorescence. Nevertheless, we found relatively strong, polarized, uncollimated emission coming from the sample. Thus, we have made a spectroscopic analysis of the state, which we are exciting in order to determine its nature. In this paper, we also present a two-photon excitation spectrum of aqueous L-trp, covering the range 680–440 nm. The analysis of this, coupled with one-photon fluorescence studies, has allowed the rationalization of the two-photon absorption cross section at 660 nm.

EXPERIMENTAL PROCEDURES

The apparatus used to measure the adsorption isotherm for L-tryptophan at the H_2O–CCl_4 interface is very similar to that used to measure the second harmonic generation of laser light in a previous experiment [10,11]. The modification for the present experiment was primarily to the sample container, shown in Fig. 1. A quartz cylinder was used to contain the two liquids. Since CCl_4 is denser than H_2O, it was the lower phase. The concentration of bulk tryptophan was altered by first removing a calibrated amount of solution from the aqueous phase and replacing it with the same amount of higher concentration stock aqueous tryptophan. Light of wavelength λ = 660 nm was used to excite the sample near the interface. The polarization of the input beam was controlled using a rhombohedral prism pair. The polarization behavior of the emitted fluorescence was monitored with a Kodak polarizing film. The same experimental setup was used to assay the polarization characteristics of both light absorption and light emission of bulk aqueous tryptophan.

An Optical-Parametric-Amplifier laser system (Spectra Physics) was employed to measure the two-

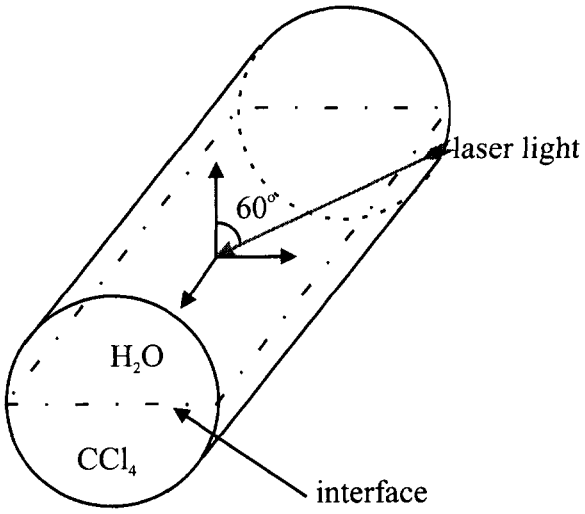

Fig. 1. The quartz cylindrical sample cell used to contain liquid CCl_4 and H_2O. The aqueous phase being less dense, is the upper. Also indicated is the optical path of the laser excitation beam. It is incident 60° from the liquid–liquid interface normal.

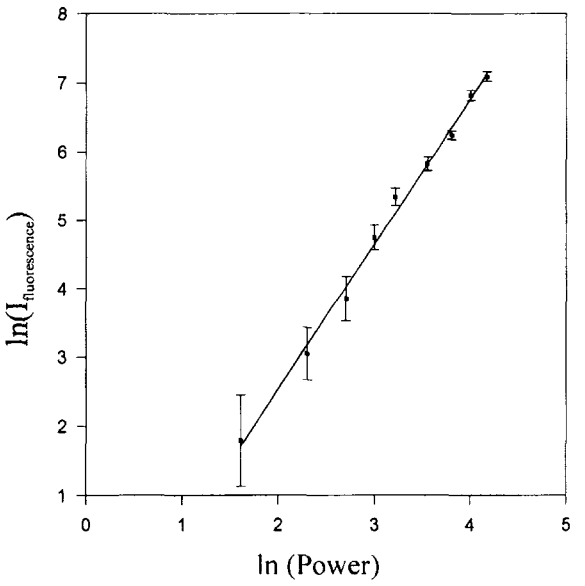

Fig. 2. A log plot of the intensity of the input laser versus the intensity of the resultant fluorescence signal for tryptophan near the interface. The straight-line fit resulted in a slope of 2.11 ± 0.06. The error bars are the RMS error on the natural log of the fluorescence signal.

photon excitation spectrum of L-tryptophan. This laser system has a continuous scanning range of 680–440 nm with a reasonably constant power output. For the spectrum shown in this paper, the output power of the laser was attenuated to 60 mW at a repetition rate of 10 Hz. The laser beam was gently focused into a 1.0 m*M* aqueous solution of L-trp using a 30-cm lens. Fluorescence, in the range 320–400 nm, was collected perpendicular to excitation using a combination of a notch filter and a short-pass filter. One-photon excitation spectra were collected at the University of Calgary on a scanning spectrofluorometer (PTI, Canada).

Chromatographic grade CCl_4 and L-tryptophan were used without further purification. High-purity distilled and deionized water was used throughout the experiment.

RESULTS

Interfacial Two-Photon Absorption and Polarization Effects

A tightly focused laser beam (focal length = 55 mm, spot radius = 3.0 μm) of λ = 660 nm was incident on the H_2O–CCl_4 interface at an angle of 60° to the surface normal [10]. The undispersed fluorescence, observed in reflection off the surface, was spatially filtered and passed through a color filter to remove the fundamental wavelength. The signal from the photomultiplier tube was found to vary as a function of the square of the input beam intensity thus confirming a two-photon absorptive process. The logarithmic plot of input laser intensity versus fluorescence intensity is given in Fig. 2. The slope of the straight line fit indicates that fluorescence intensity varies as input laser intensity to the power 2.11 ± 0.06. Two-photon absorption to both the L_a and the L_b excited states of tryptophan are allowed in the electric dipole limit [12]. In nonpolar media the L_b state is lower in energy than the L_a state, however, it has been observed that the higher dipole moment L_a state is stabilized in polar solvents such as H_2O and therefore can become the lowest-energy excited singlet state. This will be the case with bulk aqueous tryptophan and may also be the case with interfacial tryptophan. The wavelength of our incident radiation suggests that the state to which we are exciting is 30,303 cm^{-1} higher in energy than the ground electronic state. The onset of the $S_0 \rightarrow L_a$ transition in H_2O at room temperature has been measured [9] to be as low as 31,750 cm^{-1}.

To help determine the nature of the two-photon absorption process in tryptophan, a two-photon excitation spectrum of 1.0 m*M* aqueous L-tryptophan was recorded and is presented in Fig. 3. The spectrum was collected as described under Experimental Procedures. The fluorescence signal measured was found to vary quadratically with the excitation laser intensity over the entire spectrum. The small, but reproducible extended red-edge

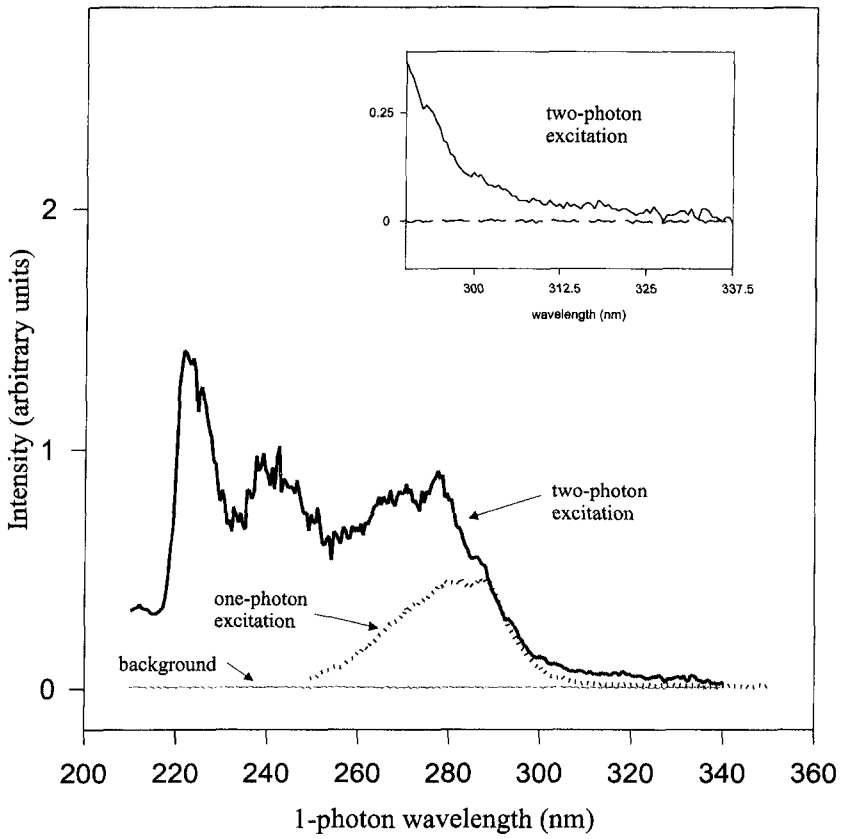

Fig. 3. The excitation spectrum of 10^{-3} *M* L-tryptophan in aqueous solution. The wavelength axis is scaled for a one-photon transition to the excited state. Thus, in the two-photon spectrum, the peaks are positioned in wavelength at the sum of the two photons absorbed. The background scan was made using neat aqueous solution. The one-photon spectrum was recorded on a spectrofluorometer as described in the text. The inset is an expanded view of the red-edge region for two-photon excitation versus background signal.

of the two-photon spectrum versus the background signal is shown in the inset in Fig. 3. The one-photon excitation spectrum of 0.05 m*M* aqueous tryptophan was recorded using a scanning spectrofluorometer and is also presented in Fig. 3. Comparing the one- and two-photon excitation spectra, there appears to be an enhancement of the red-edge excitation for the two-photon process.

Evidence for selective detection of tryptophan at or near the H_2O–CCl_4 interface comes from the excitation and fluorescence polarization behavior. In the bulk aqueous phase for tryptophan, there is no measurable variation of the fluorescence signal with input laser polarization. There is also no evidence of polarization of the emitted fluorescence. This would be expected from a sample with random orientation. However, at the H_2O–CCl_4 interface there is a dependence of the fluorescence signal on the input laser beam polarization. The signal nearly doubles for an s-polarized input beam versus a p-polarized input beam. In both cases fluorescent light was detected without a polarization selective element. When exciting with strictly s-polarized input radiation, the ratio of s-polarized to p-polarized fluorescence is 5:1. This is highly suggestive of preferential orientation of the indolyl chromophore at the H_2O–CCl_4 interface. It should be noted that these two polarization excitation schemes are not mutually exclusive since there will be a distribution of orientations at the interface. This means that p-polarized light will still excite a small fraction of the interfacial molecules. It can be asserted, however, that one can probe the interfacial chromophores more preferentially using an s-polarized excitation beam. One should also note that for all polarization schemes there will be some contribution from bulk aqueous tryptophan. The signal from the bulk aqueous tryptophan is approx-

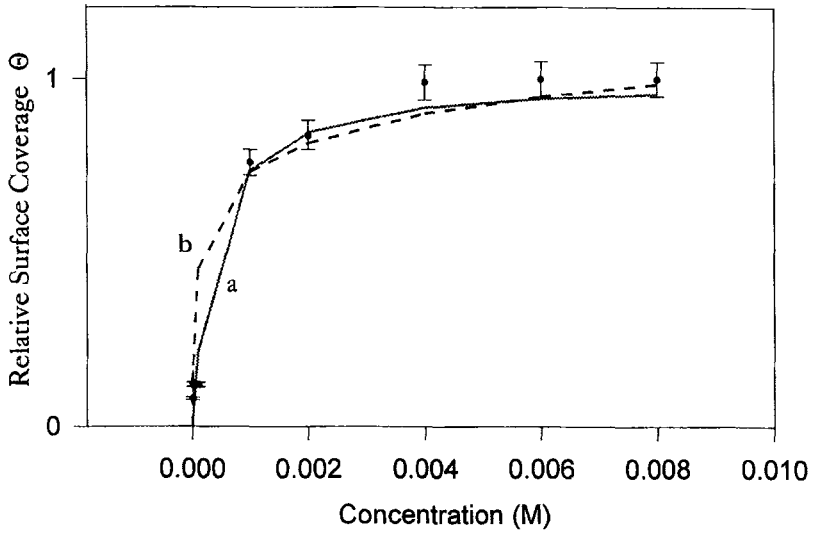

Fig. 4. A plot of the relative surface coverage of L-tryptophan at the H_2O–CCl_4 interface versus the bulk aqueous concentration of tryptophan. The error bars represent one standard deviation determined from the average of 10 data sets of 10-s collection time each. Line a represents a Langmuir isotherm fit to the data. Line b represents a Temkin isotherm fit to the data. See text for details.

imately five times smaller than the s:s (excitation:fluorescence) interfacial signal when using polarization sensitive detection.

Adsorption Isotherm

Figure 4 shows the dependence of the surface coverage, Θ, on the concentration of bulk aqueous tryptophan. The fluorescence intensity is assumed to be have a linear dependence on concentration in accordance with the Beer–Lambert approximation for the two-photon absorption process [13]. The error bars on the data points represent one standard deviation from the mean. The statistics are generated by analyzing 10 data sets for each concentration. The photons were counted for a period of 10 seconds, with zero background counts from the neat H_2O–CCl_4 interface. The lines a and b represent fits to the data points of Langmuir and Temkin adsorption isotherms, respectively [14]. It is arguable that the Langmuir isotherm represents the best fit. A Langmuir isotherm is a model in which one assumes that there is no dependence of the free energy of adsorption on the surface coverage. For the desorption process,

$$\text{Trp(interface)} \underset{\Leftrightarrow}{K} \text{Trp(aq)} \tag{1}$$

the form of the Langmuir isotherm is

$$\Theta = \frac{a_{\text{Trp}}}{a_{\text{Trp}} + K} \tag{2}$$

where a_{Trp} is the bulk activity of tryptophan and Θ is the relative surface coverage. We assume that tryptophan behaves ideally and therefore we identify the activity with the aqueous molar concentration of tryptophan. K is the partitioning constant for desorption of tryptophan from the interface. It can be thought of as equivalent to the equilibrium constant for desorption. The equilibrium constant can be used to determine the free energy of adsorption.

$$\Delta\overline{G}^{\circ}_{\text{ad}} = -RT \ln(K^{-1}) \tag{3}$$

From our model we find a value for K of $3.6 \times 10^{-4} \pm 8.0 \times 10^{-5}$, which leads to a value for the free energy of $\Delta\overline{G}^{0}_{\text{ad}} = -19 \pm 3$ kJ/mol.

DISCUSSION

Red-Shifted Two-Photon Absorption

In order to help determine the two-photon excitation mechanism involved in the interface adsorption study, a two-photon excitation spectrum of L-tryptophan in aqueous solution was recorded. Consider Fig. 3 for the following discussion. We are interested in the region

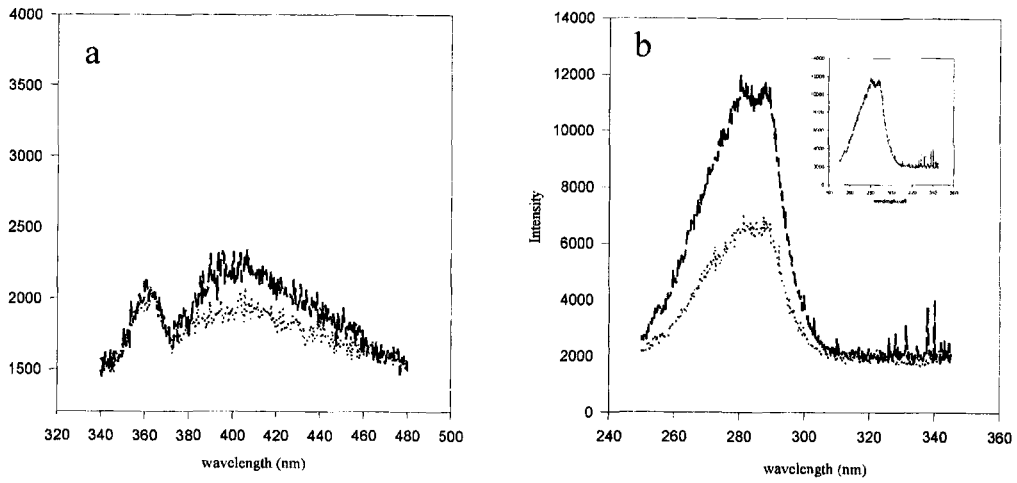

Fig. 5. (a) The fluorescence spectrum of 10^{-4} M tryptophan in aqueous solution. The dotted line represents a natural solution, whereas the darker line represents a degassed solution prepared as described in the text. The excitation wavelength was 320 nm. (b) The one-photon excitation spectrum of 10^{-4} *M* tryptophan in aqueous solution. The dark line is at 298 K, whereas the dotted line represents an equilibrium temperature of 325 K. In the inset, the 325 K spectrum has been normalized to the 298 K spectrum. The emission wavelength was 400 nm.

around 330 nm (2 × 660 nm), which is a longer wavelength than the typically quoted onset of the $S_0 \rightarrow S_1$ transition (310 nm, or 2 × 620 nm). Although excitation at a wavelength this long seems to preclude tryptophan as the carrier of the red end of the spectrum, the fluorescence spectrum resulting from this excitation is that of tryptophan. The fluorescence spectrum, presented in Fig. 5a, is extremely weak in one-photon (λ = 330 nm) excitation. Recent studies on multiphoton excitation in condensed phase have revealed significantly broadened spectra [15]. For the study in Ref. 15, the simple explanation is that excitation is induced using a femtosecond pulsed laser which necessarily has a broad energy spectrum. The spectrum of L-trp presented here was collected using a laser with transform limited pulses of approximately 5 ns. The energy spectrum of these pulses could not account for the long red absorption feature in Fig. 3. Instead, there is most likely a confluence of phenomena leading an extension of the long-wavelength region of the excitation spectrum.

The lowest electronic absorption occurs between the "vibrationless" ground electronic state and the "vibrationless" excited electronic state. This is sometimes called the 0–0 transition. Usually excitation at wavelengths longer than the 0–0 transition originates from excited vibrational levels in the ground electronic state and are termed vibrational hot-bands, because they gain intensity with increasing temperature. The equilibrium distribution of an ensemble of molecules among the various vibrational levels can be modeled using Boltzmann statistics. Thus, the relative populations, N_j and N_i, between two levels at different energies, E_j and E_i, at an equilibrium temperature, T, can be predicted with the Boltzmann equation.

$$\frac{N_j}{N_i} = \frac{G_j}{G_i} e^{\frac{-(E_j - E_i)}{kT}} \tag{4}$$

Here G_j and G_i are the number of degenerate vibrational levels at energies E_j and E_i, respectively, and k is the Boltzmann proportionality constant. The equilibrium temperature for the spectrum in Fig. 3 was 298 K. We can calculate the ratio of molecules with the excess energy sufficient to allow excitation into S_1 by absorption of two photons of 660 nm, using Eq. (4). If the 0–0 transition occurs at 300 nm, then the energy difference is approximately 3000 cm^{-1} and the ratio $N_{3000}/N_0 = 4.6 \times 10^{-7}$. If the 0–0 transition occurs at 310 nm as others suggest [16], then the energy difference of approximately 2000 cm^{-1} leads to a ratio of 6.0×10^{-5}. Both of these calculations assume a degeneracy ratio of 1. Reasonable vibrational state densities at 2000 and 3000 cm^{-1} are 30/cm^{-1} and 100/cm^{-1}, respectively. Including these numbers in the ratio calculation leads to ratios of 1.8×10^{-3} and 4.6×10^{-5}. Thus, there is likely measurable population in these vibrationally excited levels. To quote these ratios, one assumes that the Franck–Condon vibrational overlap is the same as for a one-photon transition. This is not necessarily true, especially when considering vibrational hot-bands. Nevertheless, it is

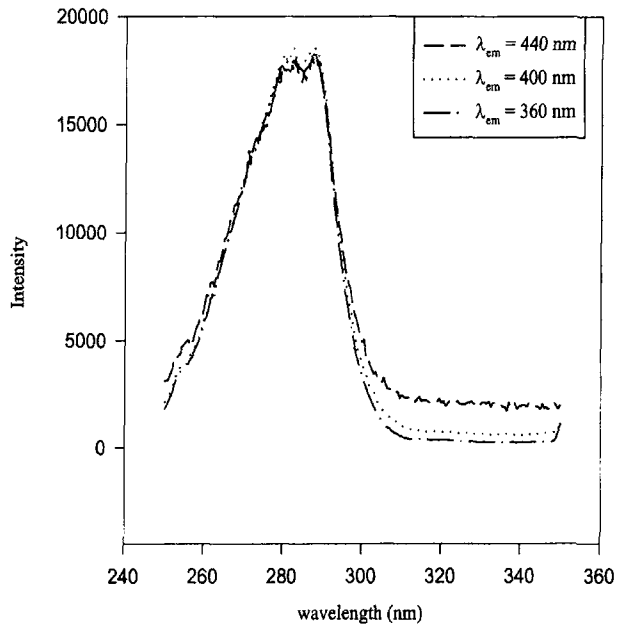

Fig. 6. The one-photon excitation spectra of 10^{-4} *M* tryptophan in aqueous solution as a function of emission wavelength.

likely that at least some of the two-photon $S_0 \rightarrow {}^1L_a$ absorption cross-section originates from vibrationally excited molecules. Evidence of this is presented in Fig. 5b. In this figure, the dependence of the excitation spectrum on temperature is plotted. There is a decrease in the 285-nm absorption coupled with a change in band shape. The ratio of absorptivities of the red edge (300–330 nm) and the maximum (285 nm) decreases with increasing temperature, but the absolute absorptivity of the red edge is not significantly effected. This suggests that the Franck–Condon overlap for transitions originating from vibrationally hot molecules into the L_a and L_b states is lower than that for transitions originating from the vibrationless ground state.

The other source of the two-photon excitation cross-section is direct absorption into the triplet manifold. The fluorescence spectrum of a 1.0×10^{-4} *M* aqueous solution of tryptophan is shown in Fig. 5a. Evident in this figure is a broad red-shifted feature suggestive of ${}^3L_a \rightarrow S_0$ emission [17,18]. After bubbling nitrogen through the standard fluorescence cuvette for 1.5 h, this feature becomes more prominent compared with the fluorescence peak at 360 nm. Further evidence that tryptophan in the carrier of both emission features is presented in Fig. 6. In this figure, the behavior of excitation spectra as a function of emission wavelength is shown. Note that as the emission wavelength becomes longer, there is an increase in the red end of the absorption feature. This is also indicative of direct excitation into a state lower in energy than the first excited singlet state. In fact, at 440 nm, it is highly unlikely that significant emission intensity originates from ${}^1L_a$. It is quite possible that we observe an enhancement of the phosphorescence signal at the H_2O–CCl_4 interface due to the heavy atom effect. The interfacial chlorine atoms could allow a stronger coupling between the singlet and the triplet states and enhance both the excitation cross-section and the quantum yield for emission.

It is notable that the two-photon excitation and one-photon excitation spectra are not as similar as previously observed in the elegant study by Callis and co-workers [16]. In the Callis paper, fluorescence polarization is used to deconvolute the ${}^1L_a$ and ${}^1L_b$ states of tryptophan. They found that the maximum for excitation into the ${}^1L_a$ state occurs around 275 nm, whereas for ${}^1L_b$ there are two maxima, at 290 and 280 nm, respectively. Furthermore, they determined that the $S_0 \rightarrow {}^1L_a$ transition extends well to the red of the $S_0 \rightarrow {}^1L_b$ transition. In our two-photon spectrum, the feature whose maximum is at approximately 275 nm is relatively stronger than in the one-photon absorption. This would suggest that the two-photon excitation (TPE) cross-section for the $S_0 \rightarrow {}^1L_a$ transition is significantly greater than that for the $S_0 \rightarrow {}^1L_b$ transition. The difference between our study and that of Callis appears to be in the two-photon excitation spectrum. Our result has a more gradual slope on the red-edge. In fact, our TPE spectrum is remarkably similar to the deconvoluted ${}^1L_a$ spectrum reported in Ref. 16. By examining the laser power level, recorded synchronously with the TPE (Fig. 3), one can see that laser power fluctuations affect the TPE only marginally.

Adsorption Phenomena: The H_2O–CCl_4 Interface

We now turn to the adsorption behavior of L-tryptophan at the H_2O–CCl_4 interface. The adsorption isotherm presented in Fig. 3 suggests that there is a propensity for tryptophan to adsorb from aqueous solution to an interface between the hydrophilic medium and a hydrophobic phase such as CCl_4. This is reasonable in light of the amphiphilicity of tryptophan and the hydrophobicity of the indolyl R-group [5]. The adsorption isotherm data confirm that an effective monolayer of tryptophan is formed at the liquid–liquid interface at a bulk concentration almost one order of magnitude lower than the bulk solubility of tryptophan in aqueous solution at 290 K, $S_{290} = 0.05$ *M*[19]. It is useful to compare our value for the change in free energy for adsorption with the hydrophobicity of the indolyl side chain calculated by Nozaki and Tanford [22]. In their work, they

determined the contribution of the indolyl side chain to the free energy for transfer of tryptophan from water into either ethanol or dioxane. They accomplished this by measuring the solubility of tryptophan in mixtures of aqueous and nonaqueous solutions of varying mole fractions. The solubility is then extrapolated to a 100% nonaqueous solution to calculate the free energy of transfer. This free energy is found to vary as the natural logarithm of the ratio the mole fraction of tryptophan in the water phase (at the saturation point) to the mole fraction of tryptophan in the nonaqueous phase (at the saturation point). From this free energy, the contribution of the zwitterionic amino acid backbone is subtracted. Nozaki and Tanford find that the amino acids with aromatic side chains display the greatest degree of hydrophobicity with the indolyl side chain of tryptophan possessing the largest free energy of transfer from aqueous to nonaqueous media. Additionally, they observe little dependence on solvent systems for their estimated value for the free energy of transfer. The value determined, $\Delta\overline{G}^0_t = -14.2$ kJ/mol, is the average over the results for water/ethanol and water/dioxane solutions. To this we compare our value for the free energy of adsorption of tryptophan to the H_2O–CCl_4 interface, $\Delta\overline{G}^0_{ad} = -19$ kJ/mol. The difference in temperature between our experiment, 290 K, and Tanford's 298 K cannot account for this difference in free energy. It is possible to rationalize the difference in values, because these free energies do not represent exactly the same process. First, our experiment was not performed at the saturation point of tryptophan in water. Since the effective monolayer has already formed well below this point, our measured value for the equilibrium constant of adsorption is reliable. Furthermore, Nozaki's measurements are for transfer to and from bulk media, whereas ours represents transfer from the bulk to an interface. Thus, the entropy change of the interface itself would also contribute to the free energy of adsorption. Second, we have measured a free energy of adsorption which contains contributions from the zwitterionic amino acid backbone. Nozaki and Tanford [22] have estimated this to be as large as 6 kJ/mol. Although one would expect there to be a large positive change in enthalpy for an ion approaching a nonpolar medium, this could be minimized because of the inherently large polarizability of CCl_4. Third, the variation between the two nonaqueous solvents in Tanford's study was as large as 1 kJ/mol. Our system of H_2O–CCl_4 may fall outside this variation. The high symmetry of CCl_4 would suggest that a nonpolar chromophore could be easily accommodated. This leads to a positive ΔS_{ad} for tryptophan. Furthermore, the solubility of the nonaqueous media in water may affect the comparison of the entropic component's contribution to the free energy in Ref. 22. Finally, in contrast to the study of Nozaki and Tanford, we find a significant dependence of the value of $\Delta\overline{G}^0_{ad}$ on the interfacial system in question. Our work on the behavior of tryptophan at the water–octanol interface revealed a $\Delta\overline{G}^0_{ad} = -11$ kJ/mol [10]. This lower value may be due to an interface which is more highly organized and therefore less entropically favorable for adsorption of an amphiphilic molecule. Given the preceding arguments, our value for $\Delta\overline{G}^0_{ad}$ compares reasonably with that for the transfer of tryptophan from aqueous to nonaqueous media derived by Tanford [22]. In both studies, the process is driven by the hydrophobicity of the indolyl side chain.

Comparison of Adsorption Behavior with the Octanol–Water Interface

We can compare the present findings with those for adsorption of L-trp to the octanol–water interface found previously [10]. By comparing the free energies of adsorption, $\Delta\overline{G}^0_{wo} = -11$ kJ/mol versus $\Delta\overline{G}^0_{wCCl_4} = -19$ kJ/mol, we find that there is a stronger propensity for L-trp to adsorb to the H_2O–CCl_4 interface. This could result from several possible sources. The simplest is that the indolyl side chain interacts more strongly with CCl_4 than with octanol. We have found 3-methyl-indole to be more soluble in CCl_4 than in octanol. This would suggest that interfacial CCl_4, although polarized, is still more hydrophobic than octanol at the interface. Additionally, there may be a lowering in entropy for rearrangement of the hydrogen-bond network at the water–octanol interface in order to accommodate each tryptophan molecule. In contrast, the CCl_4 molecules at the H_2O–CCl_4 interface are more symmetrical and interact less strongly with H_2O to begin with. Thus, the H_2O–CCl_4 interface is "freer" to accept amphiphilic molecules.

Orientation of Tryptophan at the H_2O–CCl_4 Interface

From our polarization data, we determine that the transition moment for two-photon adsorption by interfacial tryptophan must be on average closer to parallel to the interface than perpendicular to it. The evidence for this is an increase in absorbance of s-polarized excitation light versus p-polarized excitation by a factor of 1.75:1. If the chromophores are oriented preferentially at the interface, then the fluorescence polarization must also display this behavior. This is confirmed by our assay of the polarization of the fluorescence emitted from molecules near the interface. For bulk aqueous trypto-

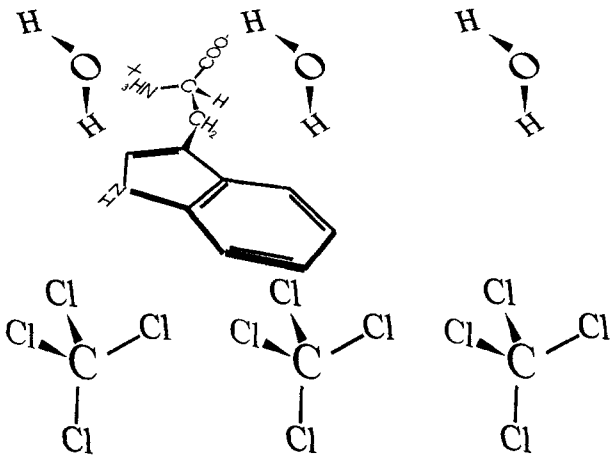

Fig. 7. The structure of the H_2O–CCl_4 interface including an adsorbed L-tryptophan molecule. The aromatic indole group of tryptophan is oriented nearly parallel to the interface. The relative orientations of H_2O and CCl_4 are assumed to be similar to those determined in Refs. 20 and 21.

phan, no polarization dependences were observed. This is expected for random molecular orientations in liquid media. The transition moments to both the L_a and the L_b states are contained in the plane of the indolyl side chain. Therefore, we suggest that these side chains are oriented in an arrangement more parallel to the interface as opposed to being perpendicular to it, irrespective of which excited state the fluorescence originates from. This is consistent with our previous studies [10,11] of other aromatic molecules at liquid interfaces. Moreover, it is consistent with the recent findings from other groups. For example, using the technique of vibrational sum frequency generation at interfaces, Gragson and Richmond [20] found a substantial degree of organization at the H_2O–CCl_4 interface, in both the presence and the absence of amphiphilic surfactants. By examining the OH stretching mode of H_2O at the interface, they found that interfacial water molecules were predominantly in a tetrahedral ice-like arrangement. Gragson and Richmond's pioneering study has corroborated the molecular dynamics prediction of the H_2O–CCl_4 interface structure calculated by Chang and Dang [21]. The structure of the interface will certainly influence the orientation of any molecule adsorbed there. Interestingly, Chang and Dang predicted a significant induced dipole moment in interfacial CCl_4 molecules. This suggests that the true hydrophobicity of CCl_4 is not apparent until well into the bulk liquid. Thus, the relatively hydrophobic indolyl moiety of interfacial tryptophan may not experience a completely nonpolar environment. Possibly the amino acid naturally orients itself such that the charged $-COO^-$ and $-NH_3^+$ groups on the interfacial amino acids minimize their free energy, resulting in an indolyl orientation more parallel than perpendicular to the interface. This interface structure is presented pictorially in Fig. 7. Moreover, by assuming a configuration where the indole's aromatic plane is parallel to the interface, the system's dispersive interactions are maximized.

CONCLUSIONS

We have introduced a two-photon excitation/fluorescence polarization technique in order to measure selectively liquid interface-specific adsorption phenomena. In this work, an isotherm for the adsorption of L-tryptophan from the bulk aqueous phase to the H_2O–CCl_4 interface was determined. Associated with this process is a change in free energy of $\Delta\overline{G}^0_{ad} = -19 \pm 3$ kJ/mol, which compares favorably with the hydrophobicity of the indolyl side chain of tryptophan determined by Nozaki and Tanford [22], $\Delta\overline{G}_t = -14.2$ kJ/mol. The difference is due primarily to differences in the interfacial systems studied. Our polarization data suggest that the indolyl chromophore of tryptophan is oriented in a parallel fashion to the liquid–liquid interface.

By comparing the free energy of adsorption in the present study to that at the water–octanol interface, we find that tryptophan binds more readily to the interface between H_2O and CCl_4. Although both processes are driven by the hydrophobicity of the indolyl side chain, there appears to be a molecular recognition of a specific type of interface. This is akin to the molecular model for recognition in biological systems, albeit in a very simple and controlled environment.

We have rationalized the two-photon excitation of L-tryptophan using 660-nm laser light. The dispersed fluorescence from excitation into a state 330 nm above the ground electronic state reveals evidence of emission from two excited states. These bands are assigned to $^1L_a \rightarrow S_0$ and $^3L_a \rightarrow S_0$ transitions, by virtue of their behavior in degassed aqueous solution. Thus, the two-photon absorption process involves both hot-band excitation into the singlet manifold and direct excitation into the triplet manifold.

ACKNOWLEDGMENTS

Financial support from the Natural Sciences and Engineering Research Council of Canada and from the Ontario Laser and Lightwave Research Centre is grate-

fully acknowledged. D.T.C. would like to thank Professor G. Liu (University of Calgary) for the use of his spectrofluorometer.

REFERENCES

1. R. E. Jacobs and S. H. White (1986) *Biochemistry* **25**, 2605–2612.
2. R. E. Jacobs and S. H. White (1987) *Biochemistry* **26**, 6127–6134.
3. R. E. Jacobs and S. H. White (1989) Biochemistry **28**, 3421–3437.
4. A. I. P. M. De Kroon, M. W. Soekarjo, J. De Gier, and B. De Kruijff (1990) *Biochemistry* **29**, 8229–8240.
5. L. Stryer (1988) *Biochemistry*, Freeman, New York.
6. C. Tanford (1980) *The Hydrophobic Effect*, Wiley, New York.
7. B. L. Smiley and V. Vogel (1995) *J. Chem. Phys.* **103**, 3140–3144.
8. A. A. Rehms and P. R. Callis (1993) *Chem. Phys. Lett.* **208**, 276–282.
9. The smallest focal spot, w_0, is calculated using $w_0 = (4\lambda f/3\pi w)$, where λ is the laser wavelength, f is the lens focal length, and w is the laser beam radius. The depth of focus, Δz, is calculated using $\Delta z = \pm(0.32\pi w_0^2/\lambda)$
10. D. T. Cramb and S. C. Wallace (1997) *J. Phys. Chem.* **B101**, 2741–2744.
11. D. T. Cramb, S. C. Martin, and S. C. Wallace (1996) *J. Phys. Chem.* **100**, 446–449.
12. S. R. Meech, D. R. Phillips, and A. G. Lee (1983) *Chem. Phys.* **80**, 317–328.
13. R. R. Birge and C.-F. Zhang (1990) *J. Chem. Phys.* **92**, 7178–7195.
14. A. W. Adamson (1990) *Physical Chemistry of Surfaces*, Wiley, New York.
15. C. Xu, W. Zipfel, J. B. Shear, R. M. Williams, and W. W. Webb (1996) *Proc. Natl. Acad. Sci. USA* **93**, 10763–10768.
16. A. A. Rehms and P. R. Callis (1987) *Chem. Phys. Lett.* **140**, 83–89.
17. T. Truong, R. Bersohn, P. Brumer, C. K. Luk, and T. Tao (1967) *J. Biol. Chem.* **242**, 2979–2985.
18. G. B. Strambini and M. Gonnelli (1995) *J. Am. Chem. Soc.* **117**, 7646–7651.
19. R. C. Weast (Ed.) (1986) *CRC Handbook of Chemistry and Physics*, CRC Press, Boca Raton, FL.
20. D. E. Gragson and G. L. Richmond (1997) *J. Chem. Phys.* **107**, 9687–9690.
21. T.-M. Chang and L. X. Dang (1996) *J. Chem. Phys.* **104**, 6772–6783.
22. Y. Nozaki and C. Tanford (1971) *J. Biol. Chem.* **246**, 2211–2217.