ELSEVIER

Biophysical Chemistry 56 (1995) 263–271

Biophysical Chemistry

Fluorescence anisotropy of tyrosine using one-and two-photon excitation

Joseph R. Lakowicz [a,*], Borys Kierdaszuk [a,b], Patrik Callis [c], Henryk Malak [a], Ignacy Gryczynski [a]

[a] *Center for Fluorescence Spectroscopy, Department of Biological Chemistry, University of Maryland School of Medicine, 108 North Greene Street, Baltimore, MD 21201, USA*

[b] *Department of Biophysics, University of Warsaw, 93 Zwirki i Wigury St., PL-02089 Warsaw, Poland*

[c] *Department of Chemistry and Biochemistry, Montana State University, Bozeman, MT 59717, USA*

Received 12 December 1994; accepted 14 February 1995

Abstract

We examined the emission spectra and steady-state anisotropy of tyrosyl fluorescence with two-photon excitation from 565 to 578 nm. The emission spectra of phenol and N-acetyl-L-tyrosinamide (NATyrA) were all the same for one-photon excitation (OPE) and two-photon excitation (TPE), and the tyrosine emission from ribonuclease A showed 10-nm shift to longer wavelengths with TPE. Surprisingly, the anisotropy of tyrosine, NATyrA and Leu^5-enkephalin in frozen solution were near zero for TPE as compared to near 0.3 for OPE. Low values of the anisotropy near 0.05 were also found for phenol and ribonuclease A. A low anisotropy appears to be a basic characteristic of tyrosine or tyrosyl residues with two-photon excitation.

Keywords: Fluorescence; Anisotropy; One-photon excitation; Two-photon excitation; Anisotropy spectra; Tyrosine; Tryptophan; Peptides; Proteins

1. Introduction

The intrinsic fluorescence of proteins is widely used in biochemical research to study their structure, functions and dynamics [1–4]. This use of fluorescence can be traced to the classic studies of Teale and Weber [5,6] which revealed the fluorescence spectral properties to the aromatic amino acids, phenylamine, tyrosine and tryptophan. It is now well known that the emission of proteins is dominated by the tryptophan residues, and that tyrosine emission is routinely observed only in proteins which lack tryptophan residues [7,8]. The spectral properties of tryptophan are known to be complex, as revealed by its complex excitation anisotropy spectrum [6]. In contrast, the spectral properties of tyrosine are less

Abbreviations: Gdn-HCl, guanidine hydrochloride; NATyrA, N-acetyl-L-tyrosinamide; OPE, one-photon excitation; OPIF, one-photon induced fluorescence; PG, propylene glycol; PPO, 2,5-diphenyloxazole; RNase A, ribonuclease A; r, steady-state fluorescence anisotropy; r_0, limiting anisotropy in the absence of depolarizing processes; TPE, two-photon excitation; Tris–HCl, tris(hydro×ymethyl)-aminomethane hydrochloride; Tyr, tyrosine

* Corresponding author.

SSDI 0301-4622(95)00040-2

complex, with the long wavelength absorption being the result of a single electronic transition [9].

To date, most studies of protein fluorescence have used the usual one-photon excitation (OPE). However, the increasing availability of picosecond (ps) and femtosecond (fs) pulsed laser has resulted in the possibility of two-photon excitation (TPE) for a variety of biochemical fluorophores [10–12], including tryptophan [13] and tryptophan-containing proteins [14]. By TPE we are referring to the simultaneous absorption of two long wavelength protons to excite the fluorophore to the first excited electronic state. While several reports have appeared on TPE of tryptophan and indoles [13–16], less information is available on tyrosine due to its weaker two-photon cross section, and the blue shift of its two-photon absorption spectrum [16,17]. In the present report we describe the emission spectra and absorption properties of tyrosine, tyrosine derivatives, and the tyrosine-containing protein ribonuclease A when excited with one UV or two long wavelength photons.

2. Materials

N-acetyl-L-tyrosinamide and L-tyrosine were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI) and was used without further purification. Phenol (loose crystals, standard Fisher Analytical Reagent) was from Mallinckrodt (St. Louis, Missouri). Leu^5-enkephalin (Tyr–Gly–Gly–Phe–Leu) in the form of acetate salt was purchased from Sigma Chemical Co. Lyophilized ribonuclease (EC 3.1.27.5, RNase A) from Bovine Pancreas, type II-A (Sigma Chem. Co.) was further purified using repeatedly Centriprep-10 Concentrators (Amicon, Inc. Beverly, MA) to exchange buffer and to remove small molecular contaminants (mol. weight < 10 kD) which showed absorption and emission in the range 250–290 and 300–380 nm, respectively. The concentration of RNase A was measured spectrophotometrically using extinction coefficient of 9800 M^{-1} cm^{-1} at 278 nm [18]. Propylene glycol (p.a.) was bought from Janssen Chimica through Spectrum Chemical Mfg Corp. (New Brunswick, NJ). Guanidine hydrochloride (ultra pure) was obtained from Schwarz/Mann (Orangeburg, NY), and Tris(hydroxymethyl)-aminomethane hydrochloride (made by E. Merck, Darmstadt, Germany) was purchased from E.M. Science Inc. (New Jersey, NJ). Tris–HCl buffer (pH 7.5) was made using MilliQ water and HCl (Aldrich). All other chemicals, reagents and materials were of the highest, spectral-grade quality commercially available. They were checked by UV absorption and/or fluorescence measurements. Absorption measurements were performed on a Perkin-Elmer Lambda 6 UV/VIS spectrophotometer (The Perkin Elmer Co., Rockville, MD). Steady-state fluorescence measurements were carried out at 20°C using a SLM 8000 photon-counting spectrofluorimeter (with 2 nm spectral resolution for excitation and emission) equipped with a Hamamatsu R928 photomultiplier. Fluorescence was excited at several wavelengths in the range 270–300 nm, and fluorescence spectra were recorded in the 280–400-nm range.

3. Fluorescence spectroscopic methods

Two-photon excitation was accomplished using the fundamental output of a cavity-dumped rhodamine-6G laser from 566 to 610 nm, which was passed through Glan-Thompson vertical polarizer and focused in the sample using a 5 cm focal length lens. The pulse full width at half-maximum was about 5 ps at a repetition rate of 3.795 MHz. The detection was based on time-correlated single photon counting [19]. We used 1.0×0.5 cm cuvettes placed in the thermostated SLM cell holder (Urbana, Champaign, IL), with a long axis aligned along the incident light path and with the focal point positioned about 0.5 cm from the surface facing the incident light. The position of the cuvette and the lens were adjusted so that the focal point of the laser excitation was located near the observation window. Fluorescence was collected by a suprasil lens, filtered from the contaminating scatter of the long-wavelength light using a two Corning 7-54 filters (2×3 mm thick) and passed through an ISA (New Jersey) grating monochromator (4 nm slit bandwidth) and a Glan-Thompson magic-angle polarizer. Then wavelength scale of the monochromator was calibrated using a SCT1 mercury lamp (Ultra-Violet Products Inc., San Gabriel, CA) as a wavelength standard. Fluorescence spectra were corrected for transmittance of the two 7-54 filters. The intensity at each wavelength was ob-

tained by integrating the time-resolved intensity decay. In addition to high sensitivity detection, this method of intensity measurement allowed us to demonstrate the absence of scattered light in the detected emission. Signal from the solvent alone was less than 0.5% and was subtracted from sample fluorescence.

For anisotropy determinations the polarized emission was observed through an interference filter centered at 313 nm (10-nm band-pass), a Glan-Thompson polarizer and two 7-54 filters. The anisotropy, r, values were determined from polarized fluorescence intensities as

$$r = (gF_{vv} - F_{vh})/(gF_{vv} + 2F_{vh}) \quad (1)$$

where F_{vv} is vertically polarized emission intensity with vertically polarized excitation, and F_{vh} is horizontally polarized emission with vertically polarized excitation. The instrumental correction factor, g, is equal to $g = F_{hh}/F_{hv}$, where F_{hh} is horizontally polarized emission intensity with horizontally polarized excitation, and F_{hv} is vertically polarized emission with horizontally polarized excitation. This g factor corrects for the small polarization bias of the optical system and photomultiplier. The g factor was usually within a few percent of 1.0. Anisotropy values presented here were obtained as an average of at least three determinations.

One-photon induced fluorescence and fluorescence anisotropy spectra were measured on the same laser system as two-photon induced fluorescence and fluorescence anisotropy (see above) equipped additionally with a 390 Frequency Doubler (Spectra Physics) and a polarization rotator. Such measurements permitted reliable comparison of the fluorescence and fluorescence anisotropy spectra resulted from these two types of excitation.

4. Results

4.1. One-and two-photon excitation of tyrosine and phenol

Emission spectra of N-acetyl-L-tyrosinamide (NATyrA) are shown in Fig. 1 for OPE at 283 nm and TPE at 566 nm. As described in Materials and methods, these spectra were recorded on the same

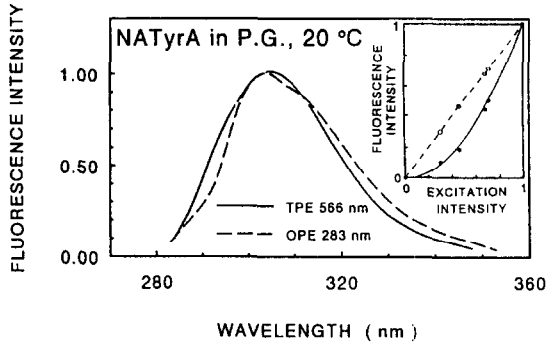

Fig. 1. Fluorescence emission spectra of NATyrA obtained for one (---) and two-photo (—) excitation. The insert shows the dependence of the one-photon (---) and two-photon induced fluorescence intensity (—) on the intensity of the 283 (○) or 566 nm (●) incident light. The maximum intensities for OPE and TPE are both normalized to unity in the insert.

instrument, except for the presence of a frequency-doubler for OPE. For OPE the emission intensity is linearly proportional to the intensity of the incident light (Fig. 1, insert, –○–). In the case of TPE the intensity depends quadratically on the intensity at 566 nm (–●–).

The quadratic dependence on the excitation intensity demonstrates that the observed emission spectra of NATyrA is due to a biphotonic process at 566. The emission spectra of NATyrA are similar for OPE and TPE (Fig. 1), which indicate that essentially the same electronic state is reached for both modes of excitation. The small difference seen in Fig. 1 is within experimental uncertainty, which is relatively high due to the weak emission for TPE of NATyrA.

4.2. Fluorescence anisotropy of NATyrA, L-tyrosine and phenol

We examined the excitation anisotropy spectrum of NATyrA and tyrosine in vitrified solution (Fig. 2). These data for TPE could only be obtained for a limited range of wavelengths where there was adequate intensity from our R6G dye laser. This wavelength range from 566 to 578 nm corresponds to a range of wavelengths for OPE when the anisotropy is known to be near 0.3 [6,20]. Surprisingly, the anisotropy for TPE of NATyr A was found to be zero or below zero (Fig. 2). Similar results were

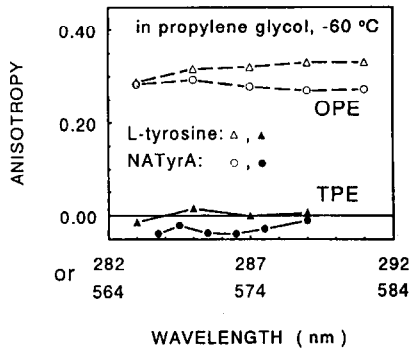

Fig. 2. Excitation anisotropy spectra of 5 mM NATyrA (△,▲) and 2.5 mM L-tyrosine (○,●) in propylene glycol (P.G.) at −60°C obtained for one (---) and two-photon (—) excitation.

found for tyrosine itself, with the usual high anisotropy for OPE, and an anisotropy near zero for TPE (Fig. 2).

To further investigate these surprising results we examine the anisotropy of phenol. Once again, the anisotropy for TPE was significantly smaller than that found for OPE (Fig. 3). The emission spectra for phenol were essentially identical for OPE and TPE (Fig. 4), and again demonstrate emission occurs from the same electronic state for both modes of excitation. At this time we do not think the minor spectral difference seen at 320 nm is experimentally significant.

As a control experiment we measured the anisotropy of PPO, which is known to display a higher anisotropy for TPE than for OPE [21]. The data for PPO displayed the expected larger

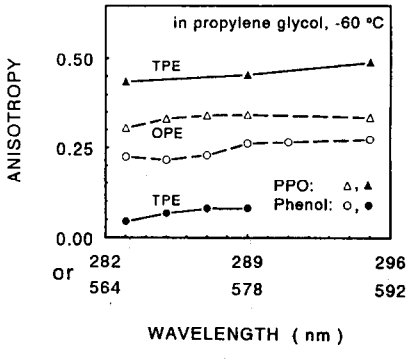

Fig. 3. Excitation anisotropy spectra of 0.54 mM phenol (○,●) and 0.54 mM PPO (△,▲) in 60% propylene glycol (P.G.) at −60°C obtained for one (---) and two-photon (—) excitation.

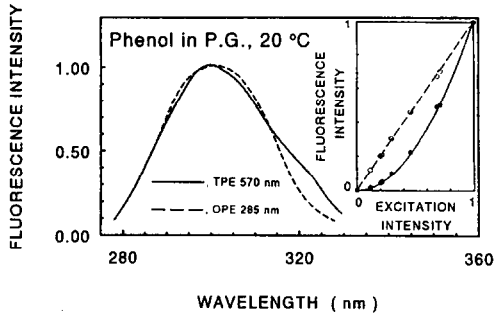

Fig. 4. Fluorescence emission spectra of phenol obtained for one (---) and two-photon (—) excitation. The insert shows the dependence of the one-photon (---) and two-photon induced fluorescence intensity (—) on the intensity of the 283 (○) or 566 nm (●) incident light. The maximum intensities for OPE and TPE are both normalized to unity in the insert.

anisotropies for TPE (Fig. 3), and thus confirm the low anisotropies observed for tyrosine and its derivatives (Figs. 1 and 2).

Because of the low cross sections for two-photon excitation it was necessary to use relatively high concentrations of tyrosine, NATyrA and phenol. Hence we question whether the low anisotropies observed for TPE could be the result of energy transfer between the fluorophores. Such an effect seemed unlikely since high anisotropies were observed for OPE (Figs. 2 and 3), but it is known that phenol can display energy transfer depolarization [6]. Hence we examined the effects of concentration on the anisotropy values for OPE and TPE. For NATyrA the anisotropy was essentially constant up to 10 mM (Fig. 5), which is a 2-fold higher concentration than

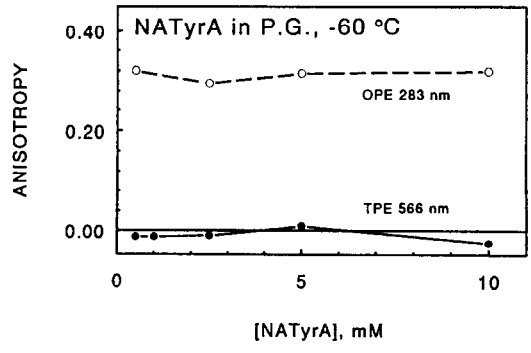

Fig. 5. Concentration dependence of the fluorescence anisotropy of NATyrA in propylene glycol (P.G.) at −60°C obtained for one (---) and two-photon (—) excitation.

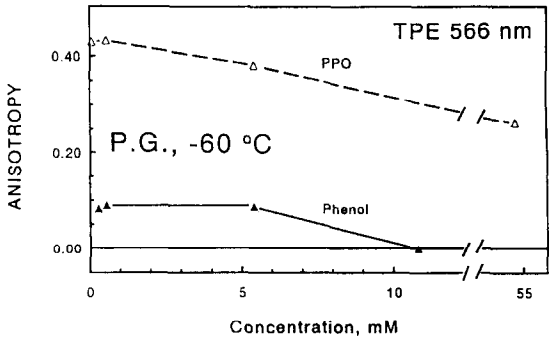

Fig. 6. Concentration dependence of the two-photon induced fluorescence anisotropy of PPO (---) and phenol (—) in propylene glycol (P.G.) at −60°C.

used to obtain the anisotropy spectra. Similarly, the anisotropy of phenol remained constant up to at least 5 mM (Fig. 6), which is nearly 10-fold larger than the 0.54 mM concentration used to obtain its anisotropy spectrum. These results suggest that energy transfer between the fluorophores is not responsible for the low anisotropy values of phenol and tyrosine derivatives observed with TPE.

4.3. *Fluorescence anisotropy of tyrosine in Leu-enkephalin and RNase A*

We next examined the anisotropy of a tyrosyl peptide, Leu^5-enkephalin, and a tyrosine protein, RNase A. For both solutions the anisotropy for OPE was near the expected value. However, for TPE, the anisotropy of Leu^5-enkephalin was near zero (Fig. 5, Fig. 6, and Fig. 7), and for RNase A near 0.06. Hence it appears that the low anisotropy of tyrosyl fluorescence seen for the model compounds persists for tyrosyl residues in peptides and proteins.

Emission spectra of RNase A for OPE and TPE are shown in Fig. 8. We were surprised to observe that the tyrosyl emission of RNase A is red shifted by 10 nm for TPE. This spectral shift disappeared upon disruption of three dimensional structure of RNase by 6 M guanidine hydrochloride (Fig. 8, bottom). At this time we do not understand the origin of the spectral shift, and we do not know if this effect occurs in other tyrosyl protein or peptides. We were unable to obtain emission spectra of Leu^5-enkephalin with TPE due to the limited availability of the peptide.

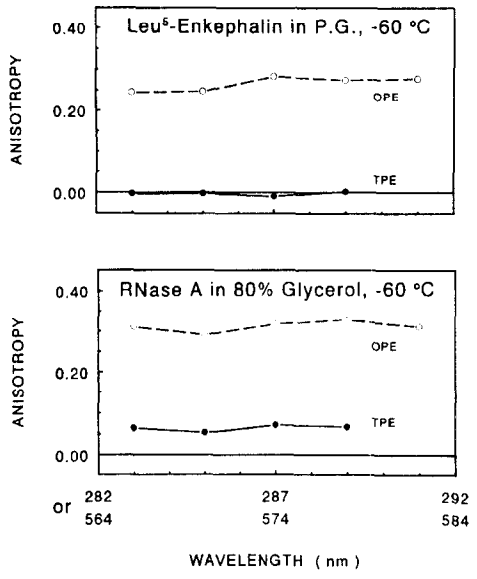

Fig. 7. Excitation anisotropy spectra of 2 mM Leu^5-enkephalin (top) in 90% propylene glycol and 2 mM RNase A (bottom) in 20 mM Tris–HCl (pH 6.0) containing 80% glycerol obtained for one (---) and two-photon (—) excitation at −60°C.

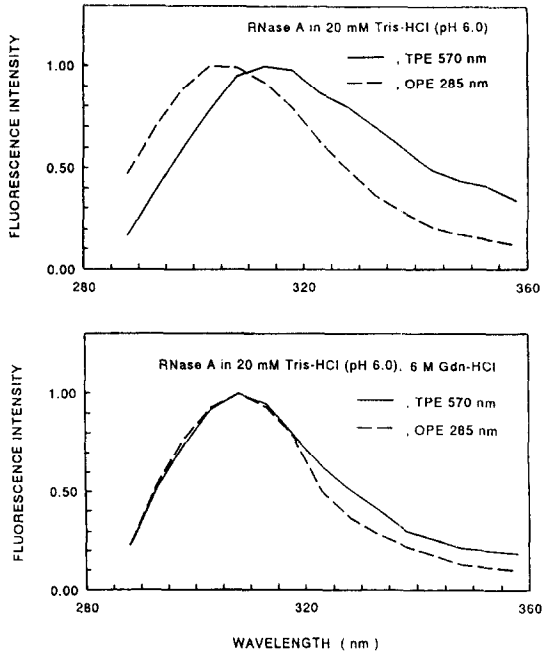

Fig. 8. Fluorescence emission spectra of 1.5 mM RNase A at 20°C obtained for one (---) and two-photon excitation (—). Top: RNase A in 20 mM Tris–HCl (pH 6.0) containing 80% glycerol. Bottom: RNase A in 20 mM Tris–HCl (pH 6.0) containing 80% glycerol and 6 M Gdn-HCl.

5. Discussion

5.1. Low two-photon anisotropy

Considering that recent reported values of TPIF anisotropy [21,22] are considerably larger than the corresponding one-photon values, it may seem surprising at first that it is so low for phenol and tyrosine. However, for S_1 of aromatic systems it is known [23,15] that low anisotropies on the order of $1/7$ should be common. Our result for phenol, which approaches 0.1, perhaps falls into the expected pattern. The result for tyrosine, however, appears to be the consequence of special circumstances involving interference of *para*-substituted groups, which is opposite the effect found in one-photon absorption [24–27,16]. Thus, the $S_0 \rightarrow S_1$ transition for tyrosine is effectively two-photon forbidden, meaning that much, if not all, of the two-photon spectrum is due to vibrationally induced intensity, just as for benzene and symmetrically *para*-substituted benzenes. This fact has already been established qualitatively from the observation that the TPE spectrum of NATyrA is similar in shape but blue shifted ca. 1600 cm^{-1} relative to that expected from the OPA [16]. The discussion in this section will first review why the anisotropy found for phenol is essentially as expected, and then will detail the interesting circumstance leading to the even lower values found for tyrosine. In the following discussion of phenol and tyrosine the molecule-fixed coordinate system is chosen so that z is perpendicular to the molecular plane and the x axis connects the *para* substituents. The one-photon transition moment, which will govern the polarization of the emitted light, is therefore in the y direction.

Phenol

Whereas one-photon absorptivity and emission is controlled by the square of a transition dipole vector, two-photon absorptivity is determined by a 3×3 tensor [28,29,15], whose elements will be explicitly related to the corresponding transition dipole only when the transition contains significant charge transfer character [30]. In general, there is little connection between the one-and two-photon parameters. In particular, for excitation to totally symmetric vibrational states of S_1 (^1L_b) of simple substituted benzenes such as tyrosine and phenol, McClain [28] has shown by symmetry arguments alone that the tensor must have the form:

$$\begin{matrix} 0 & a & 0 \\ b & 0 & 0 \\ 0 & 0 & 0 \end{matrix}$$

Providing that the substituent makes the transition strongly two-photon allowed, which is marginally the case for phenol [24,25], the xy and yx elements will dominate. In this limit the photoselection produced by excitation with linearly polarized light is compromised by the fact that maximum absorptivity occurs along *two* polarization directions in the molecule (the two directions that make a 45° angle with the x and y axes. The predicted [31,15] anisotropy, r, is therefore precisely the same as for the OPE of benzene and other symmetrical systems whose in-plane absorptivity is independent of the in-plane direction; $r = 0.1428$, independent of the direction of the fluorescence transition dipole [15]. The experimental values reported in the present paper for phenol range from 0.07 to 0.10, not far from the ideal value just mentioned. Agreement is better if one recognizes that theoretical anisotropies are seldom found experimentally, even when every effort is made to eliminate experimental artifacts. For example, the OPE anisotropy for benzene in rigid hydrocarbon glass is found to be 0.13 compared to the expected 0.14 [32]. Even more pertinent is the two-photon induced fluorescence anisotropy of benzene under these conditions [33] where e_{2g} vibronic states yield $r = 0.08$ compared to the expected 0.14, and e_{1g} states yield -0.045 when -0.07 is expected.

Tyrosine

For reasons that will shortly become clear, we begin the discussion of tyrosine by reviewing the behavior of benzene. Because of its hexagonal symmetry, the 0–0 electronic transition to S_1 (B_{2u}) is forbidden in both one- and two-photon excitation. The same is true for any benzene vibronic transition originating in the ground vibrational state of the ground electronic state and terminating in a totally symmetric vibrational state of S_1. However, benzene does have substantial UV and two-photon absorptivity to S_1 due to vibrationally induced transitions, often referred to as Herzberg–Teller vibronic cou-

pling, intensity borrowing, or intensity stealing [34]. This vibrationally induced intensity is not very strong in one-photon absorption, having an oscillator strength of about 0.001, and giving a maximum molar decadic extinction coefficient of about 200 l mol^{-1} cm^{-1}. Virtually all of the vibrationally induced intensity is due to ν_6, a 520 cm^{-1} in-plane bending mode. This means that the absorption envelope has its origin (a so called false origin) at 520 cm^{-1} above the true origin (0–0). A progression of lines consisting of successive numbers of quanta of the 900 cm^{-1} totally symmetric ring breathing mode make up the main features of the absorption band. The relative heights of the lines depends only on the Franck–Condon factors for the change in size of the ring so that the shape of the absorption envelope is the same in one-and two-photon excitation; as will be noted below, only the position of the false origins(s) will be different in the two spectroscopies.

The hydroxyl group induces about 10 times more intensity, all but overwhelming the vibronic component, which manifests itself only by the small wavelength dependence of the anisotropy. A methyl group, on the other hand, induces absorptivity only weakly, contributing at the same level as the vibronic part. A methyl added to phenol therefore has only a small effect.

The story is almost completely reversed in two-photon spectroscopy for this transition. The vibrationally induced intensity overwhelms the substituent contributions by an order of magnitude [24–27]. The methyl is about equal to hydroxyl in its ability to induce two-photon absorbance. Also of particular importance, when methyl and hydroxyl are *para* to each other their perturbations nearly cancel [24–27], leaving only the vibrational contributions as in benzene. It is seen that intuition based on UV spectroscopy is completely misleading. We conclude that *a good working model for the two-photon anisotropy of tyrosine is benzene itself.*

The vibrations which induce two-photon intensity are also different than those active in the UV excitation, and are an important part of the story. By far the major contributor is ν_{14}, a B_{2u} 1570 cm^{-1} stretching mode [35]. Thus, most of the two-photon excitation spectrum of benzene is shifted by about 1600 cm^{-1} to the blue. We believe this is the reason that the two-photon excitation curve for NATyrA is blue shifted [16]. However, this source of intensity may not be important for this discussion because our results are for excitation on the extreme red edge. Two lower frequency modes, an in-plane bend (e_{1u}, ν_{18} and an out-of-plane (oop) bend (e_{2u}, ν_{17}) with frequencies at 900 and 700 cm^{-1} respectively may be important in this case. In particular, one component of the degenerate oop bends induces a TPE tensor with only xz and zx elements. Since the emission is y-polarized while all absorption is along directions perpendicular to y, the predicted r is -0.28, an obvious source of the observed negative anisotropy. That we see only slightly negative r values ($r = -0.03$ to -0.04) is understood by the existence of overlapping sources with higher r. The other component of the e_{u2}-induced intensity has yz and zy elements in its TPE tensor while the in-plane e_{1u} bends have xy and yx elements (and xx and $-yy$ elements), all of which have $r = 0.14$.

Analysis is further complicated by at least one, and probably two additional mechanisms of TPE on the extreme red edge of the spectrum. It is known that benzene and toluene exhibit a 0–0 TPE line in solution because of the unsymmetrical solvent environment. This solvent induced origin rivals the 700 and 900 cm^{-1} vibrations in strength and is undoubtedly a main source of intensity in the work reported here [35–37]. Finally, because the methyl and hydroxyl induced intensities probably do not exactly cancel, there could be a small origin intensity in the absence of solvent induced intensity. Unfortunately, the gas phase TPE of *p*-methylphenol, which would reveal the latter source, does not seem to be known.

All but the last of the five mentioned mechanisms of TPE intensity have been documented in the elegant steady state investigation of two-photon induced fluorescence anisotropy of benzene in rigid pentane at 77K by Scott et al. [33]. Unlike the present study, the spectrum of the non-polar benzene in a non-polar solvent was sufficiently resolved that the anisotropy of the individual components could be assessed. Although they did not report the anisotropy explicitly, they reported three independent polarization ratios from which r can be recovered. Of primary interest is the oop intensity at 700 cm^{-1} above the origin for which r was observed to be -0.045, a value approaching the theoretically expected value of $r = -0.07$ (the average of -0.28 and $+0.14$). Vari-

ous unknown depolarizing influences commonly lead to such disparities in steady state studies in glasses.

In the present study, the polar nature of both solute and solvent serves to broaden the lines so that the origin and 700 cm^{-1} contributions are probably overlapped significantly at the red edge. Unfortunately, r for the solvent induced benzene origin in pentane glass was not reported [33], but it was shown to be nearly as strong as the 700 and 900 cm^{-1} intensities with linear polarization.

In addition to the two-photon anisotropy in rigid solution reported here, independent information on the two-photon tensor of NATyrA is available from the ratio, Ω, of two-photon absorptivities using circular and linear polarized light in fluid aqueous solution [16]. This ratio is defined in terms of tensor invariants,

$$\Omega = \delta_{circ}/\delta_{lin} = (-\delta_F + 3\delta_G)/(\delta_F + 2\delta_G) \qquad (2)$$

where δ_G is the sum of squares of the elements and δ_F is the square of the trace of the tensor [28,29]. Ω has been reported for many aromatic systems because it gives polarization information in fluid systems [35,38,36,37]. For NATyrA, Ω was found to approach 0.9 for excitation on the red edge and is 0.45 near the TPE maximum at 520 nm [16]. For a consistent interpretation, the tensor we deduce should simultaneously predict both the observed anisotropy and the Ω values.

Ω values expected from the five sources listed above are as follows: the allowed origin (if present) and the 700 and 900 cm^{-1} vibrations give $\Omega = 1.5$ in vapor [35,38] and the strong b_{2u} vibration gives $\Omega = 0.05$ in vapor for benzene [35]. The solvent induced origin gives $\Omega = 0.6$–0.7 for a variety of cases in various non-polar solvents [35–37] at room temperature, where it dominates the 700 and 900 cm^{-1} vibrational contributions. $\Omega = 1.2$ for the 0–0 of neat toluene [35] and toluene in ethanol solution [37] *and* for phenylalanine in aqueous solution [16], suggesting that much of the origin intensity is solvent-induced and that it is not very sensitive to solvent polarity.

The above information serves to illustrates the complexity of precisely explaining the low anisotropy observed for tyrosine. Extrapolation from benzene to tyrosine is made even more uncertain by the considerably greater solvent-induced broadening because both the solute and solvent are polar. However, it appears that a combination of the solvent induced origin and the out-of-plane vibronic contribution could account for the observed r and Ω values. The latter will definitely give the slightly negative r values seen, but by itself would give $\Omega = 1.5$, a much larger value than the observed 0.9. The solvent-induced origin is probably a significant component, and if present at the 30% level would provide the observed Ω. If its r is on the order of 0 ± 0.01, the resulting r would still be consistent with what we have observed. At shorter wavelengths near the band maximum, it is expected that the b_{2u} vibronic component will dominate and that r values near 0. 1 will be found.

5.2. *RNAase A behavior*

A possible reason for the red-shifted tyrosyl emission seen for two-photon excitation of RNAase A could be the presence of one or more tyrosinate forms in the native protein. The reason this is plausible is that, unlike tyrosine itself where the OH and alkyl moieties cancel, the O^- group overwhelms the alkyl and the resultant TPE intensity is strong. An INDO/S computation predicts the tyrosinate will have about 100 times stronger two-photon absorptivity than tyrosine. Furthermore, the absorption curve is known to be shifted to longer wavelengths, so that even one tyrosinate with an abnormal pK_a could dominate the two-photon induced fluorescence. It is expected that tyrosinate emission would be red shifted, as observed. In addition, the higher anisotropy seen is consistent with the expectation noted above for phenol.

Acknowledgements

This work was supported by grant RR-08119 from National Institutes of Health; and by grant UM-141 from the Government Committee for Scientific Research (KBN, Poland). Dr. Borys Kierdaszuk is indebted to the Fulbright Scholar Program for Advanced Research Grant. PRC acknowledges support from NIH grant GM-31824.

References

[1] A.P. Demchenko, Ultraviolet Spectroscopy of Proteins, Springer-Verlag, Berlin, 1986.

[2] J.R. Lakowicz, Proc. SPIE, Time-resolved Laser Spectroscopy in Biochemistry III, Vol. 1640, SPIE, Bellingham, WA, 1992, 807 pp.

[3] R.F. Steiner, Excited State of Biopolymers, Plenum Press, New York, N.Y., 1983.

[4] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, Plenum Press, New York, N.Y., 1983, pp. 354–363.

[5] F.W.J. Teale and G. Weber, Biochem. J., 65 (1957) 476–482.

[6] G. Weber, Biochem. J., 75 (1960) 335–345.

[7] J.W. Longworth, in R.F. Steiner and I. Weinryb (Editors), Excited State of Proteins and Nucleic Acids, Plenum Press, New York, N.Y., 1971, pp. 319–484.

[8] I. Weinryb and R.F. Steiner, in R.F. Steiner and I. Weinryb (Editors), Excited State of Proteins and Nucleic Acids, Plenum Press, New York, N.Y., 1971, pp. 277–318.

[9] J.B.A. Ross, W.R. Laws, K.W. Rousslang and H.R. Wyssbrod, in J.R. Lakowicz (Editor), Topics of Fluorescence Spectroscopy, Biochemical Applications, Vol 3, Plenum Press, New York, 1992, pp. 1–63.

[10] J.R. Lakowicz and I. Gryczynski, J. Fluoresc., 2 (1992) 117–121.

[11] J.R. Lakowicz, I. Gryczynski, J. Kuśba and E. Danielsen, J. Fluoresc., 2 (1992) 247–258.

[12] H. Szmacinski, I. Gryczynski and J.R. Lakowicz, Photochem. Photobiol., 58 (1993) 341–345.

[13] J.R. Lakowicz, I. Gryczynski, E. Danielsen and J. Frisoli, Chem. Phys. Lett., 194 (1992) 282–287.

[14] J.R. Lakowicz and I. Gryczynski, Biophys. Chem., 45 (1992) 1–6.

[15] P.R. Callis, J. Chem. Phys., 99 (1993) 27–37.

[16] A.A. Rehms and P.R. Callis, Chem. Phys. Lett., 208 (1993) 276–282.

[17] B. Kierdaszuk, I. Gryczynski, A. Modrak-Wojcik and J.R. Lakowicz, Photochem. Photobiol., (1995) submitted.

[18] M. Sela and C.B. Anfinsen, Biochim. Biophys. Acta, 24 (1957) 299–235.

[19] D.V. O'Connor and D. Phillips, Time-correlated Single Photon Counting, Academic Press, London, 1984.

[20] J.R. Lakowicz and B.P. Maliwal, J. Biol. Chem., 258 (1983) 4794–4801.

[21] J.R. Lakowicz, I. Gryczynski, Z. Gryczynski, E. Danielsen and M.J. Wirth, J. Phys. Chem., 96 (1992) 3000–3006.

[22] J.R. Lakowicz, I. Gryczynski and E. Danielsen, Chem. Phys. Lett., 191 (1992) 47–53.

[23] T.W. Scott, K.S. Haber and A.C. Albrecht, J. Chem. Phys., 78 (1983) 150.

[24] R.P. Rava, L. Goodman and J.G. Philis, J. Chem. Phys., 77 (1982) 4912–4919.

[25] L. Goodman and R.P. Rava, Adv. Chem. Phys., 54 (1983) 177–230.

[26] P.R. Callis, Chem. Phys. Lett., 107 (1984) 125.

[27] P.R. Callis, T.W. Scott and A.C. Albrecht, J. Chem. Phys., 78 (1983) 16.

[28] W.M. McClain, J. Chem. Phys., 55 (1971) 2789.

[29] D. Friedrich, J. Chem. Ed., 6 (1982) 472.

[30] G. Hohlneicher and B. Dick, J. Chem. Phys., 76 (1982) 5755.

[31] W.M. McClain, J. Chem. Phys., 57 (1972) 2264.

[32] P.G. Russell and A.C. Albrecht, J. Chem. Phys., 41 (1964) 2536–2550.

[33] T.W. Scott, K.S. Haber and A.C. Albrecht, J. Chem. Phys., 78 (1983) 150.

[34] G. Fischer, Vibronic Coupling: The Interaction Between the Electronic and Nuclear Motions, Academic Press, New York, 1984.

[35] D.M. Friedrich, J. Van Alsten and M.A. Walters, Chem. Phys. Lett., 76 (1980) 504–509.

[36] H. Faldas and K. Siomos, J. Mol. Spectr., 130 (1988) 288–302.

[37] B. Dick, H. Gonska and G. Hohlneicher, Ber. Bunsenges. Phys. Chem., 85 (1981) 746.

[38] D.M. Friedrich and W.M. McClain, Chem. Phys. Lett., 32 (1975) 541–549.