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Fluorescence Studies on Poly-α-amino Acids. Models of Protein Conformation. III. Copolymers of Tyrosine with Glutamic Acid or Lysine[1,2]

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The copolymers glutamic acid–tyrosine and lysine–tyrosine were synthetized as model protein systems for the study of tyrosyl fluorescence. The fluorescence and polarization of fluorescence of copoly-L-Glu–L-Tyr (95:5) were measured in both the α-helical (pH 4.3) and random conformation (pH 7.3), and compared to copoly-DL-Glu–L-Tyr (95:5) which was a random coil at both pH's. There was an increase in fluorescence when the concentration of the quenching species, COO^-, was lowered, and when the tyrosyl residues were enclosed in an α-helix. The carboxylate quenching of tyrosyl fluorescence was not through a hydrogen-bonded intermediate in the ground state. Copoly-L-Lys–L-Tyr (95:5) had a greater quantum yield (Q = 0.09) of fluorescence than either of the two glutamyl–tyrosyl copolymers. The Q of the L-Lys–L-Tyr (95:5) copolymer was constant from pH 1.1 to 7.3. The fluorescence emission at 303 mμ of copolymers containing a block sequence of tyrosyl residues was less than the above random sequence copolymers. These block copolymers also had a new fluorescence emission band at 400 mμ. This new emission band may be due to excimer formation among the tyrosyl moieties in the block helical sequence. The quantum yields of fluorescence of these copolymers were comparable to data previously reported for several proteins containing only tyrosine as the fluorescence residue.

Introduction

Extensive studies concerning the fluorescence and polarization of fluorescence of the tyrosine and tryptophan residues of proteins have indicated that these parameters are sensitive indicators of structural alterations.[4-13] Unfortunately, there is little quantitative knowledge of the various factors influencing fluorescence yield or fluorescence polarization in proteins. Recent studies of model compounds by Cowgill[14,15] and Edelhoch, *et al.*,[16] on small peptides and of synthetic poly-α-amino acids by Rosenheck and Weber,[17,18] Wada and Ueno,[19] and Fasman, *et al.*,[20] have indicated that this is a fruitful approach for unraveling some of the interactions of the more complex protein fluorescence systems. In this communication some earlier observations[20] are extended and some new work reported. The model systems investigated were copolymers containing small amounts of tyrosine with either glutamic acid or lysine as the hydrophilic species.

The syntheses of the copolymers were carried out by the polymerization of the desired composition of the N-carboxyanhydrides (NCA's) of the α-amino acids by base initiation.[21] The NCA's used were: γ-benzyl-L-glutamate NCA[21]; γ-benzyl-DL-glutamate NCA[22]; ε-N-carbobenzoxy-L-lysine NCA[23]; ε-N-carbobenzoxy-DL-lysine NCA[24]; and O-carbobenzoxy-L-tyrosine NCA.[25] The blocking groups were removed by the HCl–HBr treatment[23] of the copolymers in the polymerization reaction mixture.

Two types of copolymers were synthesized, one with a random distribution of tyrosyl residues among the second amino acid, *e.g.*, (L-Glu–L-Tyr), and the other with a block sequence of tyrosyl residues added to a block sequence of either lysine or glutamic acid residues, *e.g.* (L-Lys)-(L-Tyr), in the manner described by Gratzer and Doty.[26] This latter species would allow for tyrosyl–tyrosyl interaction if such existed, while the former would dilute out such interactions. Copolymers were made of either L- or DL-glutamic acid (L- or DL-Glu–L-Tyr) and L- or DL-lysine (L- or DL-Lys–L-Tyr) in order to study the effect of conformational changes on fluorescence and fluorescence polarization. The (L-Lys–L-Tyr) and (L-Glu–L-Tyr) polymers undergo a random-coil → helix transition in the pH range where the homopolymers become un-ionized. Poly-L-lysine becomes helical over the pH range 8 to 11.[27] Poly-L-glutamic acid undergoes this transition over the pH range 7.0 to 4.0.[28-30] A copolymer of (L-Glu–L-Tyr) (95:5) has also been shown to behave in a similar fashion.[31] Copolymers of (L-Glu–L-Tyr) have been shown to be right-handed helices despite differences of optical rotatory dispersion parameters, *e.g.*, b_0, from the original simple case.[32] The (DL-

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(2) For the previous paper in this series see S. S. Lehrer and G. D. Fasman, *Biopolymers*, **2**, 199 (1964).

(3) This work was done during the tenure of an Established Investigatorship of the American Heart Association. Inquiries should be addressed to G. D. F.

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Lys-L-Tyr) and (DL-Glu-L-Tyr) polymers remain in a random coil structure throughout these pH ranges (Gratzer and Doty[31] have estimated that DL polymers may be 25% helical). In the block copolymers, the tyrosyl sequence is similar to poly-L-tyrosine, whose conformation is helical at a pH below the pK_a of the phenolic ionization.[33] Thus the model system of copolymers outlined may be analogous to proteins containing only tyrosyl residues as the fluorescing species which may be in an α-helical or random-coil conformation as neighboring or widely dispersed residues.

Experimental

L-Tyrosine was obtained from Nutritional Biochemicals Co. Distilled water was used throughout. Dioxane was purified by the Fieser[34] procedure; dimethyl sulfoxide (DMSO) was obtained from Crown Zellerbach and purified by distillation from KOH *in vacuo* (2–3 mm.), b.p. 38°.

Absorbance Measurements.—A Zeiss spectrophotometer Model PMQ II was used to determine the optical densities of the experimental solutions. Solutions of optical densities lower than 0.1 at 280 mμ in a 1-cm. cell were measured in a cell with a 5-cm. path length.

Fluorescence measurements were obtained using either the Aminco–Bowman spectrophotofluorometer or the Zeiss Model ZFM 4C spectrophotofluorometer. Results were the same for both instruments; however, the Zeiss instrument was more sensitive and gave finer optical resolution. The excitation and emissionmon ochromator slits of the Zeiss fluorometer were adjusted so that the fluorescence emission at 303 mμ of a tyrosine solution approximately 5×10^{-5} *M* and excited at 275 mμ was 100 units at maximum instrument sensitivity. The emission spectra for all the polymers except the block copolymers were identical with that of tyrosine. The quantum yield of the polymers was calculated on the assumption that the quantum yield of tyrosine at neutral pH was 0.21.[6]

The formula for calculation of quantum yield was $F_1/F_2 = [(Q_1)(\text{O.D.}_1)]/[(Q_2)(\text{O.D.}_2)]$, as used by Parker and Rees,[35] where F_1 was the fluorescence of the polymer solution at 303 mμ, F_2 the fluorescence of the tyrosine standard at 303 mμ, Q_1 the quantum yield of the polymer, Q_2 the quantum yield of tyrosine, O.D._1 the optical density of the tyrosyl polymer at 275 mμ, and O.D._2 the optical density of the tyrosine standard at 275 mμ. As the emission spectra of tyrosine and the copolymers were almost identical, it was not considered necessary to use the relative areas under the curves for the calculation of Q.

Similar experimental arrangements and calculations for quantum yield determinations were used for experiments with the Aminco–Bowman fluorometer. The exit slits of the exciting monochromator were set at $^1/_8$ and $^1/_{16}$ in., and a $^1/_{16}$-in. slit was placed at the entrance of the emission monochromator. The exciting wave length was 280 ± 5 mμ and the emission monochromator was set at 308 ± 5 mμ. These settings were not at the true excitation and emission maxima but were selected for maximum sensitivity of the instrument. Quantum yields were calculated from the above formula and F values at 308 mμ were used.

The emission spectra were corrected for the relative sensitivity of the Zeiss fluorometer. The photomultiplier efficiency and monochromator dispersion both varied with wave length. A proportional photon counter[6] was used to obtain the variation of the input light of the 450-w. xenon arc lamp and monochromator dispersion. The sodium salt of 8-amino-1,5-naphthalenedisulfonic acid in aqueous solution and Rhodamine B in ethylene glycol were used as proportional photon counters. An aluminum foil reflecter was placed in the cell holder, and the input monochromator slit was set at 0.01 mm. and the emission monochromator slit at 2.0 mm. The emission monochromator was also set at the same wave length as the excitation monochromator; thus, the fluorometer was considered to have one monochromator and an aluminum mirror of constant geometry. The response of the detector system *vs.* the wave length of the reflected light was then plotted. This response was comprised of three variables: the detector system, the monochromator, and the lamp output. The monochromator dispersion curves were obtained from the manufacturer's manual. The response of the emission monochromator and photomultiplier tube with wave length could thus be calculated from the two experiments and the dispersion curve of the monochromator. We are indebted to Dr. S. Lehrer for this calibration.

Polarization of Fluorescence Measurements.—The Aminco–Bowman instrument was equipped with a Glan–Thompson prism for polarization of fluorescence measurements. Determination of the parallel ($I_\parallel$) and perpendicular ($I_\perp$) components of the emitted light were made by rotating the prism. The polarization of fluorescence (p) was calculated from the formula used by Weber[36]

$$p = (I_\parallel - I_\perp)/(I_\parallel + I_\perp)$$

An approximate correction for polarization of light inherent in the instrument was made with a tyrosine standard. The polarization of fluorescence was measured at increasing tyrosine concentrations and the value of this polarization was subtracted from the observed values since free tyrosine has a negligible polarization of fluorescence. The correction for p was on the order of 0.02 to 0.03.

Cells.—The fluorescence cells (10 × 2 mm. path length) were purchased from Pyrocell, Westwood, N. J. The absorption cells (50-mm. path length) were purchased from Quaracell Products, Inc., New York, N. Y.

Solutions for Fluorescence Studies. L-Tyrosine.—L-Tyrosine was diluted with distilled water. The optical density used (1-cm. cell) was $\text{O.D.}_{275} \cong 0.015$.

Copolymers of Glutamic Acid and Tyrosine.—Stock solutions were made up by dissolving approximately 1 mg./ml. of the copolymer in 0.2 *M* NaCl at pH 7.0. One-milliliter aliquots were diluted to 10 ml. in 0.2 *M* NaCl, adjusting the pH to 4.37 and 7.3, respectively.

Titration of γ-COOH in Copoly-L-glutamic Acid–L-Tyrosine (95:5) with Base.—The copolymer (GF10–161–25) was dissolved in DMSO–H_2O (1:1), 0.6 mg./ml. It was first dissolved in DMSO, then an equal volume of H_2O was added, and the solution was diluted to the final volume with a DMSO–H_2O (1:1) mixture. The amount of base taken up by DMSO–H_2O mixture was found to be negligible. The amount of base necessary was added in 12 aliquots, reading the optical density at 275 and 292 mμ after each addition and reading the fluorescence of the solution in a 1-cm. quartz fluorescence cell on the Aminco–Bowman spectrofluorometer.

Fluorescence Intensity and Fluorescence Polarization Measurements in DMSO–H_2O.—Solutions of copoly-L-glutamic–L-tyrosine (95:5) and copoly-DL-glutamic–L-tyrosine (95:5) were made up in the following fashion. The copolymer was dissolved in a DMSO–H_2O (1:1) mixture as described above, and 1 mg./ml. aliquot was diluted 2:5 with DMSO–3×10^{-2} *N* HCl (1:1) to give the acid solution. Another aliquot was diluted 2:5 with DMSO–3.75×10^{-3} *M* PO_4 buffer, pH 7.05 (1:1), to give a neutral solution. The measurements were made with the Aminco-Bowman spectrophotofluorometer using a 0.2-cm. cuvette thermostated at 23.5°.

Preparation of Polymers. Random Copolymers of L-Glutamic Acid and L-Tyrosine (L-Glu-L-Tyr) (95:5).—γ-Benzyl-L-glutamate NCA[21] (2.22 g., 8.455×10^{-3} mole) and O-carbobenzyloxy-L-tyrosine NCA[25] (0.152 g., 4.45×10^{-4} mole) were dissolved in dry distilled benzene (250 ml.) by warming. The solution was cooled to room temperature and the polymerization was initiated by the addition of $NaOCH_3$ (0.250 ml. of 0.355 *N*, $A/I = 100$) with stirring, and the solution allowed to stand for 4 days. A 25-ml. aliquot of the slightly viscous solution was removed and slowly added to five times its volume of *n*-hexane, with stirring. The blocked polymer which separated as a fibrous material was dried under vacuum (1 mm.) at 80° for 2 hr., $\eta_{sp}/_c$ 0.717 (0.2% in dichloroacetic acid). Anhydrous hydrogen chloride was bubbled through the remaining solution for 15 min. Precautions were taken to exclude moisture during this procedure and the following one. Anhydrous hydrogen bromide was then bubbled through the solution for 30 min. and the polymer began precipitating out. The solution was allowed to stand overnight, the supernatant was removed by decantation, and the excess solvent and HBr was removed by suction on a water aspirator.

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TABLE I

ESTIMATED MOLECULAR WEIGHTS OF TYROSINE COPOLYMERS

Sample no.		A/I[a]	$\eta_{sp/c}$[d]	η[e]	D.P.$_w$	Mol. wt.$_w$
GF10-161-25	(L-Glu-L-Tyr) (95:5)	100[b]	0.717	0.69[f]	457[i]	60,000[i]
				1.07[g]		
GF10-321-24	(DL-Glu-L-Tyr) (95:5)	200[b]		0.273[g]	173[i]	22,600[i]
GF11-406-28	(L-Glu)-(L-Tyr) (95:5)	100[b]	0.89	1.02[f]	674[i]	88,400[i]
GF11-382-13	(L-Lys-L-Tyr) (95:5)	52.5[c]		0.86[h]	442[j]	57,800[j]
GF11-444	(L-Lys)-(L-Tyr)-(L-Lys) (95:5:37)	25[c]	2.24	1.79[h]	1000[j]	130,000[j]

[a] NCA concentration 1%, initiated with $NaOCH_3$. [b] Polymerization in benzene. [c] In dioxane. [d] Specific viscosity before removal of blocking groups in 0.2% dichloroacetic acid. [e] Intrinsic viscosity of unblocked polymers. [f] At pH 12 in 0.2 *M* NaCl. [g] At pH 7 in 0.2 *M* NaCl. [h] At pH 4 in 1.0 *M* NaCl. [i] Estimated from the molecular weight calibration of M. Idelson and E. R. Blout, *J. Am. Chem. Soc.*, **80**, 4631 (1958). [j] Estimated from the molecular weight calibration of J. Applequist, Ph.D. Thesis, Harvard University, 1959.

The polymer was extracted for 3.5 hr. with anhydrous ether, repeatedly changing the ether, and dried *in vacuo* (1 mm.) at 40° for 2 hr. yielding a fine white powder, 0.95 g., 86% yield, $[\eta]^{0.2M\,NaCl}_{pH7.3}$ 1.07. The composition of this polymer was confirmed by amino acid analysis done with the Beckman Model 120 automatic amino acid analyzer.

Random Copolymer of DL-Glutamic Acid and L-Tyrosine (DL-Glu-L-Tyr) (95:5).—This copolymer was prepared in the same manner as the (L-Glu-L-Tyr) (95:5) copolymer (see Table I).

Block Copolymer of L-Glutamic Acid and L-Tyrosine (L-Glu)-(L-Tyr) (95:5).—γ-Benzyl-L-glutamate NCA (1.0 g., 3.798 × 10^{-3} mole) was dissolved in 100 ml. of dry benzene (distilled from CaH_2) by warming. The solution was cooled to room temperature and the polymerization was initiated by adding $NaOCH_3$ (0.11 ml., 0.345 *N*, A/I = 100) with stirring. After 24 hr., O-carbobenzyloxy-L-tyrosine NCA (0.068 g., 0.2 × 10^{-3} mole) dissolved in benzene (7 ml.), was added with stirring to the polymerization mixture. After 24 hr., a small aliquot was removed and the polymer recovered as in the above preparation. The specific viscosity of this blocked polymer was 0.89 (0.2% in dichloroacetic acid). The copolymer was unblocked as described above. The polymer was dissolved by shaking for 2 hr. in a saturated $NaHCO_3$ solution which had been adjusted to 30 ml. at pH 12 with 2 *N* NaOH. The solution was extracted two times with anhydrous ether, the pH was brought to 7 with 1 *N* HCl, and the solution was dialyzed *vs.* H_2O at pH 7 (Visking Co., dialyzing tubing bag size 32). The solution was filtered through a sintered glass funnel, size M porosity, and lyophilized, yielding a white spongy material. The polymer was dried under high vacuum (1 mm.) at 50° for 2 hr., giving 0.434 g., 83%, yield $[\eta]^{0.2M\,NaCl}_{pH12.1}$ 1.02.

Random Copolymers of L-Lysine and L-Tyrosine (L-Lys-L-Tyr) (95:5).—ε-Carbobenzoxy-L-lysine NCA (1.0 g., 3.27 × 10^{-3} mole) and O-carbobenzoxy-L-tyrosine NCA (0.059 g., 0.127 × 10^{-3} mole) were dissolved in dry dioxane (106 ml., 1% solution). Sodium methoxide (0.19 ml., 0.345 *N* $NaOCH_3$, A/I = 52.5) was added with stirring to this mixture to initiate the polymerization, and the solution was allowed to stand for 1.5 days. Chloroform (A.C.S., 275 ml.) was added to dilute the viscous solution and the polymer was unblocked *via* the HCl–HBr procedure described above. The reaction mixture was stirred for 90 min., nitrogen was bubbled through to remove excess HBr (90 min.), and the supernatant was removed under vacuum on a water aspirator. The polymer was dissolved in a saturated $NaHCO_3$ solution (40 ml.) by stirring. The aqueous solution was adjusted to pH 3 with 3 *N* HCl; the clear solution was then extracted with anhydrous ether and dialyzed *vs.* 0.01 *N* HCl. The clear solution was lyophilized, yielding a white spongy polymer. The polymer was dried under high vacuum at 40° for 2 hr., giving 0.370 g., 65.4% yield, $[\eta]^{1M\,NaCl}_{pH4.01}$ 0.86.

Triblock Copolymer of L-Lysine and L-Tyrosine (L-Lys)-(L-Tyr)-(L-Lys) (95:5:37).—ε-Carbobenzyloxy-L-lysine NCA (1.0 g., 3.27 × 10^{-3} mole) was dissolved in dry dioxane (100 ml., 1% solution). The polymerization was intiated by the addition of $NaOCH_3$ (0.379 ml., 0.345 *N* $NaOCH_3$, A/I = 25). After 24 hr., O-carbobenzyloxy-L-tyrosine NCA (0.0588 g., 0.172 × 10^{-3} mole) dissolved in 6 ml. of dioxane was added to the polymerization mixture. After 24 hr. ε-carbobenzyloxy-L-lysine NCA (0.391 g., 1.275 × 10^{-3} mole) dissolved in dry dioxane (39 ml.) was added to the viscous solution and the polymerization was allowed to proceed for 24 hr. A small aliquot was removed and the polymer recovered as in the above preparation. The specific viscosity of this blocked polymer was 2.25 (0.2% in dichloroacetic acid).

The polymer was debenzylated and treated in the same manner as copoly-L-lysine–L-tyrosine above, giving 0.536 g., 69.2%, yield $[\eta]^{1M\,NaCl}_{pH3.8}$ 1.79.

In Table I are listed the various polymers prepared, the conditions of the polymerization, the mole ratios of the anhydrides used, the intrinsic viscosities, the estimated weight average molecular weights, and the specific viscosities of the blocked polymers. As it has been shown that the composition of the copolymers prepared in this manner closely approximates the NCA mole ratios used, it will be assumed that these are the correct ratios. The tyrosine content of the solutions used was determined by optical density measurements. The absolute composition of the polymers is not of importance in this study.

The approximate weight average molecular weights of the copolymers used in this study were determined from viscometry measurements. The copolymers containing L-amino acids were of a higher molecular weight than those containing DL-amino acids. This is in accordance with the results of other studies which have shown that DL-polymers are of lower molecular weight than L-polymers when formed under the same anhydride/initiator ratios (A/I) and polymerization conditions. The intrinsic viscosity of the random (L-Glu-L-Tyr) (95:5) copolymer was unexpectedly pH dependent above pH 7, and the value at pH 12 was selected for the molecular weight estimations. At high pH the tyrosines are ionized and interactions with the solvent are changed. These viscosity results could also be interpreted as indicating that small sequences of tyrosine exist, forming rigid areas which upon ionization are converted to the random coil, consequently lowering the viscosity. It is not possible to make comparable measurements with the lysine copolymers, since they are not soluble at high pH and over the pH range 8–10 the conformation also changes. The high viscosity observed for the triblock copolymer (L-Lys)-(L-Tyr)-(L-Lys) probably reflects the nonrandom tyrosine sequence, and consequently the average molecular weight estimation is probably too high. This might well be true also for the (L-Lys-L-Tyr) as indicated in the case of the glutamic acid–tyrosine copolymer.

Results and Discussion

Glutamic Acid–Tyrosine Coplymers.—Teale,[7] Rosenheck and Weber,[17,18] and Feitelson[37] have demonstrated that there is a quenching of fluorescence by carboxylate groups in model compounds. To distinguish between a conformational and a quenching effect on fluorescence, a polymer such as (DL-Glu-L-Tyr), which does not undergo a helix → coil transition, was compared to a (L-Glu-L-Tyr) polymer at similar pH's to evaluate the effect of carboxyl quenching alone. The (DL-Glu-L-Tyr) polymer exists as a random coil at both pH 7 and 4.0, while the (L-Glu-L-Tyr) polymer is helical at acid pH and random at pH 7. The b_0 value for the (L-Glu–L-Tyr) (95:5) used in this study has been found to be −470 at pH 4.35, 0.2 *M* NaCl; however, this is the value for a 100% helix, as shown previously.[32,33] Figure 1 illustrates that the two copolymers (DL-Glu-L-Tyr) (95:5) and L-Glu-L-Tyr) (95:5) have the same fluorescence

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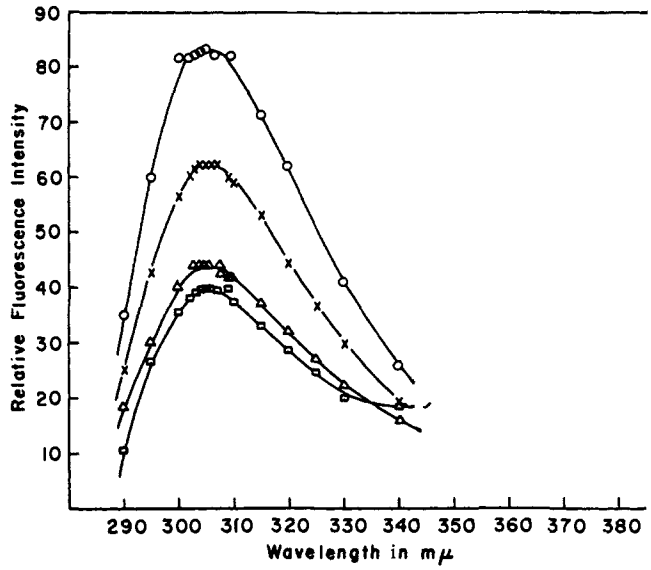

Fig. 1.—The relative fluorescence intensity of copolymers of glutamic acid and tyrosine (95:5). ○-○-○ (L-Glu-L-Tyr), pH 4.37; △-△-△ (L-Glu-L-Tyr), pH 7.32; ×××× (DL-Glu-DL-Tyr), pH 4.37; □-□-□ (DL-Glu-L-Tyr), pH 7.32. Concentration 0.109 mg./ml. in 0.2 *M* NaCl, O.D.$_{275}$ = 0.08 (1-cm. path length). Fluorescence measurements were taken in a quartz cell (1-cm. path length). 100% fluorescence intensity, L-Tyr in H_2O, O.D.$_{275}$ = 0.012.

yields (Q = 0.04) and emission spectra at pH 7.32 when the carboxyls are completely ionized and both polymers have a random conformation. At pH 4.32, where the glutamyl-γ-carboxyls are largely un-ionized, the fluorescence increased. There is a definite increase in the fluorescence yield of the (L-Glu-L-Tyr) polymer over the (DL-Glu-L-Tyr). The (DL-Glu-L-Tyr) polymer fluorescence showed a 1.53-fold greater yield for the polymer at low pH compared to pH 7.0, whereas the (L-Glu-L-Tyr) copolymer had a 1.79-fold increase (Table II).

TABLE II

FLUORESCENCE OF GLUTAMIC ACID–TYROSINE COPOLYMERS (95:5) IN H_2O[a]

Copolymer	pH[b]	Conformation	p[c]	F_{ratio}[d]
(L-Glu-L-Tyr)	4.37	Helix	0.074 ± 0.002	1.79 ± 0.09
(L-Glu-L-Tyr)	7.37	Random coil	0.056 ± 0.003	
(DL-Glu-L-Tyr)	4.37	Random coil	0.056 ± 0.005	1.53 ± 0.11
(DL-Glu-L-Tyr)	7.37	Random coil	0.056 ± 0.007	

[a] Fluorescence and polarization were measured in quartz cells (1-cm. path length); O.D. of the solutions were 0.06–0.08 for 1-cm. path length (measured in a cell of 5-cm. path length). Exciting wave length 280 mμ, maximum emission wave length 308 mμ. [b] 0.2 *M* NaCl. [c] Polarization = $(I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$. [d] (Fluorescence intensity pH 4.37)/(fluorescence intensity pH 7.37.)

Weber[4,5] has demonstrated that tyrosine in a rigid environment has a higher polarization of fluorescence than in free solution. Changes in the polarization of fluorescence therefore reflect changes in the freedom of rotation of these molecules. Variations in quantum yield, as observed in these experiments, complicate this simple relationship. The lifetime of the excited state of a molecule is directly proportional to the quantum yield.[38] The relationship between polarization, viscosity, temperature, and lifetime of the excited state is

$$1/p = 1/p_0 + [(1/p_0 - 1/3)RT/V]\tau_0/\eta$$[39]

where p = observed polarization of fluorescence, p_0 = polarization of fluorescence when $T/\eta \rightarrow 0$, T = absolute temperature, η = viscosity of solution, V = molar volume of molecule, and τ_0 = lifetime of excited state of fluorescence. Thus, the increased quantum yield resulting from the change in pH might be expected to lower the observed polarization of fluorescence. The polarization of fluorescence of the (DL-Glu-L-Tyr) random polymer did not change on lowering the pH from 7 to 4, thus indicating that there was little effect on p as a result of this quantum yield change (Table II). However, the polarization of the (L-Glu-L-Tyr) copolymer increased over this same pH change reflecting an increased rotational relaxation time for the molecule, *i.e.*, an increase in the molar volume of the molecule. Polarization of fluorescence data of the (L-Glu-L-Tyr) polymer (Table II) definitely indicates that the tyrosines in the helical polymer are in a more rigid structure than in the random-coil polymers.

Three factors to consider which may account for the difference in fluorescence yield are: (1) the extent of ionization of these polymers, since there is an effect of COO^- concentration on the fluorescence yield; (2) effective concentration of the COO^- in the polymer, since a difference in conformation may affect the proximity of the COO^- groups to the tyrosyl moiety; and (3) change of the dielectric constant of the environment around the tyrosyl residues caused by the random-coil → helix transition. In the helical conformation the environment around the tyrosyl residues is predominantly more hydrocarbon, and consequently it is in a region of lower dielectric constant. The effect of the dielectric constant on fluorescence has been reviewed recently by Van Duuren,[40] and its effect on quantum yield is questionable.[18] The first of these possibilities may be ruled out by the data of Wada[41] and Nagasawa and Holtzer,[42] which demonstrated that the carboxyl groups of both the helical and random-coil regions of poly-L-glutamic acid have the same pK_a. Thus the pK_a of the random DL-polymer and the helical L-polymer would also be similar, and the concentration of COO^- is equivalent for both copolymers. The second possibility may be tested by un-ionizing all the carboxyls, thus eliminating the quenching species. As un-ionized glutamyl polymers are water insoluble, a mixed solvent of DMSO and water 1:1 (v./v.) was selected. In this solvent the emission spectra of the polymers were similar to that of the tyrosine emission spectrum in water. The pH of the mixed solvent reflected the pH of the water component and seemed independent of the dimethyl sulfoxide. The results are summarized in Table III, and it is seen that the (L-Glu-L-Tyr) copolymer fluorescence was 2.92-fold greater in the acidic medium compared to the neutral one, while the (DL-Glu-L-Tyr) fluorescence intensity increased only 2.55-fold. The random-coil (neutral medium) fluorescence intensity of both copolymers is equal. Thus, tyrosine in a helical conformation has a higher fluorescence

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TABLE III

FLUORESCENCE OF GLUTAMIC ACID–TYROSINE COPOLYMERS (95:5) IN DMSO[a]–H_2O (1:1)[b]

Copolymer	pH	Conformation	p[c]	F_{ratio}[d]
(L-Glu–L-Tyr)	Acid[e]	Helix, $\eta^g = 0.91$	0.079 ± 0.002	2.92 ± 0.11
(L-Glu–L-Tyr)	Neutral[f]	Random coil, $\eta^g = 0.79$	0.060 ± 0.003	
(DL-Glu–L-Tyr)	Acid[e]	Random coil, $\eta^g = 1.39$	0.080 ± 0.001	2.55 ± 0.16
(DL-Glu–L-Tyr)	Neutral[f]	Random coil, $\eta^g = 0.167$	0.064 ± 0.005	

[a] Dimethlyl sulfoxide. [b] Fluorescence was measured in quartz cells (0.2-cm. path length); O.D. of the solutions used 0.25–0.35 for 1-cm. path length. [c] Exciting wave length 280 mμ. Maximum emission wave length 308 mμ. [c] Polarization = $(I_\parallel - I_\perp)/(I_\parallel + I_\perp)$. [d] (Fluorescence intensity acid)/(fluorescence intensity neutral). [e] 0.02 *N* HCl. [f] 0.002 *M* phosphate, pH 7.0. [g] Specific viscosity, $c = 0.55\%$.

emission. Therefore, of the three factors suggested to account for the fluorescence difference, the last (the change in the environment around the tyrosines) is most probably responsible.

The polarization (p) of fluorescence data in the DMSO solvent system indicates that the un-ionized helical (L-Glu–L-Tyr) polymer ($p = 0.08$) contains tyrosines in a more rigid structure than the ionized form ($p = 0.06$). The un-ionized (DL-Glu–L-Tyr) copolymer unexpectedly has a p greater than that of the ionized form. An examination of the specific viscosity of these solutions provides a possible explanation. These data are presented in Table III. The (DL-Glu–L-Tyr) polymer in the un-ionized form has a specific viscosity almost eight-fold greater than in its corresponding ionized form in DMSO–H_2O, while the (L-Glu–L-Tyr) polymer shows only a 1.2-fold difference. The (L-Glu–L-Tyr) polymer has a higher specific viscosity because of its larger molecular size (as measured in H_2O, see Table I). The un-ionized (DL-Glu–L-Tyr) polymer has a specific viscosity much greater than the larger (L-Glu–L-Tyr) polymer. The un-ionized form of the DL-polymer may be interacting with the solvent, forming an aggregate structure, which increases its viscosity. This is then reflected in the high p value for the tyrosyl residues in the polymer, since they would be less free to rotate. Since the p value is a function of T/η,[39] where η is the viscosity of the solvent, the increased p value of the DL-polymer may be due to the increase in viscosity when the polymer is dissolved in this solvent mixture.

The formation of tyrosyl glutamyl hydrogen-bonded complexes, frequently suggested for proteins[43–46] (—(O=)C—O$^-$ · · · HO—C_6H_4—) could affect the interpretation of these results. Such an association, if energetically favorable, would occur when very few of the glutamic acid residues are ionized, particularly in those polymers that contain a 20-fold excess of glutamic acid over tyrosine. The pK_a of the glutamic acid residues involved in such hydrogen-bonded structures would be lower than the other glutamic acid residues and would therefore be the first to ionize. In this study, at pH 4.3, the polymers are about 15–20% ionized; consequently, this preferred interaction resulting in a decreased fluorescence might have already occurred. To observe the initial quenching, which might be expected to be large upon ionization, it is necessary to follow the fluorescence as a function of ionization. The DMSO–water solvent system would favor this preferential hydrogen-bonding[47] interaction, as the dielectric constant of this mixed solvent is lower than that of the aqueous solution. In Fig. 2 is seen a titration curve of the glutamic acid residues of the (L-Glu–L-Tyr) copolymer. A similar curve was obtained with the (DL-Glu-L-Tyr) polymer. A large decrease in the fluorescence of the polymer upon the initial ionization is not seen, thus indicating that no preferential interaction occurred. No direct correlation of tyrosyl interaction with ionized glutamate is evident. An extrapolation of the tyrosyl fluorescence in an uncharged glutamic acid copolymer may be made from these data. At pH 4.32 a similar (L-Glu-L-Tyr) polymer is about 15% ionized.[31] Assuming that the curve of fluorescence intensity *vs.* ionization in H_2O is the same as in the DMSO–water, then the increase in fluorescence at zero ionization would also be about 15%. Thus the fluorescence of the un-ionized polymer in water would be about double that of the quenched form. This results in a quantum yield of about 7–8% for both (L-Glu–L-Tyr) and (DL-Glu–L-Tyr) copolymers.

L-Lysine–L-Tyrosine Copolymers.—These polymers were synthesized to exclude the possibility that glutamic acid in either its charged or uncharged form interacted with tyrosine and to investigate other neighboring group effects on models for protein fluorescence. The quantum yield of the random lysyl–tyrosyl polymer at neutral pH was 0.09 (see Table IV), and this correlates well with the extrapolated quantum yield of 0.08 for an uncharged glutamyl–tyrosyl polymer in aqueous solution.

TABLE IV

QUANTUM YIELD OF COPOLYMERS CONTAINING 5% (MOLE) OF L-TYROSINE[a]

	pH[b]	Q
Random sequence polymers		
(L-Glu–L-Tyr)	7.0	0.038
	3.0[c]	0.080
(DL-Glu–L-Tyr)	7.0	0.038
	3.0[c]	0.069
(L-Lys–L-Tyr)	7.0	0.090[d]
Block sequence polymers		
(L-Glu)–(L-Tyr)	7.0	0.020
	4.3	0.023
(L-Lys)–(L-Tyr)–(L-Lys)[e]	7.0	0.020

[a] Fluorescence was measured in quartz cells (1-cm. path length); O.D. of the solutions 0.06–0.10 for 1-cm. path length (measured in a 5-cm. path length cell). Exciting wave length 275 mμ. Maximum emission wave length 303–305 mμ. [b] In 0.2 *M* NaCl. [c] Extrapolated to pH 3.0. [d] Q remains constant over the pH range 7.5 to 1.1. [e] Triblock copolymer: mole ratio of blocks 95:5:37.

The fluorescence of tyrosine in a lysyl–tyrosyl

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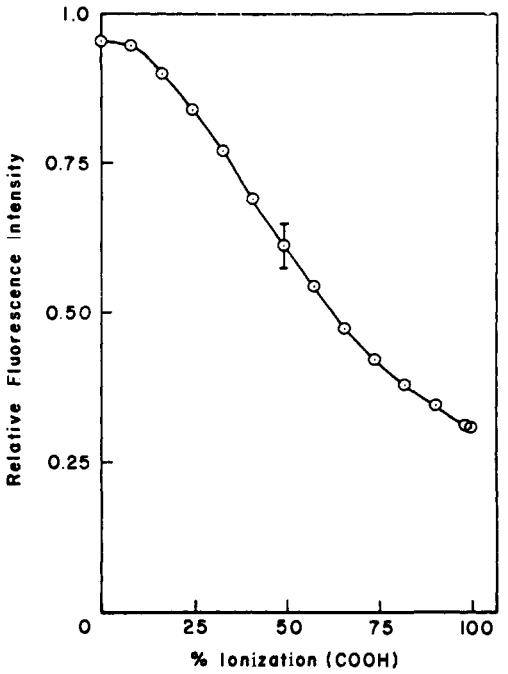

Fig. 2.—Fluorescence intensity *vs.* % ionization (COOH) for copoly(L-glutamic acid–L-tyrosine) (95:5) in dimethyl sulfoxide–H_2O (1:1); titrating agent: 1 *N* NaOH. Base was added directly into the 1-cm. quartz cell in which fluorescence intensity was read; temperature 23.5°.

polymer was investigated to examine the effect of H^+ ion on tyrosyl fluorescence. No quenching of tyrosine fluorescence was observed even at pH 1.1, the quantum yield remaining constant between pH 7.5 and 1.1, and therefore H^+ was eliminated as a quenching species during the glutamic acid helix → coil transition. This result corroborates previous work.[37] However, in alkaline solution the fluorescence of the lysyl–tyrosyl polymer is markedly quenched. The pK_a of ε-amino groups of lysyl residues is 10.0,[27] close to that of the tyrosyl phenolic OH. Ionized tyrosine does not fluoresce at 303 mμ but at 342 mμ with a much lower efficiency.[48] Also, $-NH_2$ has been shown to extensively quench tyrosine fluorescence.[18,37] Thus, in the pH range 9–10, two factors contribute to decrease fluorescence: amine quenching and loss of the fluorescing species. Thus it was not possible to separate conformational effects from other causes of decreased fluorescence in a manner similar to the glutamyl–tyrosyl polymer study.

Copolymers with Tyrosine "Blocks."—It was assumed that the copolymers studied above were reasonably random copolymers. However, the possibility exists that the copolymers were not random and the distribution was such that all the tyrosines were grouped together on one end of the molecule in a block sequence. Since the kinetics of polymerization have not been studied for the individual NCA's used in this work, this was not an unlikely complication. To exclude this possibility block copolymers were synthesized. The quantum yield of the glutamic acid–tyrosine block polymer (L-Glu)-(L-Tyr) in neutral solution was found to be very low (Q = 0.02) (Table IV) and was almost unchanged upon conversion of the glutamyl block from the random-coil conformation into a helix. Thus the glutamic acid portion of the copolymer seemed to have no effect on the tyrosine fluorescence. The triple block, (L-Lys)-(L-Tyr)-(L-Lys), also had a quantum yield (0.02) comparable to the block (L-glutamyl)-(L-tyrosyl) copolymer in neutral solution. The extra lysine block was added to increase the solubility, as these tyrosine block containing polymers were far less soluble than the random polymers. The tyrosyl residues were thus completely independent of the glutamyl or lysyl residues of the copolymer. There is a marked quenching effect which decreases tyrosine fluorescence four-fold when the tyrosines are arranged in sequence. The tyrosine block under these conditions is probably helical because of hydrophobic interactions between residues which stabilize this configuration.[53] This decrease in quantum yield may be due to concentration quenching[38] or excimer formation[49,50] among the residues. This helical structure probably facilitates the interactions mentioned above which then result in decreased fluorescence. A fluorescence study of block polymers to investigate the nature of this interaction has been discussed further in a paper by Lehrer and Fasman.[51] The absorption spectra and the emission spectra of the block polymers (L-Glu)-(L-Tyr) are, in general, similar to that of tyrosine and to that of a random copolymer of (L-Glu-L-Tyr) (Fig. 3 and 4). However, one difference in the absorption spectrum is a long absorption tail from 300 to 500 mμ that decreases slowly to zero. The fluorescence emission spectrum of the block polymer (L-Glu)-(L-Tyr) is similar to that of the random copolymer at 303 mμ, but there is another broad emission band peaking near 400 mμ. This emission maximum near 400 mμ is probably due to excimer formation.[51]

Conclusions

The test of a model system is whether or not it adequately explains the observations of the more complex, naturally occurring system it represents. From these experiments the predicted quantum yields of fluorescence of proteins[7] containing tyrosine and no tryptophan should vary between 0.09 and 0.04. Insulin has a quantum yield of 0.037,[7] malic dehydrogenase 0.038,[52] and zein 0.07.[7,53] When malic dehydrogenase is denatured, the fluorescence increases 2.2- to 2.5-fold (Q = 0.09).[52] The model system thus encompasses the range of quantum yields observed with these proteins. It does not include ribonuclease (Q = 0.017) or pancreatic trypsin inhibitor (Q < 0.01). Recently, Cowgill[54] has found that denatured ribonuclease has a quantum yield of 0.04 and, in this state, the molecule is more similar to these polymers than in the native state. This points out that there are probably other quenching effects that as yet have not been explained for proteins. The values for the copolymers reported herein are higher than those reported in an earlier study by Rosenheck and Weber,[17,18] who reported a quantum yield of 0.02 for a glutamyl–tyrosyl copolymer and 0.065 for a lysyl–tyrosyl copolymer. This difference is probably due to the difference in polymer

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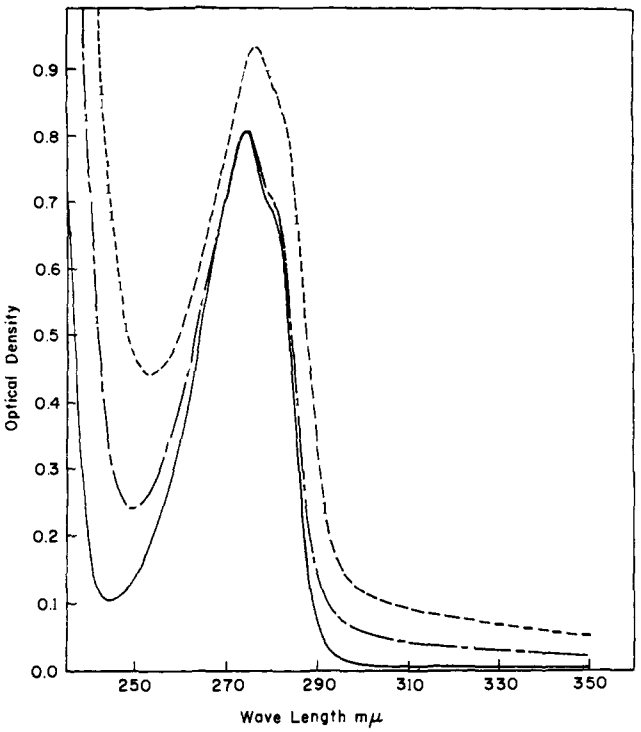

Fig. 3.—Relative absorption spectra. ——— L-Tyr in H_2O, pH 5.5; — · — (L-Glu-L-Tyr) (95:5), pH 7.5 in 0.2 *M* NaCl; - - - - block (L-Glu)-(L-Tyr) (95:5), pH 7.5 in 0.2 *M* NaCl; optical densities measured in a quartz cell (1-cm. path length). Polymer concentrations are not identical.

size and the preparative method that was employed. Another model system for tyrosine fluorescence consisting of small tyrosine-containing peptides has been reported by Cowgill.[8,9] The quantum yield, Q, varies between 0.027 and 0.103 for this system; however, some of the effects that have been studied, such as the inductive effect, probably play a small role in proteins.

Conformational changes influence the fluorescence yield of tyrosine; however, larger effects might have been expected. A 25% increase in tyrosine fluorescence due to the random-coil → helix transition was observed. However, in this helical model system the tyrosyls are not completely buried in a hydrophobic region and have areas, such as the OH group, exposed to solvent which are probably solvated.

Large changes in the polarization of fluorescence occurred as a result of the random-coil → helix transition. This parameter followed the changes in rigidity of the tyrosine environment; an increase in the polarized fluorescence indicated a more rigid molecule. The rigidity may be due to the prevention of free rotation of tyrosines in a helical structure and consequently they rotate with the whole molecule, while in the random coil the tyrosines are free to rotate independently. An additional factor which might contribute to an increase in polarization is the change in rotational relaxation time of the molecule upon assuming a helical structure.

An extremely important influence is exerted by neighboring COO^- groups, which markedly decrease tyrosine fluorescence yield. Carboxylate ion from glutamic acid and aspartic acid is probably the most likely quenching species of protein fluorescence as this species is present at neutral pH.

Formation of ($COO^- \cdots HO\text{-}C_6H_4\text{-}$) hydrogen bonds

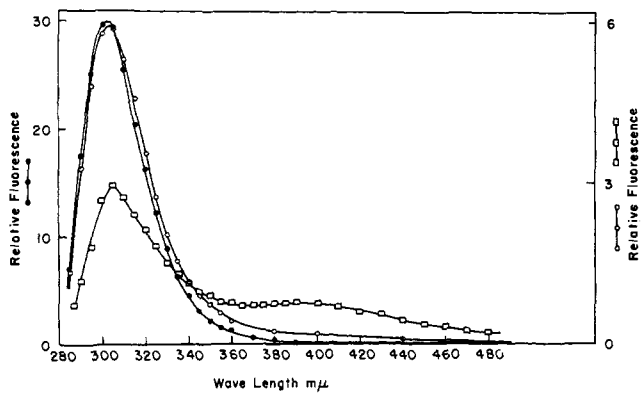

Fig. 4.—Emission spectra. ●-●-● L-Tyr in H_2O, pH 5.5; O-O-O (L-Glu-L-Tyr) (95:5), pH 7.5 in 0.2 *M* NaCl; □-□-□ block (L-Glu)-(L-Tyr) (95:5), pH 7.5 in 0.2 *M* NaCl; 1-cm. path length, exciting wave length 275 mμ; O.D. = 0.048 at 275 mμ (1-cm. path length) for all three solutions.

is not energetically favorable in this system, and preferential quenching by this ground-state interaction may be disregarded. Feitelson[37] has concluded that the quenching effect of weak acid anions is due to the general base catalyzed phenolic OH dissociation in the excited state. This decreases the concentration of the excited species responsible for fluorescence and consequently weak acid anions act as quenchers. However, no association in the ground state is necessary or implied. This explanation adequately explains the fluorescence titration curve observed in DMSO-H_2O.

In neutral solution lysyl–tyrosyl copolymers have quantum yields about double those of glutamyl-tyrosyl copolymers. The higher quantum yield (Q = 0.09) is comparable to the extrapolated Q of glutamyl-tyrosyl copolymers in an un-ionized state in aqueous solution. Thus carboxyl tyrosine interaction causes a two-fold decrease in tyrosine fluorescence. Hydrogen ion does not influence the fluorescence of the lysyl-tyrosyl copolymer even at pH 1.1, and probably may be disregarded as a quencher of tyrosine fluorescence in proteins. At alkaline pH, both $-NH_2$ and ionized tyrosine are formed in the lysyl-tyrosyl copolymer which results in a large decrease in fluorescence.

Block tyrosyl copolymers have a remarkably low quantum yield, about one-fourth of that of the unquenched tyrosyl copolymers (Q = 0.02). One phenomenon resulting in decreased fluorescence at 303 mμ is excimer formation resulting in a new broad fluorescence band at about 400 mμ. To our knowledge, this band has not been seen in proteins.[7] The orientation and distances between residues necessary for formation of this species in a protein may make its formation improbable, and the enormous tryptophan fluorescence in most proteins may have rendered it invisible. However, the transfer of energy by such interactions, although difficult to observe, may be responsible for the low quantum yields reported for many proteins.

Thus, from this model system study the most important influences on tyrosine fluorescence are: (1) carboxylate ion under neutral conditions, (2) the role of conformation, and (3) the interaction between tyrosyl residues (excimer formation). However, it is felt that the present model does not envelop the tyrosine sufficiently in a hydrophobic region to represent a truly buried moiety in a protein.