Tryptophan Fluorescence Studies of Subunit Interaction and Rotational Dynamics of Human Luteinizing Hormone†

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Received August 5, 1986; Revised Manuscript Received December 1, 1986

ABSTRACT: Human luteinizing hormone (hLH) has a single tryptophan residue occurring in the β-subunit (βhLH). This provides an intrinsic fluorescent probe, in native hLH and βhLH, that is unambiguously assigned. The fluorescence intensities of hLH and βhLH are, however, significantly different. This difference has been utilized in studying the interaction of fluorescent βhLH with the nonfluorescent α-subunit. The accessibility of the tryptophan residue in native hLH and βhLH has been assessed by measuring the rate of collisional fluorescence quenching and by solvent perturbation (D_2O/H_2O) of fluorescence. Fluorescence anisotropy measurements have been used in studying the intramolecular dynamics and segmental tryptophan mobility in hLH and βhLH. Lifetime-resolved anisotropy, measured by the technique of oxygen quenching of fluorescence, has revealed the presence of segmental tryptophan motion. These data can be satisfactorily explained in terms of fast segmental tryptophan motion and rotational diffusion of the whole protein and do not require that intersubunit motion be invoked for intact hLH as it was suggested earlier on the basis of fluorescence depolarization of fluorescein-labeled hLH [Bishop, W. H., & Ryan, R. J. (1975) *Biochem. Biophys. Res. Commun. 65*, 1184–1190].

Human luteinizing hormone (hLH) is a glycoprotein composed of two nonidentical subunits of nearly equal size, designed α and β (Pierce & Parsons, 1981). The subunits are held together by noncovalent interactions. The affinity between the subunits is assumed to be very high in view of the low physiological concentrations of the intact hormone in circulation (Ryan et al., 1970). However, surprisingly high equilibrium dissociation constants have been reported for ovine LH (0.42 μM) and porcine LH (0.06 μM), suggesting that hormonal integrity in vivo is under kinetic, rather than thermodynamic, control (Strickland & Puett, 1982). The question of the rigidity (or lack thereof) in the interaction between two subunits was addressed by Bishop and Ryan (1975). Their measurement of rotational motion was based on fluorescence depolarization experiments using fluorescein-labeled hormone. The data yielded a rotational correlation time (ϕ), for native hLH, which was faster than the expected value based on hydrodynamic calculations assuming either a rigid sphere or a prolate ellipsoid model for the shape of the hormone molecule. For the acid-dissociated subunits, on the other hand, the correlation times obtained from fluorescence depolarization data and hydrodynamic calculations (spherical model) were very similar.

In interpreting these data, Bishop and Ryan (1975) suggested that the apparent ϕ (ϕ_A) for native hLH derived from fluorescence depolarization measurements reflected an influence of intersubunit motion (fast) on the overall motion (slow) of the native hormone. This interpretation was based on the assumption that fluorescein was rigidly bound and experienced only the overall rotational diffusion of the 14 000-dalton subunits or 28 000-dalton intact hormone. The possibility of segmental motion(s) of the fluorescein-bound region(s) was not considered in this interpretation, although the data would not have excluded such motions if they were present. The local or segmental motions in proteins are expected to be faster than the overall motion of the protein and could lead to a ϕ_A shorter than the actual ϕ (ϕ_p) for the protein. Furthermore, the number of fluorescein binding sites was not known, and the possibility of fluorescein–fluorescein energy transfer could not be ruled out.

In order to circumvent the problems of multiple site labeling and possible structural perturbations with extrinsic fluorescent ligands, we took advantage of the intrinsic tryptophan fluorescence of hlH in studying intersubunit interactions and rotational dynamics of hLH. The only tryptophan residue of hLH occurs in position 8 of the β-subunit (β hLH) (Pierce & Parsons, 1981). The presence of a single tryptophan is especially attractive because the assignment of the steady-state fluorescence signal can be unambiguously made, although the possibility of heterogeneous lifetime even for a single tryptophan certainly exists. If this tryptophan residue is rigidly frozen in the protein matrix, its steady-state depolarization would be determined by the overall rotational motion of the protein. However, Lakowicz and co-workers (Lakowicz et al., 1983) presented evidence for local motional freedom of tryptophan within the protein matrix in several proteins, using lifetime-resolved anisotropy measurements. In the study reported here, we used the same technique, which involves steady-state fluorescence anisotropy measurements under conditions of oxygen quenching, to examine the possibility of segmental tryptophan motion in native hLH and in βhLH. Furthermore, we measured the temperature dependence of tryptophan fluorescence anisotropy to estimate the correlation times for rotational diffusion of hLH and βhLH.

The study of subunit interaction in hLH was further aided by our observation that the relative fluorescence intensities of the same tryptophan residue were markedly different when intact hLH and the subunit βhLH were compared. The ki-

† Supported by NIH Grants HD 9140 C3 (R.J.R.) and GM 34847 (F.G.P.) and by the donors of the Petroleum Research Fund, administered by the American Chemical Society (G.S.).

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0006-2960/87/0426-1860$01.50/0

netics of dissociation of hLH into subunits and reassociation of subunits were thus conveniently monitored by using this difference in fluorescence intensity as a signal. A comparative analysis of changes in tryptophan fluorescence spectra of hLH and human follicles-stimulating hormone (hFSH), that accompanied their dissociation into respective subunits, suggested significant differences in the tryptophan environments of these two glycoprotein hormones.

Materials and Methods

Highly purified hLH and hFSH were prepared by using methods described earlier (Ryan, 1968; Bishop & Ryan, 1973). The subunit βhLH was isolated and purified according to the method of Bishop and Ryan (1973). The homogeneity of the preparation was examined by sodium dodecyl sulfate gel electrophoresis. The potency of the preparations was monitored by radioimmunoassays (Faiman & Ryan, 1967; Prentice & Ryan, 1975) and radioreceptor assays (Lee & Ryan, 1973). Protein concentrations were determined by the weight of lyophilized samples on a Cahn 25 electrobalance. All other chemicals used were of reagent grade. Native hLH samples were buffered at pH 7.0 with 20 mM *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES)–100 mM NaCl. Ultraviolet (UV) absorption spectra were recorded on an SLM-Aminco DW 2C spectrophotometer.

Fluorescence spectra were measured by using an SLM Smart 8000 spectrofluorometer. For tryptophan emission spectra and intensity measurements, hLH samples were excited at 295 nm because excitation at 280 nm resulted in a tyrosine-dominated emission spectrum (see Results). Excitation and emission bandwidths were respectively 2 and 8 nm. The spectra were corrected for the wavelength dependence of phototube response.

Tryptophan fluorescence anisotropy of hLH and βhLH was measured in an SLM 4800 spectrofluorometer according to the method described earlier (McDowell et al., 1985). The exciting light (at 300 nm) was vertically polarized and passed through a slit with 1-nm band-pass. The fluorometer was operated in the "L" format. A polarizer was also placed in the emission path. The intensities of vertically and horizontally polarized emission (I_V and I_H) were selected by using an interference filter of 5-nm band-pass centered at 350 nm. Steady-state anisotropies (r_{ss}) were calculated by using the equation $r_{ss} = (I_V - I_H)/(I_V + 2I_H)$.

Fluorescence lifetimes (τ) were measured by the phase-modulation method (Spencer & Weber, 1969), using an SLM 4800 phase fluorometer. The modulation frequency was 30 MHz, and the excitation wavelength was 295 nm. *p*-Terphenyl (τ = 0.93 ns) was used as a fluorescence lifetime reference solution to minimize wavelength-dependent and targeting artifacts in the value of τ (Lakowicz et al., 1981).

Acrylamide quenching of fluorescence intensities was achieved by progressive additions of 5- or 10-μL aliquots of a 5 M buffered acrylamide solution to 2.0-mL samples of the fluorescent materials. The measured fluorescence intensities were corrected for dilution and for the inner filter effect at high acrylamide concentrations. The bimolecular quenching constants (k_q) were obtained from Stern–Volmer plots using the relation $F_0/F = 1 + k_q\tau_0[Q]$ where F_0 and F are the total fluorescence intensities in the absence and presence of quencher, respectively, τ_0 is the lifetime in the absence of quencher, and [Q] is the quencher concentration.

Lifetime-resolved anisotropy measurements were carried out by reducing the excited-state lifetime of tryptophan in hLH samples using oxygen as a quencher with concomitant determination of the increased anisotropy (Lakowicz & Weber, 1980). Samples were equilibrated with increasing pressures of oxygen. A time period of 90 min was allowed for equilibration at each pressure. This equilibration period was selected on the basis of a study of quenching as a function of equilibration time at each oxygen pressure employed in our experiments. Excitation was at 300 nm, and anisotropy was measured as described above. Total intensity (F) values were calculated from the polarized emission intensities obtained in the same experiment using the relation $F = I_V + 2I_H$. The ratio of F/F_0 was used to calculate the lifetime (τ) for each oxygen pressure, after the lifetime in the absence of the quencher (τ_0) had been independently determined (see above). The plot of $1/r_{ss}$ vs. lifetime yielded the lifetime-resolved anisotropy [$r(0)$] and also the apparent rotational correlation time (ϕ_A) using the relation $r(0)/r = 1 + \tau/\phi_A$. F_0/F values were used to calculate bimolecular quenching constants (k_q) using Stern–Volmer plots. The value of $r(0)$ should equal the anisotropy of the "frozen" or "immobile" protein molecule (r_0) if the loss of anisotropy in the above experiment were only caused by a single overall motion of the protein.

Rotational correlation times were also measured by studying the temperature dependence of tryptophan fluorescence anisotropy. In this method, the dependence of the steady-state anisotropy (r_{ss}) on temperature (T) and viscosity (η) is monitored, and a Perrin plot of $1/r_{ss}$ against T/η is constructed (Lakowicz, 1983). The intercept of the Perrin plot yields a limiting anisotropy denoted $r(0)'$ (the prime is used for distinction from the measured lifetime-resolved anisotropy) which should equal r_0 if r_{ss} were solely governed by the rotational motion of the whole protein molecule. With the knowledge of lifetime, an apparent correlation time denoted ϕ_A' (the prime is used to distinguish this from the correlation time measured by the oxygen quenching technique) can be estimated by employing the relation $r(0)'/r = 1 + \tau/\phi_A'$.

Rotational motion data are expressed in this paper as correlation times (ϕ's). For comparison with papers [for example, see Bishop & Ryan (1975)] where protein motion is reported as rotational relaxation time (ρ), it should be noted that $\rho = 3\phi$.

Results

Intrinsic Fluorescence of hLH and βhLH. For many proteins that contain tryptophan and tyrosine residues, excitation at 280 nm yields predominantly a tryptophan emission spectrum frequently because of energy transfer from tyrosine to tryptophan (Lakowicz, 1983). As Figure 1A shows, upon excitation at 280 nm intact hLH yields a tyrosine emission spectrum with a maximum at 305 nm. Interestingly, βhLH, the subunit that contains the only tryptophan (tryptophan-8) residue of hLH, yields a tryptophan fluorescence spectrum upon excitation at 280 nm when examined as the isolated subunit (Figure 1A). The α-subunit contains four tyrosine residues (and no tryptophan), and βhLH has two tyrosines (and one tryptophan).

The tryptophan fluorescence spectrum of hLH was obtained by exciting the sample at 295 nm. This resulted in a tryptophan emission spectrum with a maximum at 336 nm (Figure 1B). It should be pointed out that the phenomenon of tyrosinate fluorescence emission (maximum at 345 nm), that is excitable at 295 nm, has been reported for some proteins containing tyrosines but no tryptophan (Szabo et al., 1978). The strongest evidence to support that the observed hLH spectrum for 295-nm excitation is indeed a tryptophan emission spectrum was obtained by reacting hLH with *N*-bromosuccinimide (NBS), a reagent specific for tryptophan (Spande & Witkop, 1967). We observed a 40% reduction of the

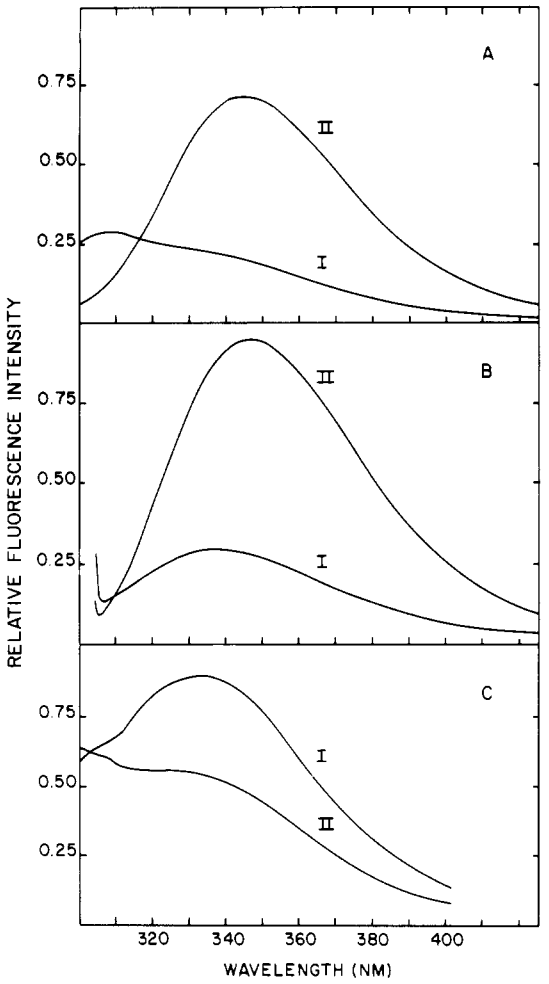

FIGURE 1: Intrinsic fluorescence spectra, in the 300–420-nm wavelength range, of hLH, βhLH, hFSH, and βhFSH (A). Spectra obtained upon excitation at 280 nm of (I) intact hLH and (II) isolated βhLH, both samples being at pH 7 and at identical concentrations (4.2×10^{-5} M). (B) Spectra obtained upon excitation at 295 nm of (I) intact hLH and (II) isolated βhLH, at pH 7 and for identical sample concentrations (4.2×10^{-5} M). (C) Spectra obtained upon excitation at 280 nm of (I) hFSH (pH 7) and (II) acid-dissociated hFSH (neutralized to pH 7), for identical sample concentrations (3.5×10^{-5} M). Excitation and emission band-passes were 2 and 8 nm, respectively. Note that spectra I and II within a single panel (A, B, or C) can be directly compared for relative fluorescence intensities. However, the relative intensities of spectra from one panel cannot be compared with those of the spectra from another panel.

fluorescence intensity of hLH at 336 nm upon reaction with NBS. This agrees with the finding of Giudice et al. (1978) of partial (40%) reaction of hLH with NBS and consequent diminution of the tryptophan circular dichroic signal (at 293 nm).

The tryptophan fluorescence emission spectra of intact hLH and free βhLH for 295-nm excitation are compared in Figure 1B at identical sample concentrations and under identical instrumental conditions. The βhLH spectrum showed an emission maximum of 344 nm which was red shifted by 8 nm relative to the peak of the hLH spectrum. The peak emission intensity of βhLH is 2.9-fold higher compared to that of hLH. In agreement with this observation, we have found that acid dissociation of hLH causes a similar increase in fluorescence intensity accompanied by red-shifted emission. This observation formed the basis of monitoring the kinetics of dissociation of hLH into its subunits (see below).

Contrasting Effects of Dissociation on hLH and hFSH Fluorescence. The intrinsic fluorescence spectra of native hFSH and acid-dissociated hFSH are shown in Figure 1C.

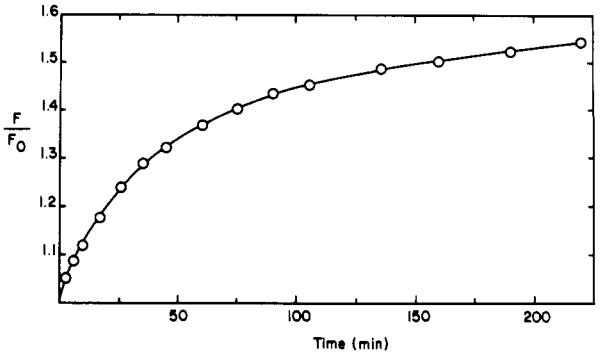

FIGURE 2: Time course for acid dissociation of hLH as measured by increase in fluorescence intensity. F_0 is the fluorescence intensity of hLH at pH 7.0 before addition of acid. F is the fluorescence intensity at a given time following treatment of the sample with HCl to lower the pH to 2.1. hLH concentration was 3.5×10^{-5} M. The sample was excited at 295 nm (band-pass = 2 nm), and emission was monitored through an interference filter centered at 350 nm with a band-pass of 5 nm. Kinetic analysis showed the dissociation process to be first order.

Two important contrasts with hLH are found. First, hFSH yields a tryptophan fluorescence spectrum upon excitation at 280 nm. Second, the acid-induced dissociation of hFSH into subunits is accompanied by a decrease in fluorescence intensity without a change in the wavelength of maximum emission (338 nm). Furthermore, the fluorescence spectrum of the acid-dissociated subunit of hFSH shows evidence of tyrosine emission in addition to tryptophan emission. Similar to hLH, the lone tryptophan of hFSH occurs in the β-subunit (βhFSH). There are nine tyrosine residues in βhFSH compared to two in βhLH. The α-subunit is identical for both hormones containing two tyrosine residues.

Kinetics of Acid Dissociation of hLH. We have found that the time course for the dissociation of hLH into its subunits can be directly monitored by following the increase in tryptophan fluorescence intensity accompanying dissociation. This is shown in Figure 2 where the relative enhancement of intensity following acid treatment of hLH is plotted as a function of time. The change in fluorescence intensity reaches 90% of completion (total increase was 2.1-fold) in 3.5 h. Sodium dodecyl sulfate (SDS) gel electrophoresis of hLH sample after 3.5 h of incubation in HCl and pH 2 showed no detectable amount of intact hLH but a virtually total conversion into subunits. The acid dissociation of hLH follows first-order kinetics with a rate constant of 0.013 min^{-1}. This agrees with the rate constant reported by Ingham et al. (1973), who used a noncovalently bound fluorescent probe, 8-anilino-1-naphthalenesulfonate (ANS), to monitor dissociation and reassociation of the hormone. We have also followed the reassociation kinetics of the acid-dissociated α- and β-subunits following titration of the dissociated mixture to pH 7 (data not shown). A decrease in tryptophan fluorescence intensity as a function of time was observed as expected. The total decrease in intensity was 1.9-fold. However, the data appeared to contain two or more kinetic phases and did not satisfactorily fit either first-order or second-order kinetics. The latter observation is in agreement with data reported by Ingham et al. (1973), who used ANS as a fluorescent probe of the reassociation kinetics.

We have evidence from near-ultraviolet CD spectra and radioreceptor assays that the acid-dissociated subunits do not reassociate to the active native hLH upon pH neutralization, although they do recombine to yield an αβ-disubunit molecule. All of the results that we report on βhLH were obtained with purified βhLH and not with the acid-dissociated subunit.

Table I: Emission Maxima, Anisotropies, Lifetimes, Solvent Perturbation, and Acrylamide Quenching Constants for Tryptophan Fluorescence in Intact hLH and Free βhLH[a]

sample	λ_{max}^{em} (nm)	r_{ss}[b] at 25 °C	τ[c] (ns)	F_{D_2O}/F_{H_2O}[d]	K_{sv} (M^{-1})[e]	$k_q \times 10^{-9}$ (M^{-1} s^{-1})[f]
hLH	336	0.137	3.6	1.2	5.9	1.6
βhLH	344	0.140	2.6	1.5	8.2	3.2

[a] All measurements were in an aqueous buffer containing 20 mM TES and 100 mM NaCl at pH 7.0, except for measurements of spectra in D_2O and H_2O (see footnote *d*). [b] r_{ss} represents steady-state anisotropy values obtained at 25 °C for an excitation wavelegth of 300 nm and an interference filter of 5-nm band-pass centered at 350 nm placed in the emission path. [c] Lifetime (τ) values were measured by using the phase-modulation method for a modulation frequency of 30 MHz. The average of the phase and the modulation times (τ_p and τ_m) was taken. [d] Fluorescence spectra were taken in H_2O and in 99.8% D_2O. The ratio of the intensities at the wavelength of maximum emission was calculated. D_2O did not change this wavelength. [e] K_{sv} is the slope of the linear Stern–Volmer plots (Figure 5) and represents the inverse of acrylamide concentration at the point of 50% quenching. [f] k_q is the rate constant for quenching of the tryptophan fluorescence by bimolecular collisions with acrylamide. These were calculated from the Stern–Volmer plots (Figure 5) by using the equation given under Materials and Methods.

Effect of D_2O on hLH and βhLH Fluorescence Spectra. The lifetime of a completely solvent-exposed tryptophan fluorescence is increased about 2-fold when the solvent is changed from H_2O to D_2O (Gudgin et al., 1981, 1983). Thus, the effect of D_2O on the tryptophan fluorescence of a protein can be used as a measure of the degree of exposure of the tryptophan residue to solvent. As shown in Table I, the effect of D_2O on the fluorescence intensity of hLH at 336 nm (emission maximum) is 1.2-fold. The effect of D_2O on the fluorescence intensity of βhLH at 344 nm (emission maximum) is 1.5-fold. Thus, the tryptophan residue in βhLH is more solvent-exposed than in intact hLH. D_2O did not change the wavelength of maximum emission in either case.

Accessibility of Tryptophan of hLH and βhLH to Acrylamide. We studied the relative accessibility of tryptophan in the intact hLH and βhLH to acrylamide, an efficient quencher of tryptophan fluorescence. The stern–Volmer plots, assuming that quenching is dynamic (collisional), are linear as shown in Figure 3. The slopes of these lines (Stern–Volmer constants or K_{sv}) are 5.9 and 8.2 M^{-1}, respectively, for native hLH and βhLH. Using fluorescence lifetimes of 3.6 and 2.6 ns, respectively, for hLH and βhLH, we calculated the bimolecular quenching constants (k_q) to be 1.6×10^9 M^{-1} s^{-1} and 3.2×10^9 M^{-1} s^{-1} (Table I). Thus, the acrylamide accessibility of the tryptophan residue in βhLH is 2-fold higher compared to that in native hLH. This finding is consistent with the larger D_2O effect on and the relative red shift of βhLH fluorescence compared to hLH fluorescence.

Fluorescence Anisotropy of hLH and βhLH. The tryptophan fluorescence anisotropy of hLH was measured at 25 °C to be 0.137 ± 0.002. Interestingly, the tryptophan anisotropy was not decreased in isolated βhLH, for which a value of 0.140 ± 0.001 was obtained at 25 °C. In principle, this is surprising because the molecular volume of βhLH is half of that of hLH. Thus, the anisotropy of βhLH is expected to be less than that of hLH. The explanation of this result is a decreased lifetime of the tryptophan in the β-subunit. We found the tryptophan lifetimes of intact hLH and isolated βhLH to be respectively 3.6 and 2.6 ns. Since a decrease in anisotropy is a result of rotational diffusion during the lifetime of the fluorophore, a reduction in the lifetime will, to a degree, compensate this decrease. If the tryptophan lifetime for βhLH were the same as that of hLH (3.6 ns) after dissociation, the anisotropy of βhLH would decrease to 0.114, assuming a calculated rotational correlation time of 5.2 ns for a 14000 molecular weight subunit and a limiting anisotropy [$r(0)'$] value of 0.192 obtained from temperature dependence of anisotropy data (see below).

Estimation of Tryptophan Motion in hLH and βhLH. Oxygen quenching of tryptophan fluorescence has been used to estimate segmental rotational mobility (ϕ_s) and the intramolecular dynamics of proteins (ϕ_p) (Lakowicz & Weber, 1973; Lakowicz et al., 1983). The fluorescence anisotropy is

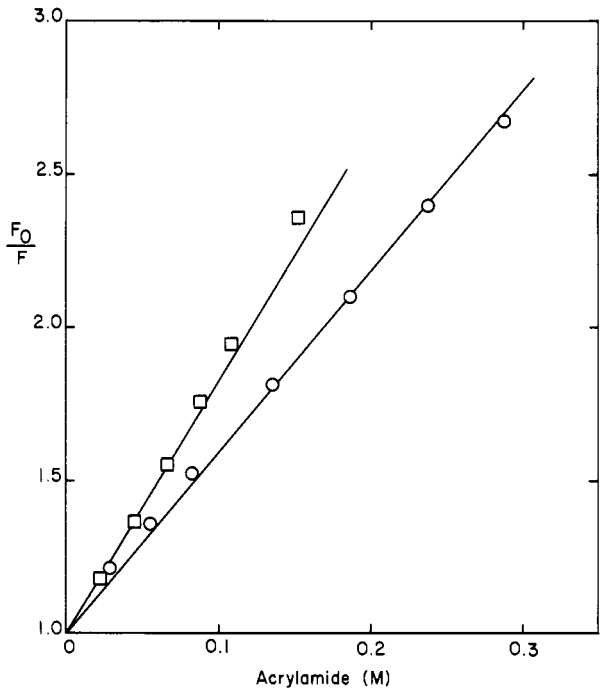

FIGURE 3: Stern–Volmer plots for acrylamide quenching of tryptophan fluorescence of intact hLH (○) and free βhLH (□). F_0 is the fluorescence intensity in the absence of acrylamide, and F is the fluorescence intensity at the indicated concentration of acrylamide. Sample concentrations were 3.6×10^{-5} M, and the pH was 7.0 in each case. The samples were excited at 295 nm (band-pass = 2 nm), and the emitted light was passed through an interference filter of 5-nm band-pass centered at 350 nm.

inversely proportional to the fluorescence lifetime which means that the anisotropy values must increase as the extent of quenching by oxygen increases. If the tryptophan residue experiences significant local freedom of motion, the results of these so-called lifetime-resolved anisotropy measurements yield an apparent correlation time (ϕ_A) which is a superposition of the segmental or local fluorophore motion on the overall motion of the protein. In principle, if the rotational motion of the protein is known from other hydrodynamic data, the contribution of segmental mobility to the measured anisotropy can be calculated directly by assuming a particular model of tryptophan side chain mobility (Lipari & Szabo, 1980; Lakowicz et al., 1983). On the other hand, if the rotational correlation time of the protein is not known a priori, then an appropriate method must be used to deconvolute the lifetime-dependent anisotropy values. Theory predicts that a modified Perrin plot of $1/r(\tau)$ vs. τ should be curvilinear with the curvature being greatest at short lifetimes presumably representing the fast local motions of segments or of the indole side chain (Lipari & Szabo, 1980; Lakowicz et al., 1983). Experimentally, however, such short lifetimes (<1 ns) are often inaccessible, and consequently, the curvature is not observed.

Table II: Rotational Correlation Times and Limiting Anisotropies for Intact hLH and Free βhLH Estimated from Tryptophan Fluorescence Anisotropy Measurements[a]

sample	M_r	ϕ_A[b] (ns)	ϕ_A'[c] (ns)	ϕ_p[d] (ns)	$r(0)$[b]	$r(0)'$[c]	r_0[e]
hLH	28 000	6.0	10.6	10.1	0.221	0.177	0.273
βhLH	14 000	4.9	6.5	5.2	0.212	0.192	

[a] Rotational correlation times were obtained at 25 °C and pH 7.0. [b] Apparent correlation time (ϕ_A) and limiting anisotropy [$r(0)$] were obtained from steady-state anisotropy measurements under oxygen quenching conditions. [c] Apparent correlation time (ϕ_A') and limiting anisotropy were obtained from Perrin plots for temperature and viscosity dependence of anisotropy. [d] Calculated rotational correlation time for the protein (ϕ_p) based on hydrodynamic data. [e] Fluorescence anisotropy of motionally frozen tryptophan of hLH measured at −70 °C in a vitrified solution containing 85% glycerol.

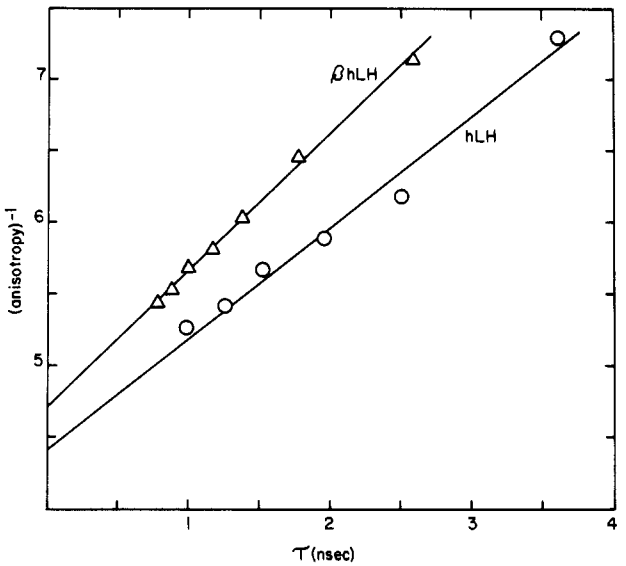

FIGURE 4: Lifetime-resolved tryptophan anisotropy plots for intact hLH and free βhLH (pH 7.0, 25 °C). Steady-state anisotropy values were measured at six oxygen pressures ranging from 0 to 1660 psi. An equilibration time of 90 min was allowed for each oxygen pressure. The unquenched tryptophan lifetimes of hLH and βhLH tryptophan were 3.6 and 2.6 ns, respectively. The quenched lifetime at each oxygen pressure was obtained on the same sample (see Materials and Methods). The sample was excited at 300 nm (band-pass = 1 nm) with vertically polarized light. Vertically and horizontally polarized emissions were observed through an interference filter of 5-nm band-pass centered at 350 nm.

In all of our experiments, the modified Perrin plots were linear, implying that we were observing only the influence of whole protein rotation in the measured anisotropy (Figure 4). However, as Lakowicz et al. (1983) have suggested, the linear plot of $1/r(\tau)$ vs. τ when extrapolated to the y axis should yield a value of r at $\tau = 0$ (nominally). This value, denoted $r(0)$, should be equal to the maximum limiting anisotropy (r_0) obtained for the fluorophore in a rigid medium. However, such consonance is seldom found, and the difference between r_0 and $r(0)$ may be related to the extent of motional freedom experienced locally by the fluorophore.

In three independent experiments where the lifetime (τ) dependence of steady-state anisotropy (r_{ss}) was measured, we obtained values of 6.0 ± 0.3 ns for ϕ_A of native hLH at 25 °C (pH 7.0). The lifetime-resolved anisotropy, $r(0)$, averaged to (r_{ss}) 0.221 ± 0.005 from these experiments. A representative plot of these data is shown in Figure 4. This value of $r(0)$ is appreciably less than the r_0 of 0.273 that we measured for presumably "immobile" tryptophan in hLH frozen in 85% glycerol at −60 °C. The latter r_0 is in agreement with the r_0 values reported for *N*-acetyl-l-tryptophanamide and several single tryptophan containing proteins (Lakowicz & Weber, 1973; Lakowicz et al., 1983). The most plausible interpretation of $r(0)$ being less than r_0 is a certain degree of motional freedom of the tryptophan residue in the protein matrix. Table II shows the comparison of ϕ_A derived from oxygen quenching experiments in comparison with the expected rotational correlation time for hLH. Assuming a hydrated rigid sphere model and using a value of 0.3 for hydration, we determined that the calculated rotational correlation time (ϕ_p) for hLH is 10.1 ns and the ratio of ϕ_p/ϕ_A is 1.68 ± 0.12. This suggests that while there is some contribution to ϕ_A from segmental motion (correlation time ϕ_s), the major contribution to ϕ_A is from ϕ_p. If the tryptophan is assumed to be an isotropic rotator with a single lifetime, the fraction of anisotropy lost due to segmental motion is calculated (Lakowic et al., 1983) from our data to be 0.19 [using mean $r(0) = 0.221$ and $r_0 = 0.273$]. This corresponds to an average angular displacement of 21° for tryptophan. It should be noted that from the data on the lifetime dependence of anisotropy, as shown in Figure 4, the correlation time for segmental motion alone cannot be directly derived. This is because the data do not extend to lifetimes shorter than 1 ns.

The steady-state anisotropy values for the purified βhLH were also measured under conditions of oxygen quenching of 25 °C and pH 7.0. The lifetime-resolved anisotropy, $r(0)$, obtained from a linear plot of $1/r_{ss}$ against τ, was 0.212 (Figure 4). This is less than the r_0 value of 0.273 for "frozen tryptophan", suggesting a limited degree of segmental tryptophan motion. Assuming an isotropic rotator model with a single lifetime and fast segmental motion, the fraction of anisotropy lost due to segmental motion is approximately 0.22, and the corresponding averae angular displacement of tryptophan is 22°. The ϕ_A obtained from the (anisotropy)$^{-1}$ vs. lifetime plot is 5.0 ns (using our measured lifetime of 2.6 ns). This agrees with the ϕ_p of 5.2 ns calculated from hydrodynamic data. Thus, while the smaller value of $r(0)$ compared to r_0 suggests segmental motion, ϕ_s for this motion is probably faster than even the shortest quenched lifetime.

Temperature Dependence of Anisotropy. The effects of variation of solvent temperature (and viscosity) on the steady-state anisotropies of native hLH and βhLH were studied to determine the rotational correlation times of these proteins by means of the Perrin plots. These data are shown in Figure 5. The plots are linear. The intercepts of the Perrin plots should yield the reciprocal anisotropy in the apparent absence of rotational diffusion [$1/r(0)'$]. For native hLH and βhLH, we obtain $r(0)'$ values of 0.177 and 0.192, respectively. These values are significantly lower than our measured r_0 values for frozen tryptophan in these proteins, again reflecting segmental tryptophan mobility. However, as pointed out by Lakowicz (1983) for fast segmental motion ($\phi_s < \tau$), the slope and the intercept of the Perrin plot are equally affected, canceling the effect of this motion on the calculated correlation time (ϕ_A'). From the Perrin plots, we obtain a ϕ_A' of 10.6 ns for native hLH (using a τ of 3.6 ns) and a ϕ_A' of 6.5 ns for βhLH (using a τ of 2.6 ns). These values are consistent with the calculated values of 10.1 and 5.2 ns, respectively, based on the assumptions of a rigid sphere model and using a value of 0.3 for hydration. As shown in Table II, the $r(0)'$ and ϕ_A' values obtained from the temperature-dependent Perrin plot are

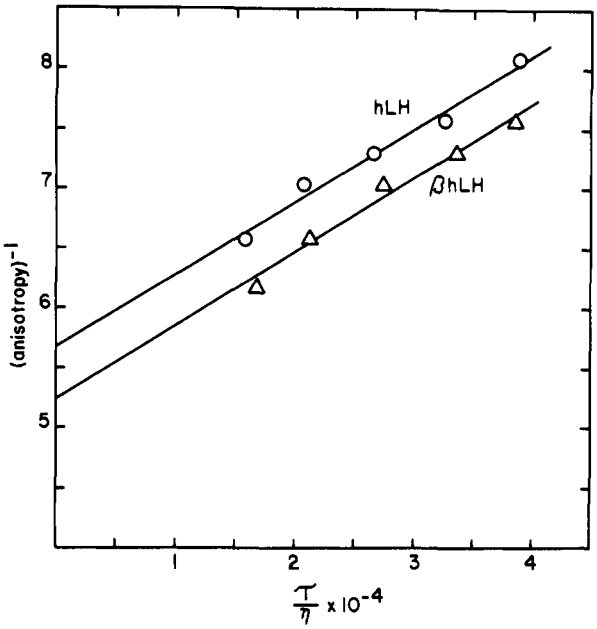

FIGURE 5: Perrin plots for the dependence of steady-state anisotropy on solvent temperature. The samples were excited at 300 nm (band-pass = 1 nm) with vertically polarized light. Vertically and horizontally polarized emissions were observed through an interference filter of 2.7-nm band-pass centered at 340 nm.

somewhat different from the $r(0)$ and ϕ_A values derived from the lifetime-resolved anisotropy data; $r(0)' < r(0)$ and $\phi_A' > \phi_A$. While these differences are not large, one possible explanation is that the shortest quenched lifetime in the oxygen quenching experiment may be approaching ϕ_s. Furthermore, changing the temperature, in studying the temperature dependence of anisotropy (Figure 5), could change the relative ^1L_a and ^1L_b energy levels of indole, thereby affecting the limiting anisotropy (Lakowicz & Weber, 1980).

Discussion

The single tryptophan residue of hLH shows markedly different fluorescence properties depending on whether the tryptophan-containing βhLH subunit is free or associated with αhLH. We have shown in this report that the dependence of the intrinsic tryptophan fluorescence intensity on the state of association can be conveniently used to monitor the kinetics of dissociation and reassociation of the hLH subunits. In some earlier studies on subunit interactions, advantage was taken of the sensitivity of fluorescence intensity of the noncovalently bound extrinsic probe ANS to the association state of the hormone (Ingham et al., 1973, 1975a). Induction of oligomer formation was, however, recognized as a problem in this method (Ingham et al., 1975b). The relative fluorescence intensity of βhLH is much greater than that of intact hLH in spite of the tryptophan lifetime in βhLH being shorter (2.6 ns) than that in hLH (3.6 ns). The increased emission intensity and red-shifted emission maximum of βhLH suggest a greater degree of solvent exposure for tryptophan in the free subunit. The latter contention is supported by the greater D_2O-induced increase in intensity and greater susceptibility to acryamide quenching observed for βhLH compared to that found for intact hLH fluorescence. Our observation that upon excitation at 280 nm the intact hLH yields tyrosine fluorescence as opposed to the tryptophan emission exhibited by free βhLH suggests that the fluorescence from the two tyrosine residues of the α-subunit is not quenched in hLH, thereby greatly masking the tryptophan fluorescence. In contrast, for hFSH which has an α-subunit identical with that of hLH, the tyrosine emission is suppressed, and a tryptophan fluorescence spectrum is obtained upon excitation at 280 nm. These observations suggest that the energy-transfer process from tyrosines in the α-subunit to the single tryptophan in the β-subunit is more efficient in hFSH compared to hLH. This is further supported by our finding that removal of the α-subunit causes a decrease in the fluorescence intensity of hFSH as opposed to a fluorescence enhancement in the case of hLH. These spectral differences between hLH and hFSH possibly reflect a difference in the relative distances and orientations between the single tryptophan and multiple tyrosine residues in the tertiary structures of the two proteins. The three-dimensional structure of none of these glycoproteins has been elucidated. However, the tryptophan residue occurs in different locations of their β-subunits, at positions 8 and 33 in hLH and hFSH, respectively (Pierce & Parsons, 1981). It appears from our data that tryptophan-33 of βhFSH is more suitably located to serve as an energy acceptor for the two possible tyrosine donors of the α-subunit.

We have determined the rotational correlation times for hLH and βhLH using the single tryptophan residue in βhLH as the intrinsic fluorescence probe. Furthermore, our data indicate a contribution (about 20%) from a fast (subnanosecond) local tryptophan motion in the decay of fluorescence anisotropy of βhLH. A similar extent of contribution from a fast motion in the tryptophan anisotropy decay of intact hLH leads us to believe that the limited motional freedom of tryptophan is retained when βhLH associated with the α-subunit. Local tryptophan motion, independent of the overall rotational diffusion of the protein, has been found in several other proteins to cause angular displacement of the tryptophan residue by 0–37° in subnanosecond time ranges (Lakowicz et al., 1983). Such local motions in the 0.2–10-ps time scale had been predicted from molecular dynamics calculations for tryptophan (Ichiye & Karplus, 1983) and tyrosine (McCammon et al., 1979; Levy & Szabo, 1982). These segmental motions must be taken into account in interpreting fluorescence depolarization of proteins.

The apparent rotational relaxation times ($\rho_A = 3\phi_A$) for hLH and its subunits (acid-dissociated) were also determined in an earlier study by Bishop and Ryan (1975) from the temperature and viscosity dependence of fluorescence depolarization. However, as opposed to the intrinsic tryptophan fluorescence utilized in the work we report here, they used the fluorescence signal from the extrinsic probe fluorescein covalently bound to hLH. Despite this difference in these two approaches, some important observations can certainly be made about the results of Bishop and Ryan (1975) especially in light of our new data. The data of Bishop and Ryan (1975) yielded a ρ_A for intact hLH (19 ns) which was 42% faster than the relaxation time (ρ_p) calculated from hydrodynamic data assuming a hydrated sphere model (33 ns). For the acid-dissociated subunits, ρ_A (14 ns) agreed more closely, within 15%, with ρ_p (16.5 ns). The data were interpreted to indicate that the subunit retained a significant degree of relative mobility in the intact hormone. The faster subunit motion was then thought to have influenced the determination of ρ_A for the intact hormone by providing an alternative means for the decay of anisotropy. The alternative possibility of segmental motional freedom of the fluorescein-bound regions influencing the data was ruled out. Fluorescein was assumed to be rigidly bound at all sites (the number of fluorescein binding sites is not known). We note, however, that the Perrin plots reported by Bishop and Ryan (1975) yield limiting anisotropy, $r(0)$, values of 0.182 for both intact hLH and its (acid-dissociated)

subunits. This value is substantially less than the r_0 for motionally frozen fluorescein which we determined in a vitrified glycerol solution at −70 °C to be 0.364. This suggests the presence of segmental fluorescein motions in the individual subunit(s) and thus makes a case for the interpretation of the data on intact hLH in terms of segmental mobility (of one or more of the fluorescein-bound sites) as an alternative to intersubunit mobility.

We reemphasize that in the analysis of our fluorescence anisotropy data we have assumed that the tryptophan fluorescence of hLH decays with a single lifetime as an isotropic rotator. This is a simple but limited model which allowed us to gain some valuable insight into the problem that we addressed here. The distribution of conformational state(s) of this single tryptophan residue of hLH and the resultant fluorescence decay pattern are currently being investigated.

Added in Proof

More recent multifrequency phase fluorometry experiments using 20 modulation frequencies ranging from 8 to 200 MHz have shown heterogeneity in the lifetime of the single tryptophan in both βhLH and hLH. These data yield an average tryptophan lifetime of 3.2 and 4.2 ns, respectively, for βhLH and hLH. Thus, the relatively shorter average lifetime for βhLH reported in this paper has been validated, and the general conclusions of this paper remain unaltered.

Acknowledgments

We thank Kathleen Kitzmann and Kenneth Peters for expert technical assistance. We are grateful to the late Julie Kisiel and Sharon Gormley for preparing the manuscript.

Registry No. hLH, 9002-67-9; L-tryptophan, 73-22-3.

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