Luminescence of the Tryptophan and Tyrosine Residues of Papain in Solution*

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ABSTRACT: The luminescence of papain in solution has been investigated at temperatures near 25° and at liquid nitrogen temperatures. The fluorescence emission properties of papain are sensitive to changes in temperature, pH, and choice of excitation wavelength. The presence of 5 mM cysteine, an activating agent, or high concentrations of guanidine in the solutions also caused significant changes in the emission behavior of papain; 99% D_2O had little or no effect. Evidence for considerable energy transfer from tyrosine to tryptophan residues near neutral pH and from tryptophan to tyrosinate residues at pH 11.5 has been obtained. Measurements of the fluorescence and phosphorescence at 100°K in ethylene glycol–aqueous buffer glasses show no pH dependence over the range 5–8, in contrast to the results at room temperature. A splitting of the low-temperature fluorescence band is detectable.

Tyrosine phosphorescence is observed in the presence of 6 M guanidine, and tyrosinate phosphorescence is seen under sufficiently alkaline conditions. Phosphorescence lifetime determinations yield results compatible with the emission spectra obtained. The interpretations of the luminescence experiments together with some additional spectroscopic data are discussed in relation to the tertiary structure of crystalline papain as obtained by others from X-ray diffraction studies.

Consideration of the intrinsic fluorescence emission of proteins probably dates from the observations of Teale (1960) upon a number of globular proteins. Although the fluorescence emission from tryptophan and tyrosine residues in a protein is, in principle, a sensitive probe of the environments of these residues, the detailed interpretation of protein fluorescence in relation to the molecular conformation in solution and the locations of tryptophan and tyrosine moieties has been hampered by the multiplicity of fluorogenic environments encountered in a typical protein. A general method of attack upon this complex situation was developed by Weber (1961) in the form of a matrix analysis of absorbing and emitting species in a protein system; he was able to distinguish between tryptophan and tyrosine emission in several cases, but not, at that time, between different classes of tryptophan residues.

More recently, Lehrer and Fasman (1967), Lehrer (1967), and Elkana (1968) have made significant experimental progress in the solution of this problem by demonstrating that the broad tryptophan emission typical of proteins can, in several cases, be resolved into contributions from at least two types of tryptophan emitters. Studies of the kind pursued by Longworth (1968) on very small proteins with only a few tryptophan residues may also prove helpful in the application of our knowledge of the fluorescence of model compounds to the problem of emission from native proteins.

At low temperatures, proteins also phosphoresce, as was initially observed by Debye and Edwards (1952). Subsequent to that work, observations of the phosphorescence of several proteins have been made (Longworth, 1961, 1966, 1968; Nag-Chaudhuri and Augenstein, 1964; Churchich, 1966; Truong *et al.*, 1967), but the development of phosphorescence as a probe of the conformation of native proteins has greatly lagged that of fluorescence. This is due in part, no doubt, to difficulties in an assessment of the relevance of deductions based on luminescence behavior in a mixed-solvent glass at liquid nitrogen temperatures to protein properties in aqueous solution at temperatures near 25°.

We report here the results of a study of the luminescence of papain in solution. This enzyme seemed an attractive choice for this investigation because (1) it is a protein of relatively low molecular weight (*ca.* 22,000) and hence presents manageable, though perhaps considerable complexity; (2) it contains a reasonable number of tryptophan groups (five) and enough tyrosine residues (nineteen) to absorb a large fraction of the 280-nm radiation; (3) the tertiary structure of the crystalline enzyme has been recently obtained by X-ray diffraction methods (Drenth *et al.*, 1968); (4) while there has been great interest in the kinetic properties and catalytic mechanism of this enzyme, there have been surprisingly few reports dealing in depth with the application of the numerous available optical and magnetic methods to the elucidation of the solution conformation (Hill *et al.*, 1959; Glazer and Smith, 1961; Barel and Glazer, 1969), and with a subsequent comparison of the conformational implications of the solution experiments in relation to the crystal structure; and (5) the fluorometric titration curves for papain obtained by Shinitsky and Goldman (1967) indicated an imidazole-linked tryptophan fluorescence enhancement and further stimulated our interest in the luminescence properties of this protein.

Experimental Section

Materials. Papain was a recrystallized product of Worthington Biochemical Corp. (Freehold, N. J.), and was used

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without further purification. Tryptophan (Sigma Chemical Co., St. Louis, Mo.), L-cysteine (free base, Sigma), and D_2O (Volk Radiochemical Co., Silver Spring, Md.) were used as obtained. Ultra-Pure guanidine hydrochloride was a product of Mann Research Labs (New York, N. Y.). Chromatoquality Reagent ethylene glycol was employed for the mixed-solvent measurements. Water was glass redistilled, and buffer constituents were Certified Reagent Grade chemicals from Fisher Chemical Co. (Silver Spring, Md.). Buffers were 0.05 M sodium acetate or potassium phosphate titrated to the indicated pH. All buffer solutions contained 5×10^{-5} M EDTA.

Determination of pH. The pH of solutions was determined with a Radiometer pH meter and a glass electrode. For the fluorometric titrations, pH was varied by adding small amounts of concentrated KOH to the sample solution containing enzyme and sodium acetate, and initially at pH 5.1. Alkaline pH readings were not corrected for sodium ion error, but this error should be small for the ion concentration present and the pH range (5–12) surveyed.

The pH values reported for ethylene glycol–0.05 M phosphate (1:1, v/v) solutions are those read directly on the pH meter at room temperature (see Weinryb and Steiner, 1968) after appropriate titration.

Absorption Spectra. Absorption spectra and difference spectra were obtained with a Cary Model 14 spectrophotometer. A dynode tap setting of two was routinely used. Jacketed cell compartments were employed when temperature control was necessary. Measurements of absorbance at a single wavelength were made with a Gilford Model 200 photometer, which could also be adapted for variable temperature experiments.

Fluorescence Measurements. The variation of fluorescence intensity with pH or temperature was monitored with an Aminco–Bowman spectrofluorometer which gave results uncorrected for photomultiplier response or monochromator transmission. For the fluorometric titrations, the fluorescence was measured relative to a sample maintained at pH 5.1, to minimize errors arising from instrumental instability. Similarly, the variation of fluorescence intensity with temperature was computed relative to a sample kept at 20–21°. The variation of fluorescence quantum yield with temperature was determined by comparison with a tryptophan standard, assuming a quantum yield for tryptophan of 0.14, the approximate average of the results of Bridges and Williams (1968) and Chen (1967).

Fluorescence yields for papain were determined relative to tryptophan samples of equivalent absorbance and were corrected for inner filter effects (Weill and Calvin, 1963). Another Aminco–Bowman spectrofluorometer, equipped with a spectral compensation attachment, was used to obtain complete emission spectra. Use of this accessory results in spectra which are *energy corrected* for photomultiplier response (R136) and monochromator transmission. The data thus obtained are expressed in terms of the energy yield of fluorescence of papain relative to that for tryptophan. This ratio is equal to the relative quantum yield of fluorescence if the spectral distributions of emission for enzyme and tryptophan are identical. For different emission maxima, the ratio can be approximately corrected by multiplying by the ratio of wavelengths of maximum emission, $\lambda_{max}(F,enzyme)/\lambda_{max}(F,tryptophan)$. In our experiments this ratio is always between 0.92 and 1.00.

Fluorescence lifetimes were measured with a TRW nanosecond spectral source system. The instrument was calibrated frequently during the course of the experiments with both a reference scattering sample (Chen *et al.*, 1967) and with a solution of zwitterionic tryptophan ($\tau_F = 2.8 \pm 0.3$ nsec). A 290-nm interference filter intercepted the excitation radiation, and appropriate glass filters the emitted radiation. Our instrument was modified by the inclusion of a 1P28 photomultiplier and a thermostattable cell holder arrangement.

Luminescence Measurements. Fluorescence and phosphorescence spectra at liquid nitrogen temperatures were obtained with the compensated Aminco-Bowman spectrofluorometer, adapted for use at low temperatures (Steiner and Kolinski, 1968). An automatic liquid nitrogen level controller was utilized. The sample temperature at equilibrium was approximately 100°K.

Measurements of phosphorescence lifetime were made excluding the compensation accessory. A spring-operated rotating shutter was employed to first admit the exciting light to the sample, and then at zero time, to rotate by 90°, so as to pass sample phosphorescence to the detector. The decay data were registered directly in a PDP 8/S computer by means of a data acquisition program written by Dr. E. P. Kirby and then printed out on a teletype. Due to the averaging procedures written into the program, satisfactory decay curves could be obtained using slit widths comparable with those utilized for total emission spectra.

Enzyme Activity. The activity of the papain preparations used was estimated according to the procedure of Bender *et al.* (1966), with α-*N*-benzoyl-L-arginine ethyl ester (Cyclo Chemical Corp., Los Angeles, Calif.) as substrate. Enzyme in the presence of EDTA but in the absence of added cysteine was inactive. Addition of 5 mM cysteine to the assay solution (pH 5.1) resulted in the activation of 60% of the enzyme present. Papain activations of up to 80% could be achieved by preincubation with 5 mM cysteine at neutral pH and 4°. These degrees of activation are comparable with those reported by Bender *et al.* (1966) and by Williams and Whitaker (1967).

Results

Fluorescence Titration Curves at Room Temperature. The initial experiments consisted of measurements of the pH profile of fluorescence intensity for papain at 25° and were directed toward a confirmation and extension of the results of Shinitsky and Goldman (1967). During the course of these investigations, Barel and Glazer (1969) presented additional data. Since our results essentially confirm theirs, the discussion here will be confined to a few brief comments.

The pH dependence of relative fluorescence intensity observed for unactivated papain was in good qualitative and quantitative agreement with that reported by Shinitsky and Goldman (1967). In the presence of 10 mM cysteine, results similar to those of Barel and Glazer (1969) for activated papain were obtained (Figure 1). The intensity increased from pH 5 to 8, the midpoint being near pH 6.8. The region of change corresponds to that of the titration of the imidazole group of histidine. At more alkaline pH the fluorescence intensity decreased sharply for both activated and unactivated papain; an inflection point occurred for both systems at pH 10.1 ± 0.1.

The pH dependence of fluorescence intensity for activated mercuripapain in 10 mM cysteine and 10^{-5} M EDTA was equiv-

TABLE I: Perturbation Difference Spectra for Papain.

pH	$E_{290.5}$	$E_{284.5}$	No. of Exposed Tryptophans	No. of Exposed Tyrosines
5.2	1130	1690	2.0 ± 0.2	13 ± 2
7.6	1140	1580	2.0 ± 0.2	13 ± 2

alent to that of activated papain, as was reported by Barel and Glazer (1969) and by Arnon and Shapira (1969).

Perturbation Spectra. The technique of perturbation difference spectroscopy was employed to gain an estimate of the number of tryptophan and tyrosine residues which were exposed to solvent. The neutral solvent perturbant used was 20% ethylene glycol. In computing the apparent numbers of exposed tryptophan and tyrosine residues, the molar absorptivity difference values for acetyl-L-tryptophan ethyl ester and for acetyl-L-tyrosine ethyl ester reported by Herskovits and Sorenson (1968) were utilized.

Maxima in the perturbation difference spectra of inactive papain were observed at 290.6 nm and at 284.5 nm. Within experimental uncertainty the magnitudes of the perturbation spectra were the same at pH 5.2 and 7.6. At each pH the number of exposed groups was computed by solution of the following simultaneous equations, using the absorptivity difference values of Herskovits and Sorenson:

$$E_{290.5} = 305x + 32y$$

$$E_{284.5} = 189x + 92y$$

where $E_{290.5}$ and $E_{284.5}$ are the molar absorptivity differences at the indicated wavelengths and x, y are the numbers of exposed tryptophans and tyrosines, respectively. The results are cited in Table I. They have only a formal significance, as it is difficult to distinguish between partially and completely exposed chromophores by this approach. The apparent numbers of exposed groups are the same at the two pH values.

In contrast, the fluorescence intensity of papain at both pH 5.1 and 8.1 was relatively insensitive to the presence of 20% ethylene glycol, in agreement with Barel and Glazer (1969). The wavelengths of excitation and emission were 290 and 330 nm, respectively. The former wavelength was chosen so as to minimize possible complications arising from radiationless transfer from tyrosine, which becomes significant for excitation at lower wavelengths, as will be discussed below. Under these conditions the ratio of fluorescence intensities in the presence and absence of 20% ethylene glycol is 1.025 ± 0.015 at pH 5.1 and 1.00 ± 0.015 at pH 8.1. This result will be considered further in the Discussion.

The Thermal Dependence of the Emission and Absorption Spectra of Papain. The quantum yields of fluorescence of tryptophan and tyrosine are very temperature dependent, for both the free amino acids and for oligopeptides and proteins into which they are incorporated (Steiner and Edelhoch, 1962; Gally and Edelman, 1962, 1964; R. F. Steiner and E. P. Kirby, 1969, unpublished data). The fluorescence intensity of the

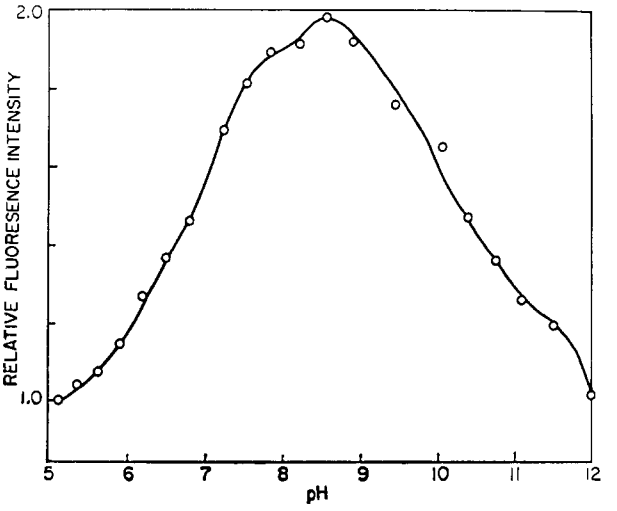

FIGURE 1: The pH profile of relative fluorescence intensity for papain (10^{-5} M), activated by the presence of 10 mM cysteine, in 0.05 M sodium acetate at 25°. The excitation and emission wavelengths are 290 and 350 nm, respectively.

aromatic amino acids decreases monotonically with increasing temperature, while the thermal behavior of proteins may be complex if conformational changes occur.

The temperature dependence of the quantum yield of an aromatic amino acid arises primarily from variations in the radiationless deactivation processes which compete with the direct emission of fluorescent radiation. In general

$$Q = k_f/(k_f + k_0 + \sum_i k_i) \quad (1)$$

or

$$Q^{-1} - 1 = \frac{k_0}{k_f} + \frac{1}{k_f}\sum_i k_i = \frac{k_0}{k_f} + \frac{1}{k_f}\sum_i f_i E^{-E_i/RT} \quad (2)$$

where Q is the fluorescence quantum yield at temperature T; k_f is the (temperature independent) rate constant for emission of fluorescence; k_0 is the sum of the rate constants for all the temperature-independent deactivation processes; and the k_i represent the rate constants for the other nonradiative mechanisms for dissipating excitation energy, each with activation energy, E_i, and frequency factor, f_i.

The magnitude of k_0 is not known in the present case, but for other tryptophan-containing systems k_0/k_f is of the order of magnitude of unity (R. F. Steiner and E. P. Kirby, 1969, unpublished data). For the system under consideration this term is sufficiently small compared with $Q^{-1} - 1$ so that eq 2 may be approximated by

$$Q^{-1} - 1 = \frac{1}{k_f}\sum_i f_i e^{-E_i/RT} \quad (3)$$

or, if only one temperature-dependent deactivation process is important,

$$Q^{-1} - 1 = \frac{f}{k_f} e^{-E_i/RT} \quad (4)$$

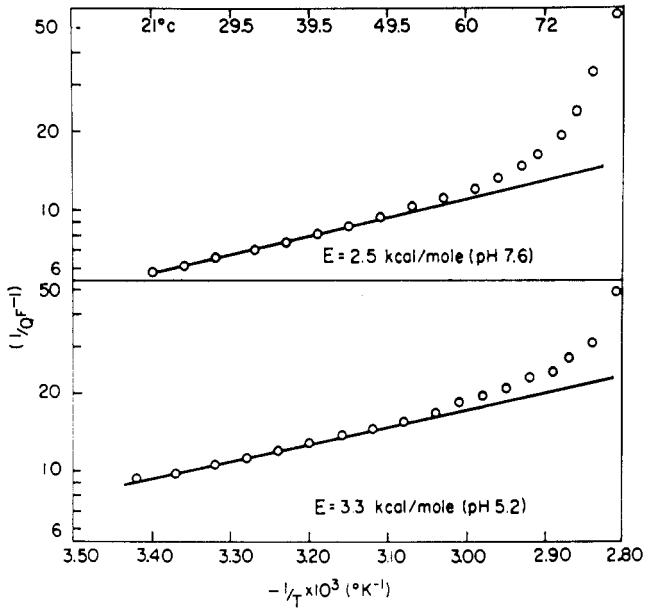

FIGURE 2: The dependence of fluorescence quantum yield upon temperature for inactive papain excited at 290 nm and monitored at 340 nm (pH 5.2) or 350 nm (pH 7.6), uncorrected for detector response or monochromator transmission.

Logarithmic plots of $Q^{-1} - 1$ *vs.* $1/T$ for unactivated papain at pH 5.2 and 7.6 are shown in Figure 2. The quantum yield at 25° (for excitation at 290 nm) was taken as 0.095 at pH 5.2 and as 0.14 at pH 7.6, as determined using tryptophan as a reference standard (see Experimental Section). Over the temperature range 20–55°, the variation is linear, corresponding to constant energies of activation of about 3.3 kcal/mole at pH 5.2 and 2.5 kcal/mole at pH 7.6. These activation energies are substantially lower than the 6–8 kcal/mole found for free tryptophan (Gally and Edelman, 1962, 1964; Eisinger and Navon, 1969; R. F. Steiner and E. P. Kirby, 1969, unpublished data).

At temperatures above 60°, the data of Figure 2 depart from linearity. This is most reasonably ascribed to the onset of thermally induced conformational changes of the protein. At temperatures above 70°, the upward curvature becomes very pronounced. At pH 5.2, cooling from about 80 to 20° results in the recovery of about 80% of the initial fluorescence; at pH 7.6, about 60% is regained. It is of interest to compare the thermal profiles of fluorescence and absorption of papain (Figure 3). A qualitatively parallel variation of the two sets of data is evident from Figures 2 and 3, the absorbancy at 286 nm decreasing sharply above 70°. The initial absorbance is not recovered upon cooling to temperatures below 30°.

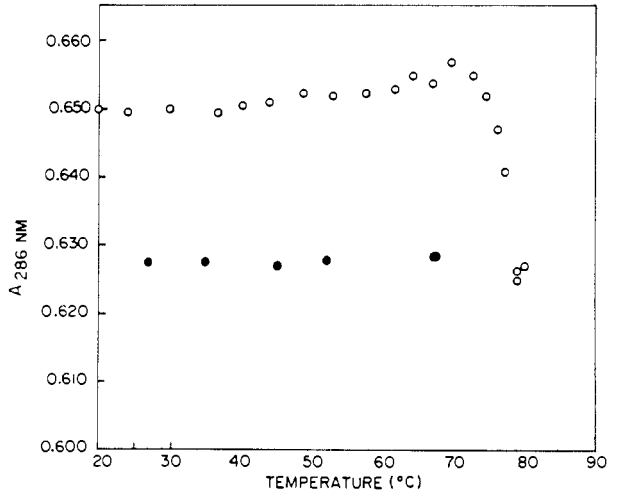

FIGURE 3: Thermal dependence of the absorbance of inactive papain pH 5.2, followed at 286 nm. Solid points represent cooling from 80°.

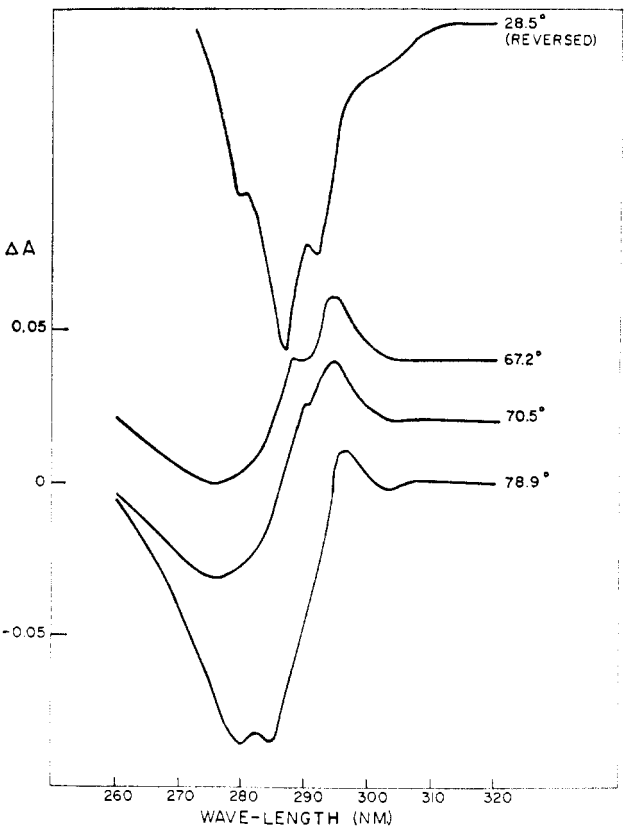

FIGURE 4: Thermal difference spectra (260–320 nm) of inactive papain, pH 5.2. $A_{280\ nm}$ is 1.33 for sample and reference protein solutions. The reference solution is maintained at 27°. The three upper curves are displaced upward for greater visibility. ($\Delta A = 0$ for wavelengths above 320 nm in each case).

Figure 4 shows the thermal difference spectra obtained for papain at a series of temperatures at pH 5.2. (The reference solution is the protein solution at 27°.) Below 70°, the changes are relatively minor; a small positive peak appearing above 290 nm and a negative difference spectrum at lower wavelengths. These changes may largely reflect the influence upon the solvent-accessible chromophores of the alteration in solvent dielectric constant with increasing temperature. No important change occurs at higher temperatures in the positive band above 290 nm, its magnitude decreasing slightly. However, a major increase in intensity of the negative difference spectrum below 290 nm occurs, with considerable structure appearing. Minima occur near 280 and 285 nm, with a shoulder near 290 nm. Upon cooling to room temperature a highly structured difference spectrum persists (Figure 4).

TABLE II: Effect of pH, D_2O, Cysteine, and Guanidine on the Fluorescence of Papain.[a]

pH	Solvent[b]	Solute	Max F(nm)[c]	$\tau_{F(ns)}$[d]	Relative Overall Fluorescence Energy Yield[e]	Relative Tryptophan Fluorescence Energy Yield[f]
5.2	Ac–H_2O	Tryptophan	353	2.9	1.00	1.00
		Papain	333	3.4	0.72	0.79
	Ac–D_2O	Tryptophan	353	(5.5)[g]	1.00[h]	1.00[h]
		Papain	334	3.4	0.76	0.84
	Ac–H_2O +	Tryptophan	354	2.7	1.00	1.00
	5 mM cysteine	Papain	327	2.6	0.59	0.65
	Ac-H_2O +	Tryptophan	355	1.8	1.00	1.00
	4 M guanidine	Papain	338	2.1	0.72	0.79
7.6	PO_4–H_2O	Tryptophan	355	2.9	1.00	1.00
		Papain	343	4.5	1.05	1.15
	PO_4–D_2O	Tryptophan	353	(5.5)[g]	1.00[h]	1.00[h]
		Papain	340	4.4	1.00	1.10
	PO_4–H_2O +	Tryptophan	355	2.7	1.00	1.00
	5 mM cysteine	Papain	339	4.6	0.89	0.98
	PO_4–H_2O +	Tryptophan	355	2.4	1.00	1.00
	4 M guanidine	Papain	341	3.8	1.16	1.27

[a] Temperature = 25 ± 2°. Excitation wavelength is 290 nm. [b] Buffer is 0.05 M sodium acetate or potassium phosphate plus 5×10^{-5} M EDTA. [c] Estimated precision: ±2 nm. [d] Estimated precision: ±0.3 ns. [e] Yields compared at equivalent concentrations using total solute absorbance at 290 nm. Estimated precision: ±8%. [f] Yields calculated on basis of tryptophan absorbance at 290 nm; equal to (overall yield)/0.91. Estimated precision: ±8%. [g] Lifetime for tryptophan in D_2O at pH 6.2. [h] Reference tryptophan solution in Ac–H_2O.

A comparison of these results with the difference spectra of the aromatic amino acids and several proteins presented by Foss (1961) suggests that the thermal difference spectrum developed by papain above 70° results primarily from perturbation of the tyrosine chromophores as a consequence of a major unfolding of the secondary and tertiary structure. This is not rapidly reversed upon cooling.

The Fluorescence of Papain at Room Temperature. Table II summarized the fluorescence parameters observed for papain at pH values on either side of the transition midpoint at pH 6.8. Table II includes data obtained in 99% D_2O and in the presence of 5 mM cysteine and 4 M guanidine hydrochloride.

Papain contains 5 tryptophan, 19 tyrosine, and 4 phenylalanine residues (Drenth *et al.*, 1968), so that at the wavelength of excitation, 290 nm, about 91% of the total exciting radiation, is absorbed by tryptophan (Wetlaufer, 1962). The relative energy yields cited in Table II were calculated on the basis of both the total protein absorbance and that of the tryptophan residues alone.

We find for papain at pH 5.2 in acetate–EDTA buffer an emission maximum at 333 nm ± 2 nm (for excitation at 290 nm), and for phosphate–EDTA buffer of pH 7.6, at 343 nm ± 2 nm. This is in contrast to the reported finding of Barel and Glazer (1969) that the maximum of fluorescence emission occurs at 352 nm and is independent of pH over the range 4–9. The use of an instrument which yields spectra uncorrected for monochromator and detector response could explain the different emission maximum they obtained; their finding of pH independence is, however, puzzling. Both the relative energy yield and the fluorescence lifetime of papain were found to be greater at pH 7.6 than at pH 5.2.

Although tryptophan shows almost a doubling of fluorescence quantum yield when D_2O is substituted for H_2O (R. F. Steiner and E. P. Kirby, 1969, unpublished data), the results of Table II indicate that the effect of D_2O substitution for H_2O upon the fluorescence properties of papain is small. For this set of experiments, energy yields relative to tryptophan in H_2O were determined, permitting direct comparison with enzyme energy yields in the latter solvent. Most of the changes observed fall within the experimental uncertainty of the measurements.

The presence of 5 mM cysteine does result in significant changes in the emission parameters for papain. At higher concentrations, cysteine is a powerful quencher of tryptophan fluorescence (R. F. Steiner and E. P. Kirby, 1969, unpublished data). At a cysteine concentration of 5 mM, however, tryptophan showed a normal fluorescence lifetime, and hence no *intrinsic* quenching by 5 mM cysteine is expected in these studies. Nevertheless, at pH 5.2, the presence of 5 mM cysteine lowers the energy yield of papain about 20% and the wavelength of maximum emission by about 6 nm. The fluorescence lifetime also drops slightly. At pH 7.6, energy yields for papain are also reduced by about 20%, and the emission maximum for papain shows a 4 nm blue shift. At this pH, the lifetime for papain is not significantly altered from its value in the absence of cysteine.

The effect of high levels of guanidine hydrochloride upon the fluorescence behavior of papain was also examined. Ear-

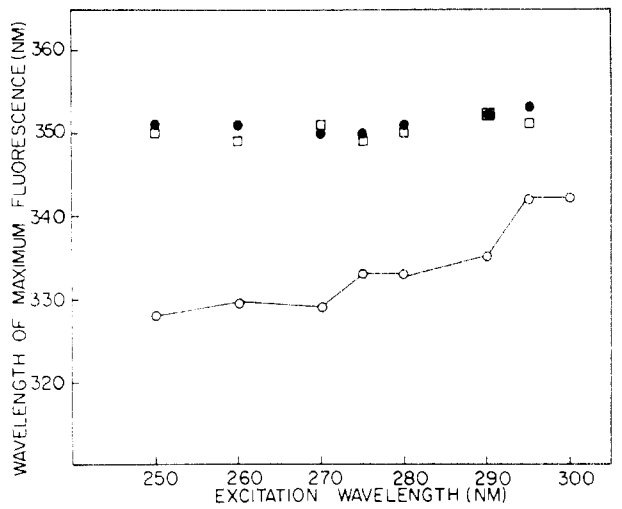

FIGURE 5: The dependence of the wavelength of maximum fluorescence emission (corrected) upon excitation wavelength for inactive papain; (○) pH 5.2, (●) pH 5.2 in the presence of 6 M guanidine, (□) pH 11.5.

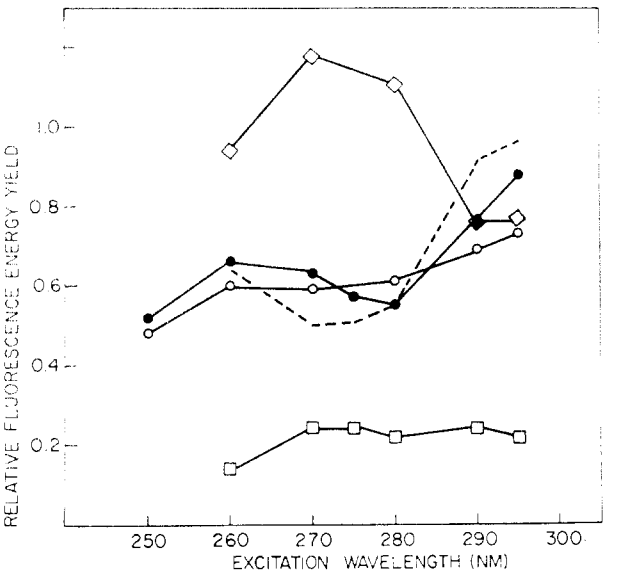

FIGURE 7: The dependence of the relative fluorescence energy yield (corrected) upon excitation wavelength for inactive papain; (○) pH 5.2, (●) pH 5.2 in the presence of 6 M guanidine, (□) pH 11.5, (◇), pH 5.2, calculated on the basis of tryptophan absorption alone. The dotted line represents the wavelength dependence of the fractional light absorption by tryptophan in an aromatic amino acid mixture of the same composition as papain.

lier optical rotation studies of papain in 4 M guanidine had sugested that the molecule was largely unfolded in this solvent (Hill *et al.*, 1959). Table II cites the energy yields relative to those of tryptophan in this same medium. There is some intrinsic quenching of free tryptophan by this level of guanidine, as is indicated by the reduced lifetime (Table II).

At pH 5.2, the emission maximum for papain was red shifted by about 5 nm. The absolute value of the lifetime decreases significantly, although the yield relative to tryptophan in the same solvent is essentially unchanged.

At pH 7.6, the only important change for papain was a reduction in fluorescence lifetime. The fact that the emission maximum did not shift at either pH to near 350 nm, its wavelength for most unfolded proteins (Teale, 1960), suggests the unfolding may be incomplete in 4 M guanidine. At pH 5.2 in 6 M guanidine, the emission maximum for papain was at about 352 nm.

Variation of Emission Properties with Excitation Wavelength. Teale (1960) found that the fluorescence quantum yield of many proteins was a function of excitation wavelength over the 270–300-nm range. This is to be expected if tyrosine and tryp-

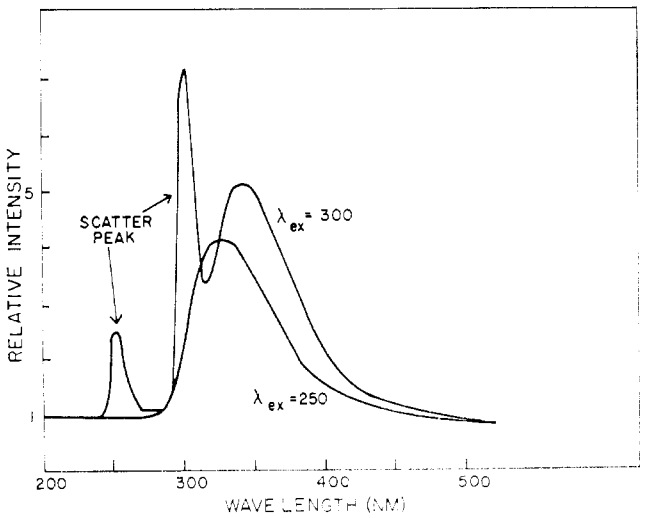

FIGURE 6: Fluorescence emission spectra (corrected) for inactive papain (10^{-5} M, pH 5.2) excited at 250 and 300 nm.

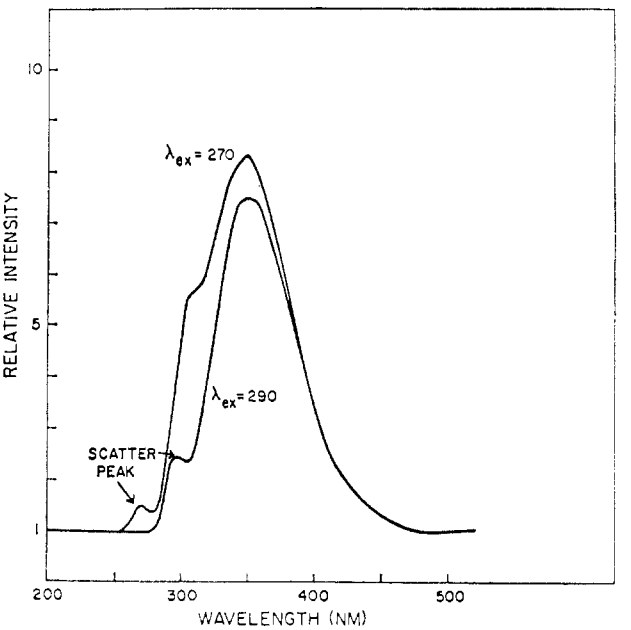

FIGURE 8: Fluorescence emission spectra (corrected) for inactive papain (10^{-5} M, pH 5.2) in the presence of 6 M guanidine, excited at 270 and 290 nm.

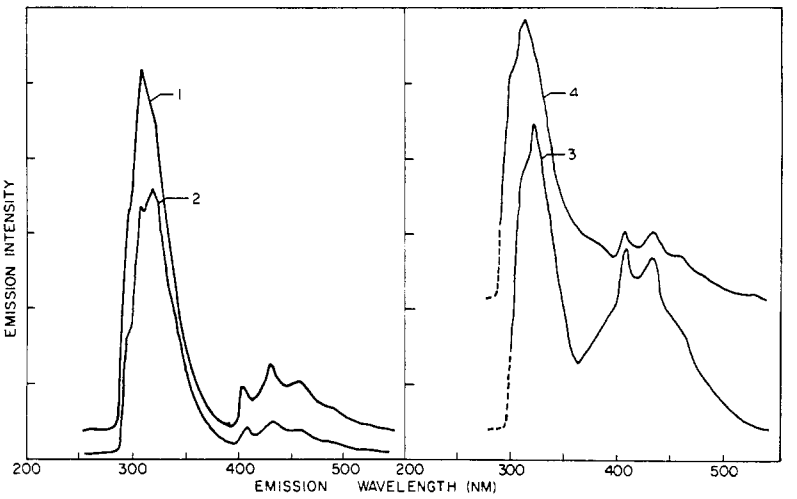

FIGURE 9: Total emission spectra (corrected) at 100°K in 1:1 (v/v) ethylene glycol–aqueous buffer glasses. Excitation wavelength 280 nm. 1, tryptophan at pH 5.2 and 11.5; 2, papain at pH 5.2 and 7.6; 3, papain at pH 11.5; 4, papain at pH 5.2 in the presence of 6 M guanidine.

tophan do not contribute equally to the emission, since their absorption spectra are not equivalent. In addition, the wavelength of maximum emission may shift (Weber and Teale, 1965).

In contrast to the report of Barel and Glazer (1969) that the emission maximum of papain fluorescence is independent of excitation wavelength, we have found a shift in the emission spectrum to lower wavelengths as the excitation wavelength decreases from 295 nm (Figure 5). The displacement of the maximum is almost 15 nm upon comparing excitation at 250 and at 295 nm (Figure 6). No shoulder near 300 nm could be detected in the emission spectra.

The energy yield for papain at pH 5.2 decreases significantly as the excitation wavelength is varied from 295 to 250 nm, with most of the change occurring between 290 and 280 nm and below 260 nm. Figure 7 includes the wavelength dependence of the fraction of the total absorbance arising from tryptophan for an aromatic amino acid mixture of the same relative composition as papain (dotted line). The energy yield dependence upon excitation wavelength for papain at pH 5.2 is less pronounced than would be expected if no transfer of excitation energy from tyrosine to tryptophan occurred. In such a case, if no emission occurred from tyrosine, the relative energy yield, as computed from the total absorbance at each excitation wavelength, would vary as the fractional absorbance of tryptophan, while the relative yield computed from the tryptophan absorbance alone would be constant. In actuality the former is almost constant, while the latter increases for excitation below 290 nm.

In 6 M guanidine, in which papain is largely, or completely, unfolded, the emission behavior changes considerably. The position of the emission maximum is close to 350 nm for excitation wavelengths from 295 to 250 nm. The relative energy yield, computed from total absorbance, varies with excitation wavelength in approximate parallel to the fractional tryptophan absorption (Figure 7).

For excitation below 280 nm in 6 M guanidine, a short-wavelength fluorescence band appears at about 310 nm as a shoulder to the main emission peak, whose maximum remains at 352 nm (Figure 8). In view of the probable loss of tertiary structure in this denaturing solvent, the shoulder at 310 nm is unlikely to arise from a set of tryptophan residues of a distinct microenvironment. It seems more reasonable to ascribe this component to direct emission from tyrosine residues, whose contribution is suppressed in the native papain molecule.

The presence of the emission band at 310 nm for excitation wavelengths above 280 nm could not be ascertained for denatured papain in aqueous solution because of interference from the scattered exciting radiation. However, for a 50% ethylene glycol solvent containing 6 M guanidine, the scatter peak was sufficiently suppressed to permit demonstration that a partially resolved band at about 305 nm occurs with 280-nm excitation, but that excitation at 290 nm gave no evidence of a species emitting in that wavelength region. This is a strong indication that the short-wavelength emission band represents tyrosine fluorescence.

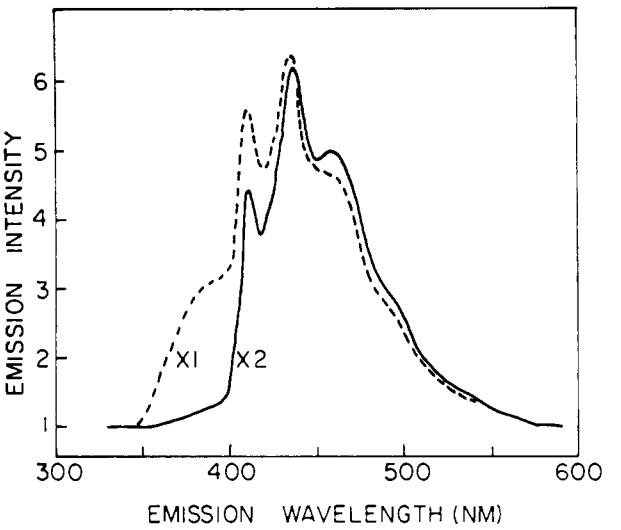

FIGURE 10: Phosphorescence spectra (corrected) at 100°K in ethylene glycol–aqueous buffer glasses obtained with a rotating shutter. Excitation wavelength 280 nm. Papain in the absence (solid line) and presence (dotted line) of 6 M guanidine, pH 5.2. The numbers indicated for each spectrum represent relative instrumental sensitivity settings.

TABLE III: Emission Properties of Tryptophan and Papain at 100°K.

Solvent	Compound	Room Temp (°C), pH	Fluorescence λ_{max} (nm)[a]	Phosphorescence λ_{max} (nm)[a]	Relative F:P[b]
Ethylene glycol–H_2O (1:1, v/v)	Tryptophan	5.2,	(295)[c]	404	1.0
		11.5	310	431	
			(322)	456	
				(485–490)	
	Papain	5.2,	(295)	408	1.5
		7.6	310	434	1.6
			320	(455–460)	
			(335)	(485–490)	
		11.5	(295)?[d]	(390)	0.25
			(310)	408	
			320	433	
			(335)	(455–460)	
				(485–490)	
0.5% Glucose	Papain	5.2	(295)	407	1.5
			310	434	
			320	(455–465)	
			(335–340)	(485–490)	
		11.5	(295)?[d]	(385–395)	0.28
			(310)	407	
			320	434	
			(335–340)	(455–460)	
				(485–490)	
Ethylene glycol–H_2O (1:1, v/v), +6 M guanidine	Tryptophan	5.2	(295)	403	0.9
			310	430	
			(320)	455	
				(485–490)	
	Papain	5.2	(300)	(375–380)	0.75
			312	405	
			(325)	432	
				(455–460)	
				(485–490)	

[a] Excitation at 280 nm. Estimated precision: ±2 nm for peaks, ±2 nm for shoulders. [b] Relative fluorescence yield:phosphorescence yield. Estimated precision: ±10%. [c] Wavelengths in parentheses indicate approximate position of shoulder. [d] Wavelength region obscured by scattered exciting radiation.

A single set of measurements were made upon heat-denatured papain produced by 5 min at 100°, followed by rapid quenching to 15°. Although the time-dependent increase of turbidity in the solution precluded meaningful calculations of energy yields, it was possible to observe that the broad emission maximum (~345 nm) showed no important shift with excitation wavelength, and a shoulder at 300–305 nm appeared for excitation wavelengths below 280 nm.

At pH 11.5, where 11–12 tyrosine groups are ionized (Glazer and Smith, 1961) the emission maximum was almost independent of exciting wavelength (Figure 5). The energy yields (Figure 7) were much lower than at pH 5.2 and were constant for excitation at 270–295 nm, a decrease occurring for excitation at 260 nm.

The Luminescence Spectra of Papain at Liquid Nitrogen Temperatures. The total emission spectra of tryptophan and papain were measured in a 50% ethylene glycol–aqueous buffer glass at temperatures close to 100°K. The initial ethylene glycol–buffer solutions were titrated to the indicated pH values at room temperature (Weinryb and Steiner, 1968). The excitation wavelength was normally 280 nm, to minimize interference from the scattered exciting radiation. The luminescence spectra at high resolution are shown in Figures 9 and 10 and the emission parameters are summarized in Table III.

The spectral distribution of emission is essentially identical for tryptophan at pH 5.2 and at pH 11.5, with a fluorescence maximum at about 310 nm and some slight indication of shoulders at 295 and 322 nm. The latter may represent indole emission fine structure. Distinct phosphorescence maxima occur near 404, 432, and 456 nm, with a shoulder at 485–490 nm. The fluorescence:phosphorescence energy yield ratio was approximately the same for the two pH values (Table III).

The total emission spectra of papain at pH 5.2 and at pH 7.6 are equivalent. This behavior contrasts with that of the fluorescence at room temperature of the protein and suggests

TABLE IV: Phosphorescence Lifetimes (τ_p) for Papain and Tryptophan.

Compound	Solvent[a]	pH[b]	Excitation Wavelength (nm)	Emission Wavelength (nm)	τ_{p_1}[c]	τ_{p_2}[c]	I_{p_1} (%)[d]
Tryptophan	Ethylene glycol–H_2O	5.2,	280	430	6.7		100
	(+6 M guanidine)	11.5	280	427	6.6		100
		5.2	280	430	5.9		100
Papain	Ethylene glycol–H_2O	5.2	280	430	5.6		100
	(+6 M guanidine)	5.2	280, 290	430	5.0		100
	(+6 M guanidine)	5.2	280, 290	375	2.1		100
	Ethylene glycol–H_2O	11.5	280	430	5.8	1.1	50
		11.5	280, 290	390	1.3		100
		11.5	290	430	4.8	0.9	45

[a] Ethylene glycol–aqueous acetate (1:1, v/v) (pH 5.2) or phosphate (pH 11.5) buffer. [b] Measured at room temperature for mixed-solvent system. [c] Estimated precision: ±0.3 sec. [d] Initial intensity percentage of component with lifetime τ_{p_1}. Estimated precision: ±10%.

that the pH-dependent fluorescence quenching observed at room temperature is of a dynamic, rather than a static character. The total emission spectra of papain are somewhat red shifted in comparison with that of tryptophan; there is also an apparent partial resolution of the fluorescence band with maxima at 310 and 320 nm. The former peak is less well resolved at pH 11.5, being manifest as a shoulder. The F:P ratio for papain at pH 5.2 or 7.6 is about 50% higher than that of tryptophan. The relative decrease in phosphorescence yield for papain probably reflects an influence of the protein conformation upon the rate of intersystem crossing, rather than a selective quenching of phosphorescence.

At pH 11.5, where a substantial fraction of the tyrosine residues are ionized at 100°K, the relative phosphorescene yield of papain increases by a factor of 6, with the appearance of an emission band near 400 nm, arising from the phosphorescence of the tyrosinate anion (Bishai *et al.*, 1967).

In ethylene glycol–water glasses containing 6 M guanidine, the emission spectrum of tryptophan is essentially equivalent to that in the absence of guanidine (Table III), except that the F:P ratio appears to be slightly lowered. For papain in the same solvent, the structure of the fluorescence band which was observed in the absence of guanidine, is largely lost (Figure 9). A single emission maximum appears at about 312 nm, with a suggestion of a shoulder at 300 nm. A new emission peak arises at 375–380 nm (Figures 9 and 10); this wavelength region is characteristic of un-ionized tyrosine phosphorescence. The emission at 375–380 nm is much reduced when 290 nm, rather than 280 nm, exciting radiation is employed, in confirmation of the conclusion that this band arises from neutral tyrosine. The F:P ratio in 6 M guanidine is reduced by a factor of two.

Comparative experiments were made for papain in buffered aqueous glasses containing 0.5% sucrose and no ethylene glycol. The quality of the glasses was poor, resulting in large scatter peaks and diminished resolution; nevertheless, it was clear that the spectral features and F:P ratios for papain were essentially unchanged from their values in 50% ethylene glycol.

Phosphorescence Lifetimes. The phosphorescence lifetime determinations for papain under various conditions are summarized in Table IV. Results for free tryptophan are included for comparison. These are in general accord with previous findings (Steiner and Kolinski, 1968; Weinryb and Steiner, 1968). A minor (10%) decrease in the lifetime of tryptophan is observed in the presence of 6 M guanidine.

The lifetime found for papain phosphorescence at 430 nm at pH 5.2 is significantly less than that of tryptophan. Attempts were made to detect tyrosine phosphorescence at this pH by lifetime determinations at an emission wavelength of 390 nm (see Longworth, 1961), which is outside the tryptophan band but close to the maximum for tyrosine phosphorescence. Measurements were hindered by very low intensities, but a lifetime close to 2.2 sec was observed, which is typical of tyrosine. The tyrosine contribution is less than about 2% of the total phosphorescence.

In 6 M guanidine at pH 5.2 a new phosphorescence band appears at about 375–380 nm. When monitored at 375 nm, the phosphorescence lifetime is close to 2.1 sec, supporting the conclusion that the new band arises from un-ionized tyrosine (Longworth, 1961; Steiner and Kolinski, 1968). The lifetime of the tryptophan component, observed at 430 nm was slightly decreased, to 5.0 sec, in this medium.

At pH 11.5, the phosphorescence decay of papain, monitored at 430 nm, could be resolved into two components of roughly equal magnitude, one of which has a lifetime of 5.8 sec, corresponding to tryptophan, and the other a lifetime close to 1.1 sec, corresponding to ionized tyrosine (Steiner and Kolinski, 1968). The decay curve observed at 390 nm could be fitted with a single lifetime, which was characteristic of ionized tyrosine (Table IV).

Discussion

The luminescence behavior of a typical protein will reflect the effects of incorporation of the fluorogenic amino acid residues into a polypeptide and of the folding of the polypeptide into the secondary and tertiary structure characteristic of the native protein, which results in a somewhat different local

microenvironment for each emitting residue. This heterogeneity of microenvironments implies a heterogeneity of effective dielectric constants for the different fluorogens. The specific interactions of individual residues, including such factors as intramolecular hydrogen bonding and the binding of small molecules, may also be important, as well as the degree of shielding from the solvent. To these factors may be added the possibility of nonradiative energy transfer between fluorogens whose emission and absorption bands overlap. This may greatly alter the relative contributions of the different classes of aromatic amino acid to the total emission (Longworth, 1968).

Phenylalanine emission cannot ordinarily be detected in proteins if the other aromatic residues are present, because of its low absorbance and low quantum yield, and presumably because of radiationless energy transfer to tyrosine and tryptophan. The contribution of tryptophan groups normally dominates the emission of proteins in which they occur, even if tyrosine accounts for a substantial fraction of the total absorption at the wavelength of excitation (Teale, 1960; Longworth, 1968).

The fluorescence yield of papain at pH 7.6 when excited at 290 nm is close to that of tryptophan and somewhat higher than the values reported for most proteins (Teale, 1960), although the values recently found for serum albumin (Weber and Young, 1964) and for pyruvate kinase (Suelter, 1967) are of similar magnitude. A determining factor for the fluorescence efficiency at 25° is the extent of shielding of the tryptophan groups from solvent, since the temperature-dependent radiationless processes which competitively deactivate the excited state of indole are less important in nonpolar media (R. F. Steiner and E. P. Kirby, 1969, unpublished data).

An X-ray diffraction study of crystalline papain has revealed that two tryptophan residues (69 and 177) are not buried, two (7 and 181) are partially buried, and one (26) is completely buried (J. Drenth, 1969, personal communication). This numbering is from the amino-terminal end of the completely determined polypeptide chain, as furnished by Dr. Drenth, which differs from the chain depicted in Figure 3 of Drenth *et al.* (1968), where consecutive numbering from the amino-terminal end yields tryptophan residues at positions 176 and 180.

There is some difficulty in interpreting the solution measurements strictly in terms of the structure deduced from the crystallographic studies. The absorption spectral perturbation results are most simply interpreted in terms of two completely exposed tryptophans. However, even the two unburied tryptophans-69 and -177 are not completely exposed in the crystal, but show their indole rings parallel to, and consequently partially shielded by, the enzyme surface (J. Drenth, 1969, personal communication). Should the same situation exist in solution, the absorption spectral perturbation results must be ascribed to major contributions from tryptophans-69 and -177, together with small contributions from the partially buried tryptophans-7 and -181.

The only slight effect upon the fluorescence intensity of high levels of ethylene glycol may suggest that the exposed residues 69 and 177 do not make a major contribution to the observed fluorescence, as does the low magnitude of the D_2O effect. This may reflect the relative enhancement of the quantum yields of the partially or completely buried groups as a consequence of their shielding from the polar solvent. It should, however, be recognized that the factors governing fluorescence enhancement by neutral solvents are incompletely understood and that it may be possible that features of the microenvironment of an exposed tryptophan are such as to prevent the effect even in the absence of shielding.

The pH profile of the quantum yield below pH 8 parallels the ionization of histidine and it is reasonable to attribute the decline in quantum yield with decreasing pH to the well-known quenching effect upon indole of the protonated imidazole group of histidine (Shinitsky and Goldman, 1967; R. F. Steiner and E. P. Kirby, 1969, unpublished data).

According to the model of J. Drenth and coworkers (1969, personal communication), two tryptophan residues (177 and 181) are fairly close to histidine-159 (158 in Figure 3 of Drenth *et al.*, 1968), which is part of the active site. The side chain of histidine-159 actually touches the side chain of tryptophan-177 (J. Drenth, 1969, personal communication). However, as noted above, there is evidence that this partially exposed tryptophan may not make a contribution to the total fluorescence sufficient to account for all of the imidazole-linked enhancement observed in Figure 1. This is suggested, subject to the above reservations, by the low magnitude of the fluorescence enhancement by ethylene glycol and by its failure to increase upon going from pH 5 to 8. There is thus an implication that residue 181 may be partly responsible for the pH variation of quantum yield. Alternatively, the possibility exists that the crystal and solution conformations of papain are not identical.

The shift of emission maximum to shorter wavelength which accompanies the quenching observed at pH 5.2 relative to pH 7.6 (Table II) further suggests that the quenched residue emits at relatively longer wavelengths and hence occurs in a more polar environment than the groups which contribute to the residual fluorescence at pH 5.2 (Lehrer and Fasman, 1967). The latter presumably include the buried residue 26.

The alternative explanation exists that a conformational change in this pH region is directly or indirectly responsible for the change in fluorescence properties. However, there is no evidence for such a structural transition; perturbation spectra fail to indicate any important change in conformation over this pH range.

The excited lifetime also decreases upon going from pH 7.6 to 5.2 (Table II). The implication is that the quenched group (or groups) has a longer lifetime than the other tryptophans and that the change in average lifetime reflects the altered weighting of the contributions to the total fluorescence.

The activation of papain by cysteine is accompanied by a significant change in fluorescence properties, including a blue shift in emission maximum, although there is no evidence for a major conformational change. These probably result from minor changes in the local microenvironment of the tryptophans in proximity to the active site.

The low magnitude of the deuterium isotope effect, which is in contrast to the major enhancement of the quantum yields of tryptophan and indole by D_2O (Stryer, 1966), also deserves comment. The suppression of the D_2O effects, whose origin is poorly understood for indole derivatives, can be at least partially attributed to the fractional or complete screening from solvent of three of the five tryptophans and to the apparently low quantum yields of the two exposed residues.

The activation energy for the thermal quenching of papain fluorescence is lower than that of tryptophan (Gally and Edelman, 1962, 1964; Eisinger and Navon, 1969) or that of other indole derivatives of comparable quantum yield (R. F. Steiner

and E. P. Kirby, 1969, unpublished data). The determination of the fluorescence by the shielded residues may again be a factor, as the activation energies for many indole and tryptophan derivatives are influenced by interactions of the excited state with solvent, and are lower in nonpolar media than in water (R. F. Steiner and E. P. Kirby, 1969, unpublished data). The rapid decrease in quantum yield above 70° probably reflects a thermally induced unfolding of the molecule and corresponds approximately to the onset of inactivation (Lineweaver and Schwimmer, 1941).

When computed on the basis of tryptophan absorption alone, the apparent quantum yield of papain is not constant with changing excitation wavelength, but shows a significant rise at excitation wavelengths below 280 nm, where the absorption of tyrosine becomes important. This arises in all probability from radiationless energy transfer at the singlet level from tyrosine to tryptophan by the Forster mechanism. The spectral distributions of absorption and emission satisfy the requirements for this kind of process, although its occurrence in proteins remained controversial for many years (Konev, 1967). Longworth (1968) has concluded that energy transfer from tyrosine to tryptophan occurs in ribonuclease T.

The transfer of excitation energy is remarkably efficient, as is reflected by the only minor variation at different excitation wavelengths of the quantum yield based on total absorbance. For excitation at 270 nm, where the fractions of the incident radiation absorbed by tyrosine and tryptophan are approximately equal, approximately 36% of the total fluorescence energy originates indirectly from tyrosine. If the real energy yield of the tryptophan residues is assumed to be constant and equal to the value for excitation at 295 nm, the relative fluorescence energy yield for tyrosine transfer to tryptophan is 0.36/(1–0.36), or 0.56. This is equal to the ratio of fluorescence energy yields for radiation absorbed by tyrosine and by tryptophan.

The variation with excitation wavelength of the spectral distribution of fluorescence can also be tentatively explained in terms of radiationless transfer, whose efficiency will vary for the different tryptophan residues according to their separation from tyrosine. The blue shift of the fluorescence band with decreasing excitation wavelength suggests that transfer increases the relative contribution of the tryptophans which are in a nonpolar environment. Crystallographic studies indicate that four of the five tryptophans are in close proximity to one or more tyrosines (J. Drenth, 1969, personal communication). The results of Figure 8 indicate that, even under the most favorable conditions, the contribution from tyrosine emission appears insufficient to blue shift the maximum of the fluorescence envelope to the extent observed (Figure 5).

The fluorescence of the tyrosine residues themselves in native papain is too weak to be readily detected. This is presumably, due, at least in part, to the deactivation of the first excited singlet state of tyrosine by radiationless energy transfer to tryptophan. In 6 M guanidine an important tyrosine contribution appears in the emission spectrum. This may reflect a considerable reduction in the efficiency of energy transfer, as a result of the unfolding which occurs in this denaturing solvent. In addition, the loss of secondary and tertiary structure would eliminate some quenching processes, such as tyrosine–carboxylate hydrogen bonding, which may occur in the native conformation.

At pH 11.5 in water, about two-thirds of the tyrosine residues are ionized (Glazer and Smith, 1961). The pronounced decrease in quantum yield under these conditions can be explained in terms of energy transfer from tryptophan to tyrosinate residues (Steiner and Kolinski, 1968).

An expecially interesting feature of the luminescence at 100°K is the splitting of the fluorescence band at high resolution into two partially resolved peaks. Since tryptophan and its oligopeptide derivatives do not show this splitting under the same conditions, it is not likely to be due to any fine structure which is not resolved at room temperature. The failure of any significant tyrosine phosphorescence to appear and the absence of tyrosine emission at room temperature both are in opposition to the assignment of one of the peaks to tyrosine fluorescence. A possible, although conjectural, explanation is that contributions from two classes of tryptophan residue have been resolved in the rigid glass at low temperatures. In terms of the proposed three-dimensional structure of papain (Drenth *et al.*, 1968), one conjectural model is that the two classes may correspond to exposed and buried tryptophans. It may be possible to test this idea by studies of the effects of selective chemical modification of the tryptophans. The loss in organized structure occurring in 6 M guanidine may be responsible for the elimination of the peak splitting in this medium. The selective quenching of the short-wavelength component of fluorescence at pH 11.5 is perhaps explainable in terms of more favorable tryptophan–tyrosinate energy transfer due to greater overlap between tryptophan emission and tyrosinate absorption (Lehrer and Fasman, 1967).

The higher F:P ratio for papain, as compared with tryptophan, indicates that the rate of intersystem crossing is curtailed in the protein as a consequence of the geometrical constraints imposed on the residues by the native conformation and the specific environmental features which influence the properties of individual tryptophans. The unfolding of the protein by 6 M guanidine abolishes these structural features and results in a decrease in F:P. The appearance of tyrosine phosphorescence under these conditions parallels the appearance of tyrosine fluorescence at room temperature.

There is, of course, no proof that the conformation of native or of guanidine-denatured papain in 50% ethylene glycol at 100°K is equivalent to those at room temperature in the absence of glycol. The similarity of the luminescence spectra observed in 50% glycol and in 0.5% sucrose at 100°K suggests that glycol itself does not cause major changes in emission properties under these conditions. It is also clear from shifts in the low-temperature fluorescence emission that major conformational changes persist in 6 M guanidine in the former medium at 100°K. It is certainly conceivable that the relative free energies of the various possible conformations might not change greatly as the system sought lower entropic states at 91°K, provided that such entropy changes made only a minor contribution to the final free energy.

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