Transfer of Singlet Energy within Trypsin†

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ABSTRACT: Transfers of singlet energy within trypsin were investigated by measuring the fluorescence absorption anisotropy of its tryptophan residues. A ratio of the anisotropy of trypsin to that for *N*-acetyl-L-tryptophanamide was determined between 306 and 250 nm. The ratio had an average value of 0.7, whether the trypsin anisotropy was measured at 228 or 296 K. However, trypsin dissolved in 5 M guanidine hydrochloride showed little fluorescence depolarization at 228 K (the anisotropy ratio was approximately equal to 0.9). Thus, there is an extensive conformation-dependent energy transfer between tryptophans in trypsin. The ratio of anisotropies of tyrpsin at 304–270 nm was used to estimate energy transfer from tyrosine to tryptophan. Ratios of 1.8 and 1.7 were obtained at 296 K for the native and guanidinium-unfolded enzyme, respectively. The comparable value for *N*-acetyl-L-tryptophanamide was 1.7. This indicates that there is little transfer from tyrosine to tryptophan in trypsin at 296 K. As confirmation, the excitation wavelength dependencies of the indole fluorescence quantum yield were the same for native and unfolded trypsin. When experiments were performed at 228 K, the 304–270-nm anisotropy ratios were 2.6 for native and 2.1 for unfolded trypsin at pH 2. This indicates that the efficiency of energy transfer from tyrosine to tryptophan increases at low temperatures. A photochemical source of error in the quantitation of the efficiency of energy transfer from tyrosine to tryptophan is also described.

Since the late 1950s photon biophysicists have been fascinated with the possibility that the detection and quantitation of electronic energy transfer among aromatic residues could provide new insights about the structure of proteins (Stryer, 1978). Two main methods for studying transfer have evolved: one method is restricted to transfer between dissimilar residues; a second method is of general application to any transfer step. Method 1 is a demonstration of a sensitization of the acceptor emission by the donor absorption. The basis of the method is to determine the relative fluorescence quantum yield of the acceptor at different exciting wavelengths (λ_{ex})[1]. Comparison with a standard absorption spectrum of donor, acceptor, and, in the case of multiple donor and/or acceptors, donor–acceptor combination then allows direct estimation of the extent of donor sensitization. Method 2 is measurement of polarization of emission to show that there is an anisotropy decrease. Since no two residues will in general have identical coordinates in the protein structure, absorption by one residue and emission by another will cause a depolarization of the emission compared to absorption and emission by the same residue. Observation of depolarization of emission from a protein-bound fluorophore compared to the free fluorophore under conditions where motional depolarization processes are eliminated immediately implies the presence of electronic energy transfers within the protein (in the case that there is no shift of overlapping bands of different polarization in the protein compared with free solution). The method applies equally to transfer between similar and dissimilar residues (homo- and heterotransfer). Kinetic analysis is also feasible but has only been rarely successful because of instrumental limitations (Longworth, 1971).

The purpose of the present work is to illustrate how fluorescence absorption anisotropy of tryptophan (Trp) residues may be used to study transfer of singlet energy within trypsin.

Experimental Section

Materials. α- and β-trypsin were prepared by equilibrium column chromatography on sulfoethyl-Sephadex from a sample obtained from Worthington Biochemical Corp. (W-trypsin) and were characterized as previously described (Ramachandran & Ghiron, 1978, and references therein). Cyclohexylcarboxamidine hydrochloride, a competitive inhibitor of trypsin, was synthesized as described (Ramachandran & Ghiron, 1978). It is used to stabilize trypsin and to inhibit autolysis at neutral and alkaline pH values. Guanidine hydrochloride (Gdn·HCl) and glycerol, spectral grade, were obtained from Mann Research Labs and Matheson Coleman and Bell, respectively.

Methods. Ultraviolet absorption measurements were performed by utilizing a Zeiss Model PM AII spectrophotometer. The absorption measurements were done with slit widths set so that the band pass of 3 nm was the same as employed in the excitation monochromator of the fluorometer. The fluorescence excitation and emission spectral data were obtained by utilizing an instrument similar in design to that described by Luk (1971).

The fluorescence polarizations were measured with an instrument built by Knopp et al. (1969). The exciting light was monochromatic. For *N*-AcTrpNH, the long-wavelength fluorescence, originating solely from the ^1L_a state, was isolated with cutoff glass color filters that passed light of wavelengths greater than either 320 or 335 nm (Valeur & Weber, 1977). For native trypsin, as can be seen in Figure 4, the fluorescence isolated using either the 320- or 335-nm filter is exclusively from Trp residues. For the guanidinium-denatured enzyme, this is only completely true when the 335-nm filter is used. The fluorescence λ_{max} of native trypsin in water is 335 nm and is blue-shifted slightly to 333 nm when the enzyme is dissolved in an equivolume glycerol–water mixture. When the latter preparation was cooled to 228 K, an additional blue shift of 3 nm is observed. The analogous values for guanidinium-

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[1] Abbreviations used: tryptophan, Trp; tyrosine, Tyr; *N*-acetyl-L-tryptophanamide, *N*-AcTrpNH or NATA; wavelength, λ; anisotropy, r; absorbance, A; temperature, T; guanidine hydrochloride, Gdn·HCl; emission, em; excitation, ex; maximum, max; Worthington trypsin, W-trypsin.

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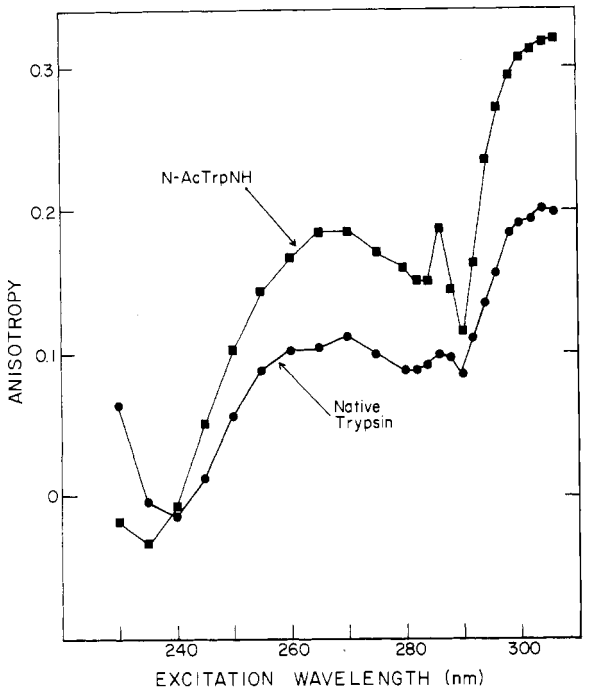

FIGURE 1: Fluorescence absorption anisotropy spectrum of *N*-AcTrpNH and trypsin. *N*-AcTrpNH (■): solvent, equivolume glycerol–water; temperature, 233 K; fluorescence isolated with a cutoff filter with a 335-nm pass. α-Trypsin (●): solvent, 10^{-3} M HCl in water; temperature, 296 K; $A^{280} = 0.8$; fluorescence isolated with a cutoff filter with a 335-nm pass.

denatured trypsin are 354, 346, and 335 nm, respectively. Polarization was determined at right angles to the excitation direction with two analyzer prisms and two matched photomultipliers (a Cornu polarimeter). The ratio of the light intensity (I) was directly determined electronically through a voltage-to-frequency conversion and a ratio frequency scaling for the two polarizations; therefore, the emission anisotropy (r) is given by $r = [(R_V/R_H) - 1]/[(R_V/R_H) + 2]$, where R equals $I_{\parallel}/I_{\perp}$ for vertical (V) and horizontal (H) excitation. Any residual imbalance in the detection efficiency of the two observation channels is thus eliminated.

The average precision of the anisotropy measurement is 6%. When the ratio of two anisotropies is determined, for example, r^{300}/r^{270} or $r^{\text{trypsin}}/r^{\text{NATA}}$, an average precision of 13% is achieved.

A threefold variation in either trypsin or *N*-AcTrpNH concentration did not influence the measured anisotropy spectrum. A sample of native trypsin dissolved in an equivolume glycerol–water mixture was repeatedly cooled to 228 K and/or held at that temperature for several hours. No significant loss of catalytic activity, using benzoyl-L-arginine ethyl ester as a substrate, was detected (Schwert & Takenaka, 1955).

The excitation wavelength dependence of the indole fluorescence quantum yield was determined in the following manner. The trypsin or Trp monomer sample was prepared such that its absorbance (A) was ~2 at 280 nm. The A of the sample was then measured at intervals in the 310–240-nm range. The sample was then diluted by a factor of 10, and the fluorescence intensity, at the appropriate emission (em) λ, was measured at excitation (ex) λ values in the 310–240-nm range. The fluorescence intensity divided by the A for a given excitation λ is then a relative measure of the fluorescence quantum yield. This ratio has a precision of 10% at λ values ≤295 nm. As the A of the sample diminishes at λ values >295 nm, the precision also decreases.

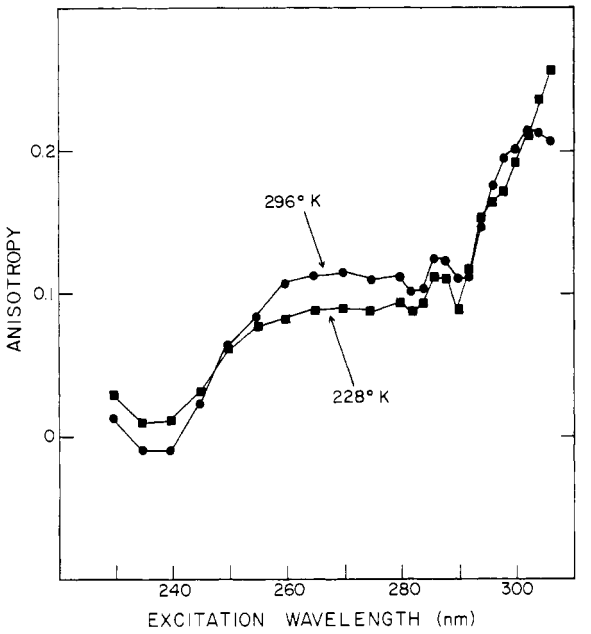

FIGURE 2: Fluorescence absorption anisotropy spectrum of α-trypsin dissolved in equivolume glycerol–water (pH 2 + inhibitor) at a temperature of 296 (●) or 228 K (■). The fluorescence was isolated with a cutoff filter with a 335-nm pass.

Results and Interpretation

Fluorescence Anisotropy of N-AcTrpNH. The fine-structured fluorescence absorption anisotropy spectrum of *N*-AcTrpNH dissolved in an equivolume glycerol–water mixture at 233 K is shown in Figure 1. The range of r values obtained, 0.32 to −0.034, is less than the theoretically maximum of 0.4 to −0.20. A number of hypotheses have been proposed to explain this discrepancy (Dale & Eisinger, 1975). The spectrum may be interpreted on the basis of three transitions: two overlapping approximately perpendicular ones in the λ range 250–306 nm and another with λ_{max} = 235 nm (Valeur & Weber, 1977).

The ratios of the r values at either 304 or 300 nm to that at 270 nm are 1.72 and 1.66, respectively.[2] These values will subsequently be used to assess the extent of energy transfer from tyrosine (Tyr) to Trp within trypsin.

Fluorescence Anisotropy of Trypsin. The fluorescence absorption anisotropy spectrum of trypsin, shown in Figure 1, is similar in form to that of *N*-AcTrpNH, having a maximum positive r value in the 300–306-nm region and a maximum negative one at ~240 nm. However, as might be expected for a multitryptophan-containing native protein, the minimum at 290 nm and the fine structure are less pronounced. The major difference is the diminished range of r values (0.20 to −0.014) obtained compared to that observed for *N*-AcTrpNH at 233 K. At 296 K, an approximate fivefold increase in viscosity, by addition of glycerol to the water solvent, has only a minor effect on the spectrum and r value range (see Figure 2). This indicates that the rotational motion of trypsin can account for only a small portion of the depolarization observed.

The measurement was repeated at 228 K in order to abolish the motion of individual residues. As can be seen in Figure 2, the general shape of the spectrum remains unchanged. The range of r values extends from 0.25 at 305 nm to only +0.01

[2] Data for both λ_{ex} = 300 and 304 nm are presented in order to show that qualitatively similar results can be obtained in a wavelength region which is prone to errors because it is near the onset of light passage by the color filter.

Table I: Fluorescence Absorption Anisotropy of Trypsin and *N*-AcTrpNH[a]

type-pH-filter (nm)	solvent	T (K)	r^{304}	r^{300}	r^{270}	r^{304}/r_{NATA}^{304}	r^{300}/r_{NATA}^{300}	r^{304}/r^{270}	r^{300}/r^{270}
NATA-320	G	233	0.320	0.304	0.183			1.75	1.66
NATA-320	G	233	0.318	0.306	0.190			1.66	1.60
NATA-335	G	233	0.318	0.307	0.185			1.72	1.66
β-3-320	H	296	0.227	0.204	0.107	0.71	0.67	2.12	1.91
	G	296	0.241	0.223	0.118	0.76	0.73	2.04	1.89
	G	233	0.237	0.204	0.085	0.75	0.67	2.79	2.40
α-3-320	M, H	296	0.125	0.104	0.060	0.39	0.34	2.08	1.73
	H	296	0.235	0.200	0.110	0.74	0.65	2.14	1.82
	G	296	0.252	0.221	0.120	0.79	0.72	2.10	1.84
	G	233	0.242	0.206	0.091	0.76	0.67	2.66	2.26
α-3-335	H	296	0.202	0.191	0.112	0.64	0.62	1.80	1.71
	G	296	0.233	0.219	0.118	0.73	0.72	1.98	1.86
	G	251	0.229	0.198	0.088	0.72	0.65	2.60	2.25
	G	231	0.246	0.198	0.091	0.77	0.65	2.70	2.18
α-2-335	G, I	296	0.215	0.202	0.115	0.68	0.66	1.87	1.76
	G, I	228	0.239	0.194	0.091	0.75	0.63	2.63	2.13
α-8-335	G, I	296	0.223	0.211	0.123	0.70	0.69	1.81	1.72
	G, I	219	0.261	0.208	0.083	0.82	0.68	3.15	2.51
α-10-335	G, I	296	0.206	0.208	0.125	0.65	0.68	1.65	1.66
	G, I	226	0.274	0.219	0.099	0.86	0.72	2.77	2.21
α-2-335	M, G	296	0.200	0.204	0.118	0.63	0.67	1.70	1.73
	M, G	228	0.277	0.279	0.130	0.87	0.91	2.13	2.15
α-10-335	M, G	296	0.211	0.200	0.128	0.66	0.66	1.65	1.56
	M, G	223	0.289	0.281	0.162	0.91	0.92	1.78	1.74

[a] Abbreviations used: (α) α-trypsin; (β) β-trypsin; (H) water; (G) equivolume glycerol-water; (I) inhibitor; (M) 5 M Gdn·HCl; (NATA) *N*-AcTrpNH.

at 235 nm. At λ values ≥295 nm, the r values are very similar to those obtained for this preparation at 296 K. However, there is an approximately constant decrease in r values at excitation λ values ≤295 nm.

The spectrum of the guanidinium-denatured (5 M Gdn·HCl) trypsin at 228 K is shown in Figure 3. Data for *N*-AcTrpNH and the native enzyme, obtained under the same experimental conditions, are also included for the purpose of comparison. The range of r values (0.29 to −0.014) and the prominence of the fine structure are almost as great as those obtained for *N*-AcTrpNH. To aid in interpreting these results, we plotted these data as the ratio of the anisotropy of trypsin, either native or denatured, to that of *N*-AcTrpNH vs. the excitation wavelength in the insert in Figure 3. It is clear that the ratio for native enzyme is much smaller than that for the denatured enzyme at all excitation λ values ≥250 nm.

Table I summarizes fluorescence absorption anisotropy data of trypsin determined for different forms, at different pH values, in the presence or absence of either a competitive inhibitor or 5 M Gdn·HCl, using either 320- or 335-nm cutoff filters and different temperatures and solvent viscosities. Data for *N*-AcTrpNH are also included for comparative purposes. It is evident that changing the cutoff filters or the form of the enzyme does not significantly change the spectrum. Additional features to be noted are the following. The ratio of the anisotropy of trypsin at 304 or 300 nm to that of *N*-AcTrpNH at 233 K (r^{304}/r_{NATA}^{304}, r^{300}/r_{NATA}^{300}) is less than 1 under all experimental conditions. As expected, the smallest value of either ratio is obtained with the denatured protein in aqueous solution at 296 K (due to rotational depolarization). Values approaching 1 are obtained for denatured trypsin at ~225 K in an equivolume glycerol–water solvent. For native trypsin, at 296 K, these ratios exhibit a slight, statistically insignificant, increase with an approximately fivefold increase in viscosity. Also, these ratios are temperature independent at all reported pH values when native trypsin is in an equivolume glycerol–water solvent. The ratio of the anisotropy of trypsin at either 300 or 304 nm to that at 270 nm (r^{300}/r^{270}, r^{304}/r^{270})

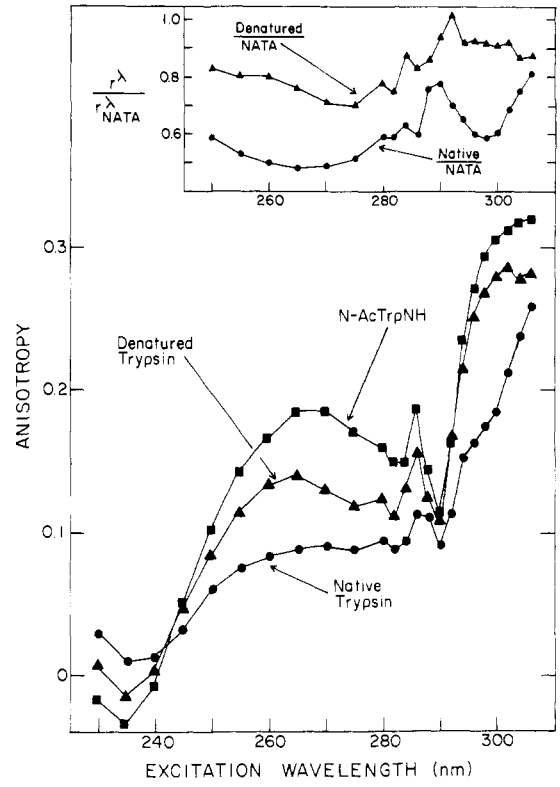

FIGURE 3: Fluorescence absorption anisotropy spectrum of denatured (5 M Gdn·HCl) trypsin (▲) and native trypsin (●): solvent, equivolume glycerol–water (pH 2); temperature, 228 K; fluorescence isolated with a cutoff filter with a 335-nm pass. Data for *N*-AcTrpNH (NATA) (■) are also shown for the purpose of comparison. Insert: anisotropy ratio, $r^{\lambda}/r_{NATA}^{\lambda}$, for denatured trypsin–NATA (▲) and native trypsin–NATA (●).

Table II: Relative Indole Fluorescence Quantum Yield

type of molecule and conditions	em λ(nm)	excitation wavelength (nm)													
		305	300	295	290	285	280	275	270	265	260	255	250	245	240
Trp, H_2O	350		1.20	1.11	1.00		1.00		1.00		0.99		0.93	0.79	0.65
Trp, 6 M Gdn·HCl, pH 3, H_2O	350		1.38	1.06	1.00		0.98		0.97		0.92		0.92	0.78	0.64
W-trypsin, H_2O, pH 3	332	1.01	0.98	1.05	1.00		0.81		0.76		0.66	0.54	0.37	0.25	0.14
W-trypsin, 6 M Gdn·HCl, pH 3, H_2O	354	0.97	1.03	1.02	1.00		0.79		0.71		0.64	0.51	0.37	0.22	0.10
W-trypsin, 341 K, pH 3, H_2O	354	1.30	1.30	1.08	1.00		0.81		0.76		0.73	0.65	0.55		0.17
β-trypsin, pH 3, H_2O	332	1.81	1.41	1.24	1.00	0.86	0.79	0.73	0.71	0.66	0.64	0.55	0.45	0.31	0.19
α-trypsin, pH 3, H_2O	332	1.20	1.43	1.23	1.00	0.86	0.78	0.73	0.70	0.67	0.62	0.53	0.46	0.28	0.18
β-trypsin, pH 3, 6 M Gdn·HCl	360	0.73	0.87	0.98	1.00	0.85	0.80	0.75	0.73	0.77	0.68	0.61	0.49	0.34	0.20

is substantially greater than that obtained for *N*-AcTrpNH at 233 K at all pH values for the native enzyme at ~230 K. When the enzyme is guanidinium-denatured at pH 2 this enhancement is, at most, partially abolished, whereas at pH 10 it is completely eliminated. At room temperature, the r/r^{270} ratios for native trypsin are marginally enhanced at acid pH values and not at all at alkaline pH values. At room temperature, the magnitude of these ratios, for denatured trypsin, at all pH values is the same as that obtained for *N*-AcTrpNH at 233 K.

Excitation Wavelength Dependence of the Trypsin Fluorescence Quantum Yield. The fluorescence emission spectra of native and guanidinium-denatured α-trypsin are shown in Figure 4. The difference in the spectra obtained upon 295-nm excitation, where only Trp residues absorb, and those obtained upon 280-nm excitation, where both Trp and Tyr residues absorb, is the tyrosin contribution to the emission. Support for this contention comes from the observation that when the pH of the solution is adjusted to 10, this difference disappears because the ionized Tyr residues of the *denatured* protein fluoresce with a greatly reduced yield and in a different wavelength region (Ramachandran & Ghiron, 1978). Thus, it is evident that the fluorescence monitored at 332 nm for the native enzyme and at 354 nm for the denatured enzyme is entirely from Trp residues. The fluorescence intensity monitored at these wavelengths was then used in the determination of the relative fluorescence quantum yield of the native and denatured proteins as a function of excitation λ. These data are reported in Table II along with those for the Trp monomer. As can be seen, the relative fluorescence yield of the monomer is excitation λ independent from 295 to 250 nm. However, the yield is reduced at excitation λ values <250 nm. The relative fluorescence yield of trypsin is found to diminish progressively by the same proportion, as the excitation λ is decreased from 295 to 240 nm, irrespective of the protein's conformational state and of the denaturing process.

The excitation spectrum of heat-denatured (341 K) trypsin is superimposable on the one obtained for the native enzyme. This indicates that heat denaturation does not perturb the absorption spectrum of the *fluorescent* Trp residue(s) of trypsin. In contrast, guanidinium denaturation has a significant perturbing effect since the excitation spectrum is blue-shifted and exhibits more prominent fine structure compared with that of the native enzyme. However, the environment of these residues must not be completely "normalized" since the excitation spectrum of the guanidinium-denatured protein is red-shifted relative to that of the Trp monomer.

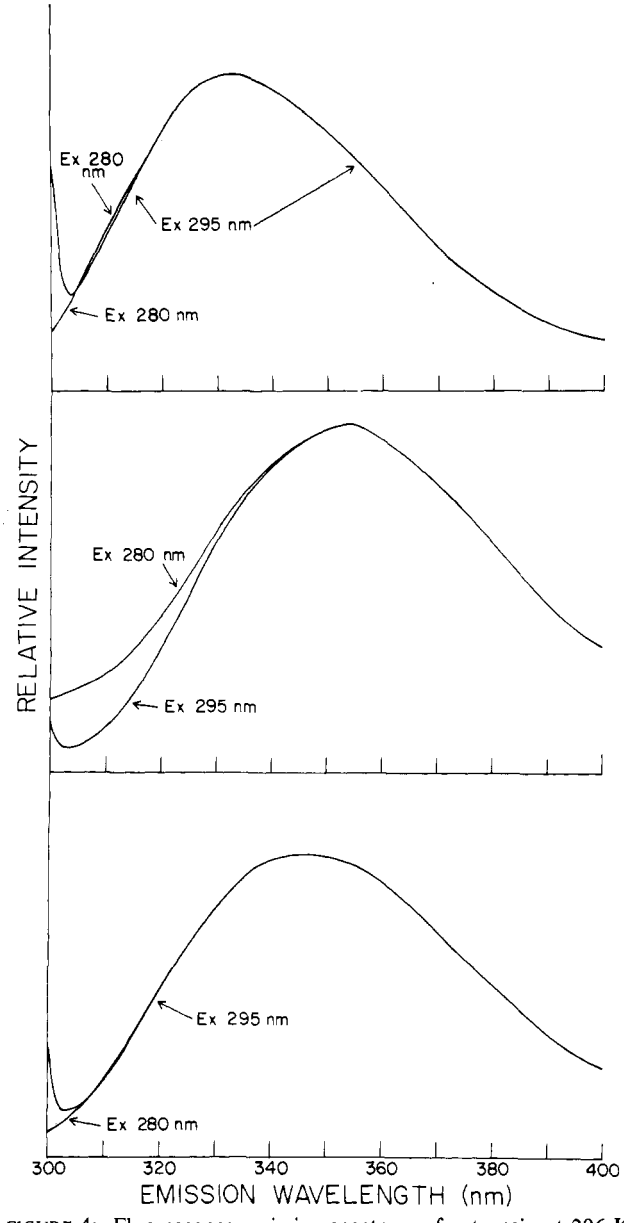

FIGURE 4: Fluorescence emission spectrum of α-trypsin at 296 K, excited (ex) at 280 and 295 nm. (Upper panel) Solvent, 10^{-3} M HCl in water; (middle panel) solvent, 6 M Gdn·HCl in water (pH 3); (lower panel) solvent, 5 M Gdn·HCl in equivolume glycerol–water (pH 10).

Discussion

The reduced magnitude of r (expressed as r/r_{NATA} in Table I and Figure 3) obtained with 295–306-nm excitation, where Trp is the only absorbing luminescent residue, may be a reflection of energy transfer among the Trp residues of native trypsin and/or motional depolarization effects. The observation that the r/r_{NATA} ratios are only slightly increased with an approximately fivefold increase in viscosity at 296 K eliminates the possibility that the rotational motion of the entire trypsin molecule is the major cause of the observed depolarization. Abolishing the motion of individual side chains by lowering the temperature to ~228 K was effective in eliminating most of the depolarization in denatured but not

in native trypsin. Thus, it must be concluded that energy transfer among the Trp residues of native trypsin is the cause of the observed depolarization.

The Förster critical distance, R_0, has been estimated by Eisinger et al. (1969) to be 7–9 nm for energy transfer between tryptophans in a protein at 300 K. Crystallographic data (Stroud et al., 1974) indicate that Trp^{51} and Trp^{237} are the only Trp residues, among the six possible pairings, with a sufficiently short interresidue distance to permit energy transfer. On the basis of these considerations and a favorable κ^2 [the orientation factor in the Förster equation; see Eisinger et al. (1969)] value, it is suggested that this pair is the one participating in the observed homotransfer.

However, if energy transfer were the sole factor determining the magnitude of r, the r/r_{NATA} ratio should have decreased as the temperature was reduced rather than remain approximately constant. This is because the expected increase in Trp fluorescence yield and the emission blue shift observed with decreasing temperature should have significantly enhanced the transfer efficiency. To account for the independence of r/r_{NATA} with temperature, it is postulated that the decrease is cancelled by a repolarization effect caused by a reduction in indole side-chain motion, and/or a conformation change leading to a decrease in the value of κ^2, and/or an increase in the interresidue (Trp) separation.

The anisotropy ratios r^{304}/r^{270} or r^{300}/r^{270} may be used to estimate energy transfer from Tyr to Trp. The magnitudes of either ratio for the native and unfolded enzyme at 296 K are comparable to the equivalent value for *N*-AcTrpNH at 233 K. This indicates that there is little energy transfer from Tyr to Trp. This conclusion is supported by the observation that the excitation wavelength dependence of the indole fluorescence quantum yield is the same for the native and unfolded forms of this enzyme. The magnitude of r/r^{270} is significantly increased for native trypsin when the measurements are performed at 228 K. Since a reduction in indole side-chain motion cannot account for this increase, it must be concluded that it is due to an increase in the efficiency of energy transfer from Tyr to Trp. This enhancement is probably caused by an increase in the Tyr (donor) fluorescence yield in combination with a blue shift in the Tyr emission spectrum (the latter producing an increase in the magnitude of the donor–acceptor overlap integral). Changes in Tyr residue mobility and/or conformation may also be involved but are probably not as important because an enhancement is also observed with denatured trypsin at pH 2. Additional evidence that the latter enhancement is due to energy transfer from Tyr to Trp comes from the observation that it disappears concomitant with Tyr ionization at pH 10. We suggest that residues 234 and 237 are participating in the transfer of singlet energy in the denatured protein because they form the closest Tyr–Trp pair in the primary sequence of trypsin (Stroud et al., 1971). In any case, in contrast to a previous report (Kronman & Holmes, 1971), it is concluded that transfer of singlet energy from Tyr to Trp occurs in native trypsin with appreciable efficiency only at low temperatures. Consequently, intramolecular quenching processes other than energy transfer from Tyr to Trp must be invoked to account for the low Tyr fluorescence yield observed in native trypsin at 296 K.

The data shown in Table II highlight some of the sources of error inherent in the quantitative estimation of energy transfer from Tyr to Trp in proteins using method 1. Knowledge of the relative contribution of Tyr, Trp, and cystinyl (if present) residues to the total absorbance of the protein in the 310–240-nm region is needed, but is usually only known to a first approximation. The cystinyl contributon has often been ignored even though it may be appreciable at λ values >295 nm and in the 260–240-nm region. Even if reasonable estimates are made for these parameters, the calculated efficiency, e, of transfer from Tyr to Trp, as defined by the formula $\Phi^{ex\,\lambda} = \Phi^{295}(f_{Trp}^{ex\,\lambda} + (e)f_{Tyr}^{ex\,\lambda})$, where Φ is the fluorescence quantum yield and f is the fractional absorbance [see Eisinger et al. (1969) for details], is found to decrease with decreasing excitation λ and ultimately becomes negative. This result may be explained by noting that the decrease in Trp monomer yield upon excitation into the second absorption band is caused by a light-induced oxidation of the indole ring (Tatischeff & Klein, 1976; Steen & Kongshang, 1976). Similar reactions occur in both native and unfolded trypsin as evidenced by a dramatic increase in the *average* indole photolysis quantum yield with decreasing illumination λ (Sellers & Ghiron, 1973; Ramachandran & Ghiron, 1979). This source of error cannot be easily taken into account because estimates of the photolysis yield of individual *fluorescent* indole side chains are difficult to obtain in multitryptophan-containing proteins.

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