Zahn, H., Gliemann, J., & Gammeltoft, S. (1976) *Nature (London) 259*, 371–373.
Scheraga, H. A. (1979) *Acc. Chem. Res. 12*, 7–14.
Shanghai Insulin Group (1978) *Sheng Wu Hua Hsueh Yu Sheng Wu Wu Li Hsueh Pao 9*, 169–173.
Steinberg, I. Z., & Scheraga, H. A. (1963) *J. Biol. Chem. 238*, 172–181.
Tanford, C. (1970) *Adv. Protein Chem. 24*, 1–95.
Waelbrock, M., Van Obberghen, E., & De Meyts, P. (1979) *J. Biol. Chem. 254*, 7736–7740.

Time-Resolved Fluorescence and Anisotropy Decay of the Tryptophan in Adrenocorticotropin-(1–24)†

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ABSTRACT: The direct time-resolved fluorescence anisotropy of the single tryptophan residue in the polypeptide hormone adrenocorticotropin-(1–24) (ACTH) and the fluorescence decay kinetics of this residue (Trp-9) are reported. Two rotational correlation times are observed. One, occurring on the subnanosecond time scale, reflects the rotation of the indole ring, and the other, which extends into the nanosecond range, is dominated by the complex motions of the polypeptide chain. The fluorescence lifetimes of the single tryptophan in glucagon (Trp-25) and the 23–26 glucagon peptide were also measured. In all cases the fluorescence kinetics were satisfied by a double-exponential decay law. The fluorescence lifetimes of several tryptophan and indole derivatives and two tryptophan dipeptides were examined in order to interpret the kinetics. In close agreement with the findings of Szabo and Rayner [Szabo, A. G., & Rayner, D. M. (1980) *J. Am. Chem. Soc. 102*, 554–563], the tryptophan zwitterion exhibits emission wavelength dependent double-exponential decay kinetics. At 320 nm $\tau_1 = 3.2$ ns and $\tau_2 = 0.8$ ns, with $\alpha_1 = 0.7$ and $\alpha_2 = 0.3$. Above 380 nm only the 3.2-ns component is observed. By contrast the neutral derivative *N*-acetyltryptophanamide has a single exponential decay of 3.0 ns. The multiexponential decay kinetics of the polypeptides are discussed in terms of flexibility of the polypeptide chain and neighboring side-chain interactions.

Polypeptide hormones in solution behave in many cases as fairly flexible chains. For example, before the recent developments in time-resolved fluorescence emission anisotropy [for review see Badea & Brand (1979)], suggestive but indirect evidence for dynamic motions in these molecules was obtained from a variety of physical techniques, including circular dichroism, optical rotatory dispersion, and steady-state fluorometry. In an elegant series of papers, Edelhoch and co-workers (Edelhoch & Lippoldt, 1969; Bernstein et al., 1969; Bornet & Edelhoch, 1971; Schneider & Edelhoch, 1972a,b) evaluated the conformational properties of model peptides and polypeptide hormones such as glucagon, adrenocorticotropin, parathyroid hormone, mellitin, and gastrin, and certain of their peptide fragments. The results confirm the notion that these are indeed flexible molecules, some of which can be induced to acquire secondary structure under a variety of circumstances. The polarization of the tryptophan fluorescence also suggested that the indole group was rotating at rates exceeding that expected if the hormones were to be considered as tumbling rigid spheres. When adrenocorticotropin and glucagon, each of which has a single tryptophan residue, were compared, it appeared that whereas the indole moiety was essentially unhindered in adrenocorticotropin, the results for glucagon suggested some steric restriction of the side chain. The NMR[1] spectrum of glucagon peptides is consistent with this notion (Bundi et al., 1976).

With the advent of time-resolved fluorescence anisotropy measurements, direct evidence was obtained for subnanosecond motions of tryptophans in several proteins (Munro et al., 1979). If it is possible to resolve the time-dependent motions in the polypeptides hormones, then a new avenue exists for exploring structure–function relationships. In this paper we present the results of one such test case, that of a biologically functional synthetic analogue of adrenocorticotropin, ACTH-(1–24) tetracosapeptide. Our results show that the single tryptophan residue rotates on the subnanosecond time scale, and furthermore this motion is the dominant factor responsible for the rapid depolarization of the tryptophan fluorescence. In addition, we find that the decay kinetics of the tryptophan fluorescence are complex and fit a double-exponential decay law which is essentially wavelength independent in neutral pH buffer. For comparison, we have measured the fluorescence lifetimes of the single tryptophan in glucagon and in its four-peptide fragment Val-Gln-Trp-Leu, which includes residues 23–26 of the 29 amino acid polypeptide chain [cf. Bromer et al. (1956)]. The decay data obtained with the polypeptides are compared with those obtained from simpler tryptophan systems under identical experimental conditions. The latter data, which include the decay kinetics of the tryptophan zwitterion, agree with the findings of Szabo &

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[1] Abbreviations used: ACTH, adrenocorticotropin hormone-(1–24); ADH, horse liver alcohol dehydrogenase; NATA, *N*-acetyltryptophanamide; NMR, nuclear magnetic resonance; ODMR, optically detected magnetic resonance.

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Rayner (1980) and Rayner & Szabo (1978).

Experimental Procedures

Materials. Synthetic adrenocorticotropin-(1–24) (ACTH) was a gift of Dr. W. Rittel of CIBA-Geigy, Ltd., Basel, Switzerland. Crystalline glucagon was obtained from Elanco Products and was purified by ion-exchange chromatography (Ross et al., 1979). A short fragment of human parathyroid hormone which has the same sequence as the 23–26 peptide in glucagon (Val-Gln-Trp-Leu) was donated by Professor K. Wüthrich of the Swiss Fereral Institute of Technology, Zurich, Switzerland. Indole, 3-methylindole, tryptamine hydrochloride, L-tryptophan, and *p*-terphenyl were purchased from Alrich Chemical Co. *N*-Acetyl-L-tryptophanamide, *N*-acetyl-L-tryptophan, L-tryptophanamide, L-tryptophan, and tryptamine hydrochloride were Vega products. Glycyl-L-tryptophan and tryptophyl-L-glycine were supplied by Research Plus. 3-Indolepropionic acid, 1,1′-binaphthyl, and Spectrograde cyclohexane were obtained from Eastman. Ultrapure sucrose was a Schwarz/Mann product and was used without further purification. Other chemicals were reagent grade.

Spectroscopy. Absorption measurements were made with a Cary 219 spectrophotometer. At neutral pH the absorption spectrum in the region above 273 nm of the polypeptide hormones glucagon and ACTH is essentially that of two model compounds, *N*-acetyl-L-tryptophanamide (NATA) and *N*-acetyl-L-tryosinamide, in a mole ratio of 1:2. Therefore, the hormone concentrations were determined by using an extinction coefficient at 280 nm of 7.78×10^3 cm^2 mmol^{-1} (Ross, 1976).[2] The absorbance of the 23–26 peptide, which contains only tryptophan, was accordingly that of NATA. For fluorescence lifetime measurements of the other indole-containing compounds, sample solutions were made at optical densities of between 0.1 and 0.25 at 295 nm, where the contribution of tyrosine absorption is negligible. Details concerning specific experiments are given in the figures, tables, and text. Aqueous solutions were not degassed.

Uncorrected steady-state fluorescence spectra, at constant temperature, were measured on a Perkin-Elmer MPF-4 spectrofluorometer, equipped with a quartz quarter-wave plate to provide natural excitation light. Typically, 4-nm bandwidths were used for excitation and emission.

The instrument used to obtain nanosecond fluorescence decay and fluorescence anisotropy decay data was constructed in this laboratory (Easter et al., 1976). The theory and practical aspects of its operation have been reviewed by Badea & Brand (1979). Excitation was at 295 nm. The characteristics of the nitrogen flash lamp (Photochemical Research Associates) and the narrow band-pass filter (Baird-Atomic) gave an effective bandwidth of 10.5 nm. The time response characteristics of the phototube [56 DUVP/03 (Amperex)] were determined with a single-exponential standard, such as *p*-terphenyl or 1,1′-binaphthyl in cyclohexane (Lewis et al., 1973; Wahl et al., 1974). Collection of the lamp, sample, and standard was typically over a period of 2 h, with 5-min alternation of the cuvettes. Optical configurations which satisfy the "magic angle" geometry provided measurement of the fluorescence lifetimes without polarization bias [see Badea & Brand (1979)]. Steady-state anisotropy values were obtained as outlined by Paoletti & Le Pecq (1969) [see also Azumi & McGlynn (1962)].

[2] The concentrations of glucagon used in our experiments (see Table II) were optimized to avoid aggregation. This particular problem has been discussed in more detail by Ross et al. (1977).

Analysis of Fluorescence and Anisotropy Decay. According to Perrin (1934, 1936), for isotropic depolarization of a spherical rotor, the emission anisotropy is described by

$$r_0/\langle r\rangle = 1 + \tau/\phi \tag{1}$$

where τ is the fluorescence lifetime, ϕ is the rotational correlation time, r_0 is the zero-point anisotropy, and $\langle r\rangle$ is the steady-state anisotropy (Jablonski, 1957, 1960), defined by the relationship

$$\langle r\rangle = \frac{I_V - I_H}{I_V + 2I_H} \tag{2}$$

I_V and I_H being the emission components parallel and perpendicular, respectively, to the linearly polarized exciting light. The total fluorescence is proportional to $I_V + 2I_H$. This sum contains the information concerning the fluorescence decay.

Depending upon the nature of the fluorophore and its interaction with its immediate environment, the emission may be complex. If a multiexponential model is assumed, the impulse response of the fluorescence is

$$s(t) = I_V(t) + 2I_H(t) = \sum_i \alpha_i \exp(-t/\tau_i) \tag{3}$$

where α_i is the relative weight and τ_i is the decay constant of the ith component. The rotational motion of nonspherical molecules will also be complex but can generally be described by a sum of exponential terms (Rigler & Ehrenberg, 1973; Small & Isenberg, 1977) so that

$$r(t) = \sum_j \beta_j \exp(-t/\phi_j) \tag{4}$$

where the zero-point anisotropy is determined by the sum of the preexponentials, β_j.

The parameters for the decay of the emission anisotropy are conventionally obtained from analysis of the difference curve $d(t)$ and the sum curve $s(t)$ generated from the decay of $I_V(t)$ and $I_H(t)$ [see Wahl (1969)]:

$$r(t) = \frac{I_V(t) - I_H(t)}{I_V(t) + 2I_H(t)} = \frac{d(t)}{s(t)} \tag{5}$$

However, the information concerning $r(t)$ is contained in both $I_V(t)$ and $I_H(t)$. As will be described in the results, the apparent time-shift artifact related to the difference in energy between the excitation and emission photons requires that for accurate determination of short rotational correlation times, the measurements of the experimental decay curve should be made contemporaneously with the single-exponential standard. Since from eq 5

$$3I_V(t) = s(t)[1 + 2r(t)] \tag{6}$$

and from eq 3 and 4

$$3I_V(t) = \sum_i \alpha_i \exp(-t/\tau_i)[1 + 2\sum_j \beta_j \exp(-t/\phi_j)] \tag{7}$$

it can readily be seen that if the fluorescence decay parameters are known, then in principle the anisotropy decay parameters can be obtained from the analysis of just $I_V(t)$ alone. The procedure we followed was first to measure in separate experiments the fluorescence decay and the steady-state anisotropy. Then recognizing that

$$\int_0^\infty s(t)r(t)\,dt = \langle r\rangle \int_0^\infty s(t)\,dt \tag{8}$$

an experimental constant C was calculated from the ratio

$$C = \frac{3\int_0^\infty I_V(t)\,dt}{(1 + 2\langle r\rangle)\int_0^\infty \sum_i \alpha_i \exp(-t/\tau_i)\,dt} \tag{9}$$

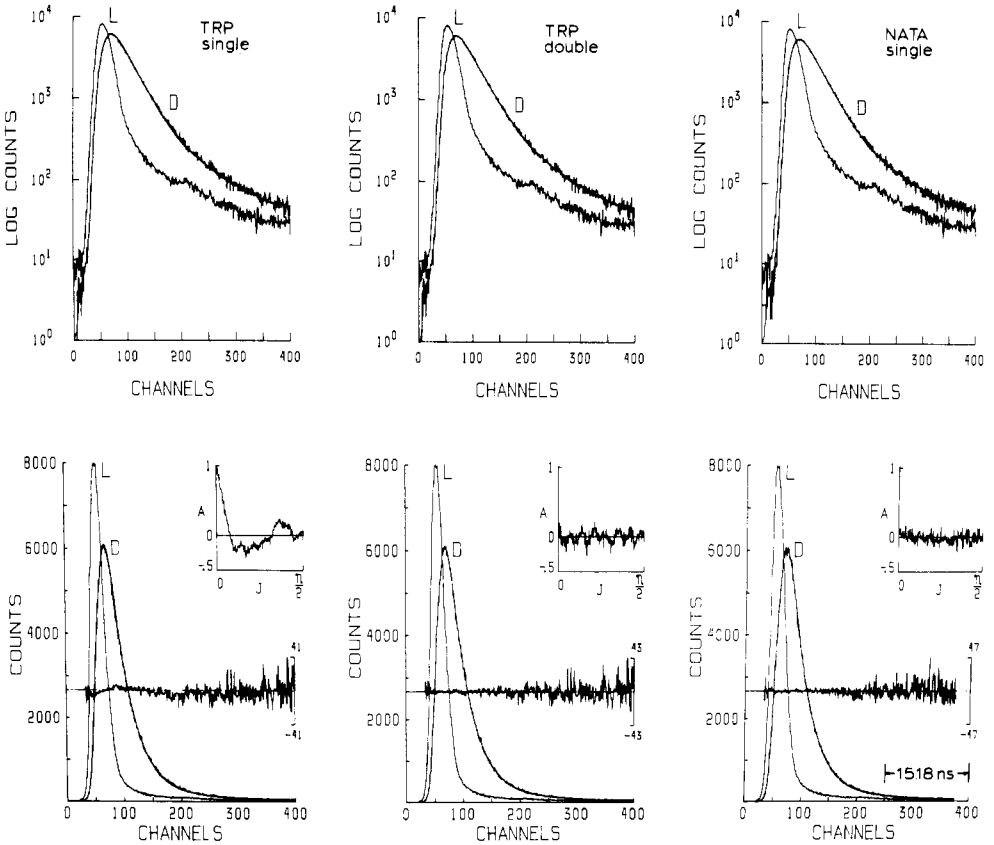

FIGURE 1: Fluorescence decay data of tryptophan and NATA, each at 1.2×10^{-4} M in 0.01 M sodium phosphate pH 7 buffer at 20 °C. Logarithmic (top row) and linear (bottom row) representations of the data (D) are shown along with the experimental lamp (L). Included with the linear data are the percent residuals and their autocorrelation. The solid noise-free curve is the theoretical parameters convolved with lamp. Excitation was with 295-nm natural light, and the emission was monitored at 355 nm through a magic angle polarizer. The best fits to these data, from right to left, were respectively $\tau = 2.99$ ns with $\chi^2 = 2.06$; $\tau_1 = 3.18$ ns, $\alpha_1 = 0.78$ and $\tau_2 = 0.84$ ns, $\alpha_2 = 0.22$, with $\chi^2 = 1.16$; $\tau = 2.99$ ns with $\chi^2 = 1.25$.

in which the independently determined fluorescence lifetime parameters (amplitudes normalized to unity) were convolved with the same experimental lamp of $I_V(t)$. Then by use of C to properly weight the amplitidues of the fluorescence decay contained in $I_V(t)$, the anisotropy decay parameters were obtained by analysis of $I_V(t)$ according to eq 7.

The decay curves were analyzed by a nonlinear least-squares method (Grinvald & Steinberg, 1974). The fit between the curves generated by the theoretical parameters and the data was evaluated from the percent residuals and the reduced χ^2. Simulations of the data were based upon both derived and hypothetical parameters to test our supposed models, as well as to examine the reliability of the anisotropy parameters recovered from the $I_V(t)$ analysis.

Results

Tryptophan Models. Table I gives the fluorescence decay kinetics of several tryptophan and simpler indole derivatives in 0.1 M phosphate pH 7 buffer at 20 °C. These experiments were done under the same conditions used with the polypeptide hormones. The fluorescence decay depends upon the nature of the molecule and is described by either a single- or a double-exponential decay law. Two kinds of average lifetimes are listed in Table I (and also in Table II): $\langle\tau\rangle = \sum_i \alpha_i \tau_i^2 / \sum_i \alpha_i \tau_i$ approximates the average lifetime measured by phase methods, provided that the product of the modulation frequency and the lifetime are much less than unity (Inokuti & Hirayama, 1965); $\bar{\tau} = \sum_i \alpha_i \tau_i / \sum_i \alpha_i$ approximates the integral of the impulse response obtained from the pulse data (Grinvald & Steinberg, 1974). These data are presented here since they are relevent to the discussion of the fluorescence decay of polypeptides and, in addition, because there has been considerable debate and little agreement in the literature regarding the decay of the tryptophan zwitterion and related molecules.

The criteria used to establish the nature of the decay law appropriate to the results are indicated in Figure 1. The data shown in this figure are for the tryptophan zwitterion and NATA. It is not immediately obvious from either the linear or the semilogarithmic representations of the data and the fit of the lamp-convolved theoretical parameters that a double-exponential fits the zwitterion decay better than does a single. However, the superior fit of the double is evident in the significantly smaller χ^2, as well as in the improved percent residuals and their autocorrelation. The fit of the NATA data presents the standard expected for a single-exponential decay.

In 0.1 M phosphate pH 7 buffer, only tryptophan, tryptophanamide, and the dipeptides exhibit double-exponential fluorescence decay kinetics. The wavelength dependence of the zwitterion decay at this pH in 0.01 M cacodylate has been previously reported by Rayner & Szabo (1978) and Szabo & Rayner (1980). Our results in general agree. The pK of the amino group of tryptophan amide is close to 7. At this pH two ionic species exist, and the double-exponential decay kinetics can be explained in this way. Szabo & Rayner (1980) have measured the decay of tryptophanamide and report monoexponential kinetics with decay times of 1.61 ns at pH 5 and 6.86 ns at pH 9. Our double-exponential decay times of 1.54 and 6.37 ns at pH 7 are in good agreement with these values. At pH 7 the decay parameters of tryptophanamide are emission wavelength independent in contrast to those of tryptophan. The double-exponential decay kinetics of tryptophylglycine at pH 7 may likewise be attributed to the

Table I: Fluorescence Lifetime Parameters of Indole and Tryptophan Derivatives[a]

sample	λ′ (nm)	α_1	τ_1 (ns)	α_2	τ_2 (ns)	$\langle\tau\rangle$ (ns)	$\bar{\tau}$ (ns)
indole[b]	360		4.54				
3-methylindole	360		8.72 ± 0.04				
3-indolepropionic acid	355		9.71 ± 0.03				
tryptamine	355		5.83 ± 0.03				
tryptophan	325	0.70 ± 0.03	3.19 ± 0.04	0.30 ± 0.03	0.85 ± 0.07	2.95	2.49
	355	0.79 ± 0.02	3.17 ± 0.01	0.21 ± 0.02	0.96 ± 0.12	3.01	2.71
	400		3.16 ± 0.01				
N-acetyltryptophanamide	355		3.00 ± 0.01				
N-acetyltryptophan	360		4.83 ± 0.06				
tryptophanamide	360	0.18 ± 0.03	6.37 ± 0.10	0.82 ± 0.03	1.54 ± 0.04	3.83	2.41
tryptophylglycine	360	0.11 ± 0.02	7.38 ± 0.13	0.89 ± 0.02	1.83 ± 0.05	3.68	2.44
glycyltryptophan[c]	340–400	0.54 ± 0.03	1.46 ± 0.02	0.46 ± 0.03	0.47 ± 0.15	1.25	1.01

[a] Solvent was 0.1 M sodium phosphate, pH 7, at 20 °C, and the samples had an absorbance at 295 nm of 0.1–0.2. The average lifetimes are $\langle\tau\rangle = \Sigma_i\alpha_i\tau_i^2/\Sigma_i\alpha_i\tau_i$ (Inokuti & Hirayama, 1965) and $\bar{\tau} = \Sigma_i\alpha_i\tau_i/\Sigma_i\alpha_i$ (Grinvald & Steinberg, 1974). [b] This datum was from a single experiment. [c] These data are the average of single determinations at λ′ = 340, 360, 380, and 400 nm; no wavelength dependence was observed.

Table II: Polypeptide Fluorescence Lifetime Parameters[a]

sample	T (°C)	α_1	τ_1 (ns)	α_2	τ_2 (ns)	$\langle\tau\rangle$ (ns)	$\bar{\tau}$ (ns)
ACTH-(1–24)	3.5	0.59 ± 0.02	2.31 ± 0.03	0.41 ± 0.02	5.93 ± 0.09	4.62	3.79
	10.0	0.57 ± 0.03	2.03 ± 0.07	0.43 ± 0.03	5.13 ± 0.11	4.06	3.36
+20% sucrose	3.5	0.62 ± 0.01	2.32 ± 0.02	0.38 ± 0.01	6.03 ± 0.05	4.60	3.73
+20% sucrose	10.0	0.67 ± 0.01	2.23 ± 0.04	0.33 ± 0.01	5.70 ± 0.04	4.16	3.38
+20% sucrose	15.0	0.66 ± 0.02	1.97 ± 0.06	0.34 ± 0.02	5.05 ± 0.09	3.72	3.02
glucagon	10.0	0.51 ± 0.01	1.66 ± 0.04	0.49 ± 0.01	5.04 ± 0.04	4.18	3.32
	20.0	0.53 ± 0.02	1.54 ± 0.06	0.47 ± 0.02	4.08 ± 0.08	3.32	2.73
glucagon-(23–26)[b] (Val-Gln-Trp-Leu)	10.0	0.31 ± 0.01	1.63 ± 0.09	0.69 ± 0.01	4.48 ± 0.01	4.07	3.60

[a] Sample concentrations were as follows: ACTH-(1–24), 8×10^{-5}–12×10^{-5} M; glucagon and glucagon-(23–26), 1.6×10^{-5} M. A solvent blank was subtracted from the glucagon and glucagon-(23–26) data since the background exceeded 1% of the fluorescence intensity. A 0.1 M sodium phosphate buffer was used for glucagon (pH 7.4) and the 23–26 peptide (pH 7.1). For ACTH-(1–24) 0.01 M sodium phosphate buffer (pH 7.0) was used. The definitions of $\langle\tau\rangle$ and $\bar{\tau}$ are in the legend of Table I. [b] The results given for glucagon-(23–26) are the average of single determinations at λ′ = 350, 355, and 360 nm; no wavelength dependence was observed over this range.

presence of two ionic species [p*K* amino ≃ 7.9, Edelhoch et al. (1967)]. Cowgill (1970) has shown that a peptide bond at the α-amino position of tryptophan causes quenching. This may explain the short lifetimes (and low quantum yield) of glycyltryptophan. We have observed excellent agreement between the relative $\bar{\tau}$ (see Table I) of NATA and glycyltryptophan and their relative quantum yields. The origin of the double-exponential decay behavior with glycyltryptophan is still obscure but may be related to the ionization of the glycyl α-amino group (pK ≃ 8.2). The fluorescence decay parameters of tryptophylglycine and glycyltryptophan are independent of emission wavelength. The tryptophan zwitterion differs from the other molecules which exhibit multiexponential decays in that its amino and carboxyl p*K*s are near 9.4 and 2.4, respectively [cf. Mahler & Cordes (1971)]. Thus, the peculiar neutral pH decay kinetics of tryptophan are of a single population of molecules.

Polypeptides. The data for all three molecules were not satisfactorily fit by a single-exponential decay law. At least a double was required. The improvement offered by a triple-exponential fit was small and statistically insignificant according to the criteria established by the best fit of the single-exponential standard. Therefore, only the double-exponential decay parameters are reported. The results obtained from the data analyses show that both the long and the short lifetimes decrease as the temperature of the solvent is increased. This is reflected in the calculated lifetime averages as well. However, there is essentially no temperature dependence in the preexponential terms. Also, we note that the calculated average lifetimes, either $\langle\tau\rangle$ or $\bar{\tau}$, yield similar values for the three peptides ($\langle\tau\rangle$ and $\bar{\tau}$ differ from each other because of the respective weightings of the time constants). In addition, the lifetime kinetics of ACTH and glucagon-(23–26) are independent of the emission wavelength. By contrast, a small wavelength dependence is found in the glucagon fluorescence decay, with the preexponential terms showing the larger percent change. The results reported for glucagon at 20 °C are comparable to those obtained by Grinvald & Steinberg (1976), who also used 0.1 M phosphate buffer. Their data included two emission windows, one covering the major portion of the fluorescence spectrum between 310 and 380 nm and the other all of the emission above 380 nm. Below 380 nm they determined that $\tau_1 = 1.1$ ns, $\alpha_1 = 0.55$, $\tau_2 = 3.6$ ns, and $\alpha_2 = 0.45$. Above 380 nm the decay constants increased by 0.2 ns, and α_1 decreased and α_2 increased by 0.03. Werner & Forster (1979) monitoring the total emission and using different conditions—0.01 M pH 8.15 carbonate buffer (undefined temperature)—obtained $\tau_1 = 0.5$ ns, $\alpha_1 = 0.65$, $\tau_2 = 3.3$ ns, and $\alpha_2 = 0.35$. To our knowledge, the fluorescence decay kinetics of ACTH and glucagon-(23–26) have not been previously reported.

Time-Resolved Anisotropy of ACTH. The fluorescence decay parameters of ACTH, used for analysis of the parallel polarized fluorescence decay (which will be referred to as the V curve), are given in Table II. Table III lists steady-state anisotropy values and the anisotropy parameters obtained from analysis of the V curve according to a double-exponential rotational correlation model. The steady-state anisotropy values for ACTH in 20% sucrose are the same at 10 and 15 °C because the decrease in the mean fluorescence lifetime at the higher temperature must be compensating for the increase in the overall Brownian motion. As reflected by the χ^2, a single exponential gives nearly as satisfactory a fit to the V curve data as does a double, especially when the viscosity is low. However, as the viscosity of the solvent is increased by the addition of sucrose, the double-exponential nature of the

Table III: Time-Resolved Anisotropy Parameters of ACTH-(1-24)[a]

T(°C)	conditions	η (cP)	β_1	ϕ_1 (ns)	β_2	ϕ_2 (ns)	r_0	$\langle r \rangle$
3.5	buffer	1.65	0.13 ± 0.04	0.92 ± 0.08	0.05 ± 0.01	4.47 ± 1.47	0.18	0.050 ± 0.004
3.5	20% sucrose	3.32	0.12 ± 0.01	0.73 ± 0.04	0.08 ± 0.01	13.13 ± 1.08	0.20	0.082 ± 0.004
10.0	20% sucrose	2.65	0.13 ± 0.02	0.69 ± 0.10	0.07 ± 0.01	6.19 ± 1.19	0.20	0.062 ± 0.004
15.0	20% sucrose	2.27	0.12 ± 0.01	0.73 ± 0.05	0.07 ± 0.01	4.94 ± 0.73	0.19	0.062 ± 0.004

[a] Samples were 8×10^{-5} M in 0.01 M phosphate pH 7 buffer. The viscosity, η, values were obtained from *Lange's Handbook of Chemistry* (1949). The steady-state anisotropy, $\langle r \rangle$, was determined by using the lifetime instrument in the steady-state photon-counting mode. The zero-time anisotropy, r_0, is the sum of the preexponential terms, $\Sigma_i \beta_i$. Excitation was at 295 nm (band-pass of 10.5 nm), and fluorescence was monitored at 355 nm (band-pass of 9.9 nm). Details concerning the data analysis are given under Results.

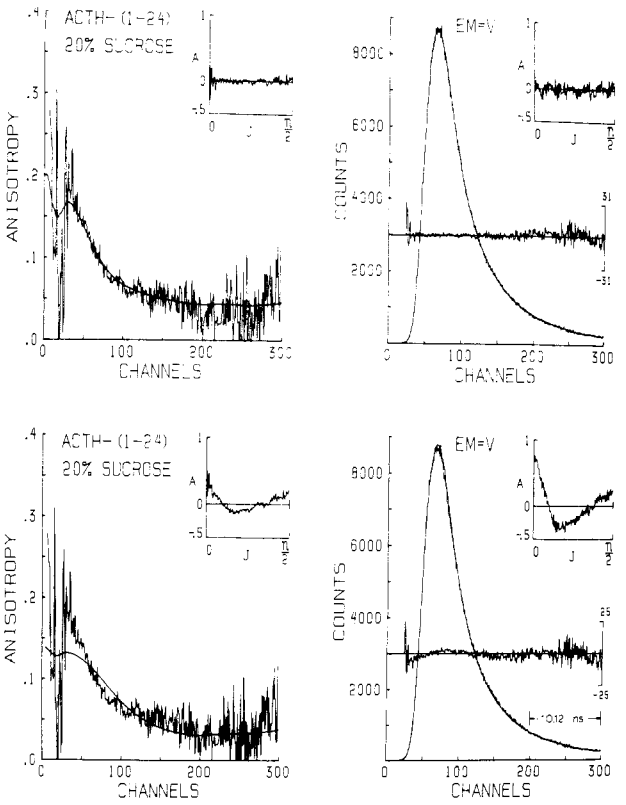

FIGURE 2: Parallel polarized fluorescence decay (EM = V) of ACTH (8×10^{-5} M) in 0.01 M sodium phosphate pH 7 buffer at 3.5 °C with 20% sucrose by weight. Excitation was with vertically polarized 295-nm light (band-pass of 10.6 nm), and the emission was detected at 355 nm (band-pass of 13.2 nm). Representation of the data in the form of the decay of the anisotropy is used to provide a visual comparison for the fit of the anisotropy parameters obtained from analysis of the V curves to either a single- or a double-exponential rotational correlation time model. The best-fit theoretical parameters reflected by the percent residuals and their autocorrelation in the top row are $\phi_1 = 0.70$ ns, $\beta_1 = 0.13$ and $\phi_2 = 13.88$ ns, $\beta_2 = 0.08$, with $\chi^2 = 1.40$. In the bottom row they are $\phi = 5.67$ ns, $\beta = 0.14$, with $\chi^2 = 1.75$. The theoretical parameters and autocorrelation of the anisotropy were calculated by starting with channel 20 of the data.

time-resolved anisotropy becomes clearly evident. This result is shown in Figure 2. The right-hand curves are the experimental data obtained by monitoring the vertical component of the emission using vertical excitation (EM = V). The left-hand curves show the convolved experimental and theoretical anisotropy together with the autocorrelation of the residuals between them. A synthetic sum curve convolved with the real lamp flash (used in the V curve analysis) was generated from the fluorescence decay parameters—there is no anisotropy information in the pure fluorescence decay. From the sum curve and the V curve the perpendicular component of the decay was calculated according to the relationship

$$\int_0^\infty I_H(t)\,dt = \left[\int_0^\infty s(t)\,dt - \int_0^\infty I_V(t)\,dt\right]\Big/ 2 \quad (10)$$

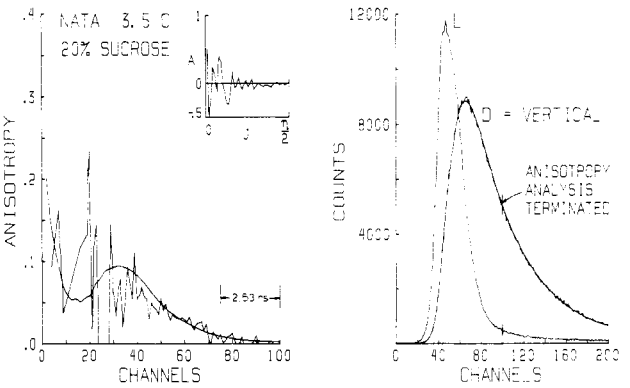

FIGURE 3: Decay of fluorescence anisotropy of NATA 0.01 M sodium phosphate pH 7 buffer at 3.5 °C with 20% sucrose by weight. The optical conditions were as described in Figure 2. The autocorrelation of the anisotropy decay was calculated by starting with channel 15. The theoretical parameters fitting the data are $\phi = 0.29$ ns, $\beta = 0.20$, with $\chi^2 = 1.83$, when analyzing the V curve from channels 19–100. Analysis to longer times (i.e., to 0.5% of the V curve peak height at channel 476) improved the χ^2 to 1.62 and gave $\phi = 0.33$ ns, $\beta = 0.19$. Analysis for a double exponential was unsuccessful.

Then the experimental anisotropy data contained in the V curve were resolved by using eq 5. The theoretical anisotropy decay, derived from analysis of the V curve, was convolved with the experimental lamp for comparison with the representation of the experimental data.

Since the anisotropy decay of ACTH was better fit by a double exponential than by a single, we also examined the emission anisotropy of the model NATA in 20% sucrose (0.1 M phosphate pH 7 buffer) at 3.5 °C. The purpose of this experiment was to evaluate the ability of our technique to study fast rotations. We found that the V curves could be equally well fit for a rotational time of ~0.3 ns with an r_0 of 0.2 (Figure 3) or, for example, 0.4 ns with an r_0 of 0.16. The data could not be successfully analyzed in terms of a double-exponential decay law.

In testing the analysis of the anisotropy parameters from simulated V curves, we found that although single-exponential subnanosecond correlation times could be recovered with reasonable accuracy, the values obtained were much less precise when we attempted to recover two correlation times. Compared with synthetic data, real data—the ACTH data—showed more scatter in the recovered parameters. However, data collected and analyzed according to the method of Wahl (1969), as outlined under Experimental Procedures, showed even greater scatter in the parameters. Since only random error was introduced into the simulated data, it appears that the meausrement of subnanosecond rotational correlation times is quite sensitive to systematic errors. In our V curve anisotropy experiments, the time-shift correction for the response of the photomultiplier was determined from a single-exponential standard collected semisimultaneously with the lamp and a single sample. But when the method of Wahl (1969) was used, in which the anisotropy parameters were recovered

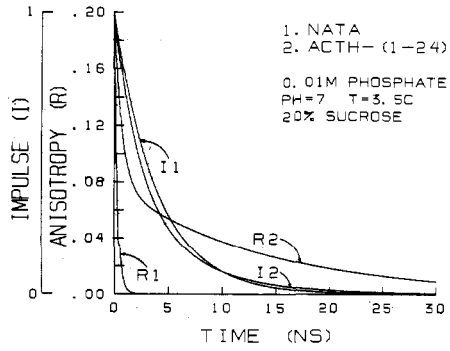

FIGURE 4: Impulse responses for fluorescence decay of ACTH and NATA compared with their anisotropy decay parameters.

from simultaneous collections of $I_V(t)$ and $I_H(t)$, the time-shift was measured in a separate experiment. Therefore, any drift in the instrument between the time of the separate sample and standard data collections would appear as additional systematic error in the anisotropy decay data. The theoretical impulse response functions for the fluorescence decay and the anisotropy decay of ACTH and NATA are shown in Figure 4.

It must be pointed out that since the rotation rates are fast and the fluorescence lifetimes are short, it was difficult to determine whether the analysis of the decay of the anisotropy for ACTH in terms of a double-exponential decay law gave significant improvement over that of a single. In spite of the fact that our statistical criteria, namely, the χ^2, residuals, and the autocorrelation of the residuals, were consistently better for a double exponential than for a single, it could be argued that these differences were just due to systematic errors. For this reason it is important to emphasize that analysis in terms of a single-exponential decay law gave rise to r_0 values close to 0.1, while analysis of a double-exponential decay of the anisotropy consistently gave r_0 values close to 0.2. An r_0 value close to 0.2, at the excitation wavelength of 295 nm, was reported for tryptophan by Valeur & Weber (1977) and for NATA by Lakowicz & Weber (1980). The wavelength region covered by the excitation bandwidth in our experiments was 290–300 nm, with the maximum throughput at 295 nm. Valeur & Weber (1977) observed a range of r_0 values from 0.13 at 290 nm, where it is near a minimum, to 0.30 at 300 nm. At 295 nm r_0 was 0.23. The values observed by Lakowicz & Weber (1980) for NATA are similar, with an r_0 of ~0.19 at 295 nm. The r_0 values we obtained at 295 nm for NATA from the time-resolved anisotropy decay agree within experimental error. The r_0 value of 0.20 for Trp-9 in ACTH, obtained from analysis of the anisotropy decay data in terms of a double exponential, is similar. An r_0 value of 0.1, obtained from analysis of the ACTH anisotropy decay in terms of a single rotational time constant, is inconsistent with all of the above data. In fact, simulations of data showed that analysis of a double-exponential rotational correlation model for a single exponential invariably yielded unrealistically low r_0 values. The correct r_0 value was obtained when the simulated data were analyzed for two time constants.

Discussion

Fluorescence decay may be simple, i.e., described by a monoexponential decay law, or it may be quite complex and show biexponential, multiexponential, or nonexponential decay. Before discussing the data presented here, it would be of value to indicate some possible origins of complex fluorescence decay behavior.

Simple aromatic hydrocarbons (containing no heteroatoms) show monoexponential decay kinetics when they are dissolved in noninteracting solvents. The fluorophores 1,1′-binaphthyl and *p*-terphenyl dissolved in cyclohexane are examples.

A mixture of different species in the ground state can give rise to multiexponential decay. Ground-state microheterogeneity may involve a gross conformational change such as a helix–coil transition in a polypeptide chain or it may involve a more subtle change such as the pH-dependent protonation of an ionizable group or the movement of a charge residue a few angstroms closer to a fluorophore.

Even in cases where the ground state is homogeneous, deviations from monoexponential decay behavior may occur due to excited-state reactions. The fluorescence decay of a system undergoing excited-state reactions may follow different decay laws depending on the mechanisms involved. Complex decay kinetics are possible even when only one of the excited-state species emits significantly. Decay parameters (α_i, τ_i) will show characteristic behavior as a function of emission wavelength depending on the nature of the excited-state interaction.

In general, complex decay kinetics will be observed if excited-state reactions occur on the same time scale as the fluorescence decay. In a two-state excited-state reaction the decay constant characteristic of each species can be reflected in the emission of either species. The decay of 2-naphthol is an example of a two-state excited-state reaction (Loken et al., 1972; Laws & Brand, 1979). With this type of system the decay times are independent of the emission wavelength. A characteristic feature of emission due to the species formed during the excited-state lifetime is that it will be associated with a negative preexponential term. However, this negative amplitude is not always observed (Gafni et al., 1976).

Excited-state solvation reactions may involve emission by a large number of species formed during the excited-state lifetime. This type of mechanism may lead to nonexponential decay laws (DeToma et al., 1976). The mean lifetime of these systems increases with increasing wavelength. As a pertinent example, NATA shows monoexponential decay in aqueous solutions where solvation occurs much more rapidly than the fluorescence lifetime. But in viscous propylene glycol the mean decay time increases across the emission band (Lakowicz & Cherek, 1980).

We now discuss the fluorescence decay data presented here in terms of the above generalizations. Indole or NATA exhibit monoexponential decay and represent limiting cases of a fluorophore in a solvent-relaxed environment. The tryptophan derivatives, such as tryptophanamide or the dipeptides which exhibit emission wavelength independent double-exponential decay kinetics, exist in two different ground-state forms at pH 7 due to the intrinsic pK of the α-amino group. However, the wavelength dependence of the amplitudes in the double-exponential decay of tryptophan as a free amino acid is anamolous since only one ground-state form predominates at neutral pH.

The first report of the pH dependence of the fluorescence decay of tryptophan, by DeLauder & Wahl (1970), indicated that at *neutrality* the emission fit a single exponential of between 3.0 and 3.2 ns. Recently, conflicting reports about the zwitterion decay kinetics have come from other laboratories. With considerable improvements in analytical methods, Rayner & Szabo (1978) unexpectedly found that a small fraction of the fluorescence decayed in ~0.5 ns, the majority of the emission having a lifetime of 3.1 ns. Furthermore, the small short-lived component had a blue-shifted spectrum with no measurable contribution above 380 nm. Then Fleming et al. (1978) found that the red edge of the tryptophan fluorescence above 370 nm could be resolved into two decay

components of 2.1 and 5.4 ns. Next, Alpert et al. (1979) reported that the fluorescence above 320 nm was adequately described by a single exponential. Our fluorescence decay results for tryptophan and several tryptophan and indole derivatives agree with those of Szabo & Rayner (1980). In a recent report (Robbins et al., 1980) the observations of Fleming et al. have been revised. The latter results are more consistent with the results presented here.

In the zwitterion there exists the possibility of an excited-state reaction between the indole ring and, depending upon the rotamer configuration, the protonated amino group or the deprotonated carboxyl group, or both groups. The rotamer model proposed by Szabo & Rayner (1980), which is based upon a considered review of the literature, has the short decay component arising from an interaction between the indole ring and the α-amino group. Whatever the nature of this quenching interaction, it is of interest that the ground-state configuration in which it occurs leads to a blue-shifted emission for a small fraction of the total fluorescence. This would be expected if the indole ring were undergoing solvation on the nanosecond time scale.

In contrast to tryptophan at pH 7, tryptophanamide at pH 5 (where it exists in one ionic form) exhibits only a single decay time of 1.5 ns. Compared with the 3.2-ns lifetime of tryptophan, the short lifetime of tryptophanamide can be attributed to the conversion of the carboxyl group to an amide. The quenching efficiency of the amide is evident from the observation that the lifetime of NATA is 1.8 ns shorter than that of *N*-acetyltryptophan. Detection of a subnanosecond component in the tryptophanamide emission would depend upon the relative amplitudes and time constants of the decay components associated with the three possible rotamer configurations.

The concept of rotamers being associated with double-exponential fluorescence kinetics in tryptophan peptides was introduced by Donzel et al. (1974), who studied the diketopiperazines cyclo(glycyltryptophyl) and cyclo(alanyltryptophyl). In each case, two fluorescence lifetimes were observed which remained constant across the emission spectrum while the preexponential factors varied. An interpretation was made in terms of two possible conformers, one folded and the other extended. By contrast, the preexponential terms for the fluorescence decay of ACTH and glucagon-(23–26) are wavelength independent. In glucagon, the kinetic components of the Trp-25 fluorescence exhibit a small emission wavelength dependence apparent in both the time constants and the amplitudes.

The factors important in the double-exponential decay kinetics of ACTH and glucagon undoubtedly differ. The steady-state fluorescence polarization data of Bernstein et al. (1969) indicate that there is a freedom of rotation for the indole ring of Trp-9 in ACTH such as would be expected in a randomly coiled polypeptide chain. Other physical evidence such as singlet energy transfer (Eisinger, 1969; Schiller, 1972), deuterium exchange (Li, 1962), and optical detection of magnetic resonance (ODMR) (Deranleau et al., 1978) supports this view. Trp-9 in ACTH is bounded by Arg-8 and Gly-10. The lack of secondary structure could permit interaction between the positive charge of Arg-8 and the indole ring of Trp-9. On the other hand, there is evidence from physical methods such as circular dichroism (Panijpan & Gratzer, 1974; Bromer, 1976), hydrogen exchange (McBride-Warren & Epand, 1972), and ODMR (Ross et al., 1977, 1980) that there is helical structure in glucagon. Trp-25 in glucagon is bounded on either side by the neutral residues Gln-24 and Leu-26. In a helix the indole group of Trp-25 can interact with Phe-22 and Asn-28.

The lack of emission wavelength dependence for the fluorescence decay parameters of ACTH and glucagon-(23–26), in contrast to glucagon, is of special note in light of the observation of Deranleau et al. (1978) that the four-peptide fragment is too short to satisfy the requirements for secondary structure. The wavelength dependence of Trp-25 in the intact hormone could arise from a fraction of the population of molecules in solution persisting in one or more energetically stable conformations during the excited state of the tryptophan residue. This argument only requires that the stable conformations have closely overlapping spectra with slightly different decay parameters from the open or random conformations. In fact, the steady-state fluorescence line width (full width at half-height) of glucagon appears to be slightly broader (1 ± 0.5 nm) that that of glucagon-(23–26) or ACTH. The line widths of the latter two molecules are identical.

While the origin of the double-exponential decay behavior of Trp-25 must be considered in terms of interactions with neutral residues, it is conceivable that Trp-9 in ACTH interacts with the guanidinium group of Arg-8. However, if this involves a two-state excited-state reaction, then a definite wavelength dependence should be observed in the amplitudes. The fact that the amplitudes and time constants of the ACTH fluorescence are wavelength independent is an argument against a two-state excited-state reaction being the origin of the double-exponential decay kinetics.

The concept of a flexible rotating tryptophan could explain the complicated decay kinetics of the polypeptides. Thus, a physical model in which rotamers, each with a different range of allowed nearest-neighbor interactions with adjacent amino acid side chains, would provide a distribution of slightly differing tryptophan environments. Different degrees of quenching of the indole excited state would lead to a distribution of fluorescence decay times and hence multiexponential decay kinetics. If this picture is correct, then we might expect a subnanosecond correlation time for the indole ring rotation. In addition, the anisotropy decay of the fluorescence would be expected to have a major contribution from the rotational motions of the rest of the hormone. We have selected ACTH to test this hypothesis.

The equations of motion for a complex system like a random coil are not satisfied by a double-exponential decay law. The terms depend upon the asymmetry, including the axis of rotation of the fluorophore relative to the rest of the molecule [see Rigler & Ehrenberg (1973)]. We would expect the tumbling of the polypeptide to be complex and certainly more sensitive to the bulk solvent viscosity than the motion of the tryptophan side group. The recovery of two or more time constants from the data would mean that one or more of the exponential terms is substantially different from the rest.

Comparing the recovered rotational correlation times from the decay of the fluorescence anisotropy of ACTH in buffer alone and with 20% sucrose, we find the following: first, the subnanosecond component shows little change over the viscosity range of the experiment, whereas the slower component becomes a factor of three longer at the highest bulk viscosity; second, the limiting zero-time anisotropy remains, within experimental error, constant over the viscosity range. Since the rotational time is directly proportional to the molecular volume of the fluorophore, the short decay component is predominated by the relatively small-volumed cone swept out by the rotation of the indole ring, and the longer component is predominated by the complex motion of the entire hormone. Nevertheless,

all rotational correlation times should change with solvent viscosity. We believe that it is the noise in the data which obscures the viscosity dependence of the faster component. The important conclusion is that the indole ring does indeed exhibit subnanosecond rotations. Then the tryptophan is capable of "sampling" a variety of local side-chain interactions. Hence, multiexponential decay kinetics might be expected. An observation also consistent with this reasoning is that the two tryptophans of horse liver alcohol dehydrogenase (ADH) which reside in different environments, one buried and one exposed to solvent, are both immobile and only rotating with the protein as a whole: the fluorescence decay times are monoexponential for each residue (Ross et al., 1981).

It happens that the fluorescence kinetics of the polypeptides discussed here can be adequately fit by a double-exponential decay law. However, cases may well exist which would require a triple-exponential fluorescence decay law for a single tryptophan residue. The wavelength independence of the time constants of Trp-9 implies that the perturbations from the nearest-neighbor interactions do not significantly shift the energy of the excited state relative to that of the ground state. Although in glucagon there is a small but significant energy shift, the interactions in the polypeptides studied here do not lead to the same kind of fluorescence kinetics observed for the tryptophan zwitterion. This is not surprising since, for example, the guanidinium group of Arg-8 does not have the same steric restrictions relative to the indole ring of Trp-9 as does the charged α-amino group of tryptophan. The sterically restricted α-amino group not only quenches but also provides a sufficiently strong electronic perturbation to the indole ring to increase the S_0–S_1 splitting.

In summary, we find that Trp-9 in ACTH has fluorescence decay kinetics which are complex, and thus similar to those found in other polypeptides. We also help resolve the conflicting data in the literature regarding the fluorescence decay of the tryptophan zwitterion by providing confirmation of the wavelength-dependent double-exponential decay of the tryptophan zwitterion, observed by Szabo & Rayner (1980) and Rayner & Szabo (1978). Using time-resolved anisotropy measurements, we have found that the indole group of Trp-9 indeed has considerable freedom of rotation. This behavior is consistent with the notion that the multiexponential decay has its origin in the differing interactions of various configurations. Thus, the wavelength independence of the fluorescence lifetimes argues that ground-state microheterogeneity characterizes the emission of Trp-9 and that excited-state reactions are not evident. If the emissions characteristic of the various environments are shifted in energy, then the amplitudes of the lifetimes will be wavelength dependent, as is the case for glucagon. From these arguments, we conclude that multiexponential decay kinetics are to be expected for flexible polypeptides. However, this statement does not imply that an *immobile* tryptophan residue in a protein will necessarily exhibit monoexponential fluorescence decay, since excited-state reactions, including dipolar relaxation, may occur.

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References

Alpert, B., Jameson, D. M., Lopez-Delgado, R., & Schooley, R. (1979) *Photochem. Photobiol. 30*, 479–481.

Azumi, T., & McGlynn, S. P. (1962) *J. Chem. Phys. 61*, 378–425.

Badea, M. G., & Brand, L. (1979) *Methods Enzymol. 61*, 378–425.

Bernstein, R. S., Wilchek, M., & Edelhoch, H. (1969) *J. Biol. Chem. 244*, 4398–4405.

Bornet, H., & Edelhoch, H. (1971) *J. Biol. Chem. 246*, 1785–1792.

Bromer, W. W. (1976) *Metab. Clin. Exp. 25*, 1315–1316.

Bundi, A., Andreatta, R., Rittel, W., & Wüthrich, K. (1976) *FEBS Lett. 64*, 126–129.

Cowgill, R. W. (1970) *Biochim. Biophys. Acta 200*, 18–25.

DeLauder, W. B., & Wahl, P. (1970) *Biochemistry 9*, 2750–2754.

Deranleu, D. A., Ross, J. B. A., Rousslang, K. W., & Kwiram, A. L. (1978) *J. Am. Chem. Soc. 100*, 1913–1917.

DeToma, R. P., Easter, J. H., & Brand, L. (1976) *J. Am. Chem. Soc. 98*, 5001–5007.

Donzel, B., Gauduchon, P., & Wahl, P. (1974) *J. Am. Chem. Soc. 96*, 801–808.

Easter, J. H., DeToma, R. P., & Brand, L. (1976) *Biophys. J. 16*, 571–583.

Edelhoch, H., & Lippoldt, R. E. (1969) *J. Biol. Chem. 244*, 3876–3883.

Edelhoch, H., Brand, L., & Wilchek, M. (1967) *Biochemistry 6*, 547–559.

Eisinger, J. (1969) *Biochemistry 8*, 3902–3908.

Fleming, G. R., Morris, J. M., Robbins, R. J., Wolfe, G. J., Thistlethwaite, P. J., & Robinson, G. W. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4652–4656.

Gafni, A., Modlin, R. L., & Brand, L. (1976) *J. Phys. Chem. 80*, 898–904.

Grinvald, A., & Steinberg, I. Z. (1974) *Anal. Biochem. 59*, 583–598.

Grinvald, A., & Steinberg, I. Z. (1976) *Biochim. Biophys. Acta 427*, 663–678.

Inokuti, M., & Hirayama, F. (1965) *J. Chem. Phys. 43*, 1978–1989.

Jablonski, A. (1957) *Acta Phys. Pol. 16*, 471–479.

Jablonski, A. (1960) *Bull. Acad. Pol. Sci., Ser. Sci., Math., Astron. Phys. 8*, 259–264.

Lakowicz, J. R., & Cherek, H. (1980) *J. Biol. Chem. 255*, 831–834.

Lakowicz, J. R., & Weber, G. (1980) *Biophys. J. 32*, 591–601.

Lang's Handbook of Chemistry (1949) 7th ed.

Laws, W. R., & Brand, L. (1979) *J. Phys. Chem. 83*, 795–802.

Lewis, C., Ware, W. R., Doemeny, L. J., & Nemzek, T. L. (1973) *Rev. Sci. Instrum. 44*, 107–114.

Li, C. H. (1962) *Recent Prog. Horm. Res. 18*, 1–40.

Loken, M. R., Hayes, J. W., Gohlke, J. R., & Brand, L. (1972) *Biochemistry 11*, 4779–4786.

Mahler, H. R., & Cordes, E. H. (1971) *Biological Chemistry*, 2nd ed., Harper and Row, New York.

McBride-Warren, P. A., & Epand, R. M. (1972) *Biochemistry 11*, 3571–3575.

Munro, I., Pecht, I., & Stryer, L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 56–60.

Panijpan, B., & Gratzer, W. B. (1974) *Eur. J. Biochem. 45*, 547–553.

Paoletti, J., & Le Pecq, J.-B. (1969) *Anal. Biochem. 31*, 33–41.

Perrin, F. (1934) *J. Phys. Radium 5*, 497–511.

Perrin, F. (1936) *J. Phys. Radium 7*, 1–11.

Rayner, D. M., & Szabo, A. G. (1978) *Can. J. Biochem. 56*, 743–745.

Rigler, R., & Ehrenberg, M. (1973) *Q. Rev. Biophys. 6*, 139–199.
Robbins, R. J., Fleming, G. R., Beddard, G. S., Robinson, G. W., Thistlethwaite, P. J., & Woolfe, G. J. (1980) *J. Am. Chem. Soc. 102*, 6271–6279.
Ross, J. B. A. (1976) Ph.D. Thesis, University of Washington, Seattle, WA.
Ross, J. B. A., Rousslang, K. W., Deranleau, D. A., & Kwiram, A. L. (1977) *Biochemistry 16*, 5398–5402.
Ross, J. B. A., Rousslang, K. W., de Haen, C., Lavis, V., & Deranleau, D. A. (1979) *Biochim. Biophys. Acta 576*, 372–384.
Ross, J. B. A., Rousslang, K. W., & Kwiram, A. L. (1980) *Biochemistry 19*, 876–883.
Ross, J. B. A., Schmidt, C., & Brand, L. (1981) *Biochemistry* (following paper in this issue).
Schiller, P. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 975–979.
Schneider, A. B., & Edelhoch, H. (1972a) *J. Biol. Chem. 247*, 4986–4991.
Schneider, A. B., & Edelhoch, H. (1972b) *J. Biol. Chem. 247*, 4992–4995.
Small, E. W., & Isenberg, I. (1977) *Biopolymers 16*, 1907–1928.
Szabo, A. G., & Rayner, D. M. (1980) *J. Am. Chem. Soc. 102*, 554–563.
Valeur, B., & Weber, G. (1977) *Photochem. Photobiol. 25*, 441–444.
Wahl, P. (1969) *Biochim. Biophys. Acta 175*, 55–64.
Wahl, P., Auchet, J.-C., & Donzel, B. (1974) *Rev. Sci. Instrum. 45*, 28–32.
Werner, T. C., & Forster, L. (1979) *Photochem. Photobiol. 29*, 905–914.

Time-Resolved Fluorescence of the Two Tryptophans in Horse Liver Alcohol Dehydrogenase†

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ABSTRACT: The tryptophan fluorescence decay of horse liver alcohol dehydrogenase, at 10 °C in 0.1 M pH 7.4 sodium phosphate buffer, with excitation at 295 nm, is a double exponential with time constants of 3.8 and 7.2 ns. Within experimental error, the two lifetimes remain constant across the emission spectrum. Only the 3.8-ns lifetime is quenched in the NAD^+–pyrazole ternary complex, and only the 7.2-ns lifetime is quenched by 0–0.05 M KI. On the basis of these results, we assign the 3.8-ns lifetime to the buried tryptophan, Trp-314, and the 7.2-ns lifetime to the exposed tryptophan, Trp-15. The steady-state lifetime-resolved emission spectrum of Trp-15 has a maximum at ~340 nm and that of Trp-15 is at ~325 nm. The total time-resolved emission, after 40 ns of decay, has a maximum between 338 and 340 nm and is primarily due to the Trp-15 emission. As a consequence of the wavelength dependence of the preexponential weighting factors, there is an increase in the average lifetime from the blue to the red edge of the emission. This increase reflects the change in the spectral contributions of Trp-15 and Trp-314. Consideration of the spectral overlap between the emission spectra of the two tryptophans and the absorption due to formation of the ternary complex, as well as the distances between the two residues and the bound NAD^+, shows that the selective fluorescence quenching in the ternary complex can be accounted for entirely by singlet–single energy transfer. The decay of the fluorescence anisotropy was measured as a function of temperature from 10 to 40 °C and is well described by a monoexponential decay law. Over this temperature range the calculated hydrodynamic radius increases from 33.5 to 35.1 Å. Evidently, the indole groups of Trp-15 and Trp-314 rotate with the protein as a whole, and there is some expansion of the protein matrix as the ambient temperature is increased.

Tryptophan fluorescence has been used as a sensitive probe for conformational perturbations in proteins and polypeptides. The emission maximum and intensity are often affected during events such as catalysis, denaturation, or intermolecular association. For example, a red shift in the spectrum is usually associated with an increase in the polarity of the local dielectric or with a quenching of the less solvent-accessible residues (Konev, 1967). In addition, collisional quenching has been used as an indicator of relative accessibility of tryptophans and to distinguish buried from exposed residues (Lehrer, 1971).

Interpretation of changes in the spectral parameters of multitryptophan proteins requires the identification of the affected residues. Time resolution of the fluorescence decay and its anisotropy has excellent potential for addressing this problem. Considerable improvements in methods of measurement and data analysis over the past decade [see review by Badea & Brand (1979)] make this approach feasible. However, since it has been found that a number of *single*-tryptophan proteins and polypeptides exhibit multiexponential decay kinetics (Grinvald & Steinberg, 1976), correlation of the fluorescence decay with individual tryptophan residues is not straightforward. In more complicated cases, such as that of pig heart lactate dehydrogenase which has six tryptophans, association of decay times with particular residues is tenous (Torikata et al., 1979). Nevertheless, progress has been made for proteins with two tryptophan residues. Privat et al. (1980) resolved the fluorescence decay of yeast 3-phosphoglycerate kinase into three components, two of which are attributed to one of the tryptophans. The single-exponential tryptophan has

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