

# THE INTRINSIC TYROSINE FLUORESCENCE OF HISTONE H1

## Steady State and Fluorescence Decay Studies Reveal Heterogeneous Emission

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**ABSTRACT** In wavelength-resolved steady state spectra we observe three different kinds of emission from histone H1, a class A protein with only a single tyrosine residue. Unfolded H1 emissions that peak at ~300 and 340 nm can both be excited maximally at ~280 nm. Another, peaking much further to the red at ~400 nm, can be excited maximally at ~320 nm. The 300-nm fluorescence can be resolved by lifetime measurements into three components with decay times of ~1, 2, and 4 ns. On sodium-chloride-induced refolding of H1, simplification of the emission properties occurs. The 340 and 400-nm components disappear while the two shorter lifetime components of the 300-nm band diminish in amplitude and are replaced by the 4-ns decay. We believe that the 340-nm emission is tyrosinate fluorescence resulting from excited-state proton transfer. The origin of the 400-nm emission remains uncertain. We assign the 1 and 2-ns components of the 300-nm emission to two states of tyrosine in denatured H1 and the 4-ns decay to fluorescence of the single tyrosine residue in the globular region of refolded H1. Our results support the contention that salt induced folding of H1 is a cooperative two state process, and permit us to better understand the previously reported increases in fluorescence intensity and anisotropy on salt-induced folding.

### INTRODUCTION

Histones are class A proteins. Unlike class B proteins, which also contain tryptophan, class A proteins contain only the aromatic amino acids tyrosine and phenylalanine, and are unique in that their fluorescence derives solely from the tyrosine. (See references 1 and 2 for reviews on the fluorescence properties of class A proteins.)

Tyrosine fluorescence has been used extensively to study histone conformational changes and cross complexing (3–5). More recently we have used tyrosine emission of the inner histones to study conformational changes induced in nucleosome core particles by changes in ionic strength and pH (6–8). These studies made use of changes in the steady state intensity and anisotropy of the fluorescence, but to date the sources of these changes have not been understood. The experiments on fluorescence properties of histone H1 described here were undertaken in order to better understand how conformational changes affect tyrosine fluorescence.

Fluorescence lifetime measurements form an essential part of this work. Although numerous fluorescence lifetime studies on class B proteins have been reported, only a handful of studies have reported on the decays of tyrosine containing peptides (9–11) and class A proteins (11–13). This lack of decay work on tyrosine has been primarily due to instrumental and data analysis difficulties. It has been difficult in the past to measure tyrosine decays, because

tyrosine in native proteins has a low quantum yield, usually in the range of 0.02 to 0.05 (1), and because tyrosine also has a relatively low extinction ( $\epsilon_{275} = 1,340 \text{ M}^{-1} \text{ cm}^{-1}$ ) (14). Since traditional light sources for decay instruments have low intensities in the ultraviolet (UV) region where tyrosine absorbs, it has been difficult to obtain adequate signal levels for measuring high quality lifetime data. These difficulties are now largely overcome by new laser sources and by new data analysis procedures like those used in this work.

Here we report our studies on the intrinsic tyrosine fluorescence of histone H1, a class A protein with a single tyrosine residue near position 72 within the highly conserved globular region of the molecule (15, 16). H1 in water is denatured, but on the addition of salt it undergoes a cooperative refolding to a more ordered structure (17, 18). This refolding is accompanied by increases in tyrosine fluorescence intensity (17, 19) and anisotropy (17). Because H1 contains only a single tyrosine residue, we expected to find relatively simple fluorescence properties. Instead the results are complex. By using a combination of wavelength-resolved steady state and fluorescence lifetime measurements, we have been able to resolve five different emitting species.

### MATERIALS AND METHODS

Crude H1 was prepared from calf thymus chromatin by the method of Johns (20), yielding an acetone-dried powder. Sodium bisulphite was used

to inhibit protease activity. The H1 was purified on a large ( $7.5 \times 450$  cm) Sephadex G-100 column equilibrated with 0.01 N HCl. The middle fractions of the H1 band were lyophilized and stored desiccated at  $-80^{\circ}\text{C}$ . A sodium dodecyl sulfate (SDS) polyacrylamide gel of the H1 is shown in Fig. 1. Only with severe overloading could any minor bands be observed and we estimate these bands to constitute  $<1\%$  of the total protein. Stock solutions of H1 were first prepared in water and were diluted by the addition of appropriate solutions to reach the final conditions of ionic strength and pH. The final H1 concentration was  $7 \times 10^{-5}$  M. Other experimental details can be found in the figure captions.

Steady state fluorescence intensity and anisotropy were measured on a computer controlled polarization spectrometer that has been modified considerably since its original description (21). Two arc lamps are used as excitation sources. A 450-W xenon lamp with a relatively smooth wavelength profile is used for measuring spectral changes and a 1,000-W mercury-xenon lamp is used to excite samples with high intensity at individual wavelengths. Tandem Jarrell-Ash quarter meter monochromators (Jarrell-Ash Div., Fisher Scientific Co., Waltham, MA) are used to limit the excitation bandwidths and to reduce spurious light. Emitted fluorescence is measured through additional monochromators and appropriate cut-off filters.

The steady state instrument is under the control of a dedicated PDP 8/e computer (Digital Equipment Corp., Maynard, MA). Polarizer positioning and wavelength scanning are driven by stepping motors controlled by the computer. Computer programs automatically correct for photomultiplier dark current and the differential sensitivity of the two arms of the instrument, which measure the two polarization directions. Data are transferred to a PDP 11/34 computer (Digital Equipment Corp.) for processing, wavelength-dependent corrections, and plotting.

Fluorescence lifetime data were obtained on a monophoton decay fluorometer, which has been recently described in the literature (22). Samples were excited with frequency doubled output from a Spectra Physics synchronously pumped cavity-dumped dye laser system (Spectra-Physics Inc., Mountain View, CA) pumped by a mode-locked argon ion laser. Fluorescence was detected using an RCA C31000M photomultiplier (RCA, RCA New Products Div., Lancaster, PA). (We have recently shown that the more commonly used RCA 8850 photomultiplier

gives rise to a luminescence background when used at these ultraviolet (UV) wavelengths, and that this background can effect the resolution of decays (23). Fluorescence decay data were analyzed using the method of moments (24) with moment index displacement (MD) (25-27), and the results were interpreted using lambda invariance plots (28).

## RESULTS

The steady state fluorescence of chromatographically pure calf thymus H1 is surprisingly complex, consisting of three distinct emitting species. We will refer to them as the 300-nm, 340-nm, and 400-nm components, based on observed maxima in the emission spectra. The 300-nm component has excitation and emission properties characteristic of normal tyrosine fluorescence. The unusual 340-nm and 400-nm components cannot be removed by dialysis against 8 M urea followed by removal of the urea; they are thus covalently attached to high molecular weight molecules. Both are observed for H1 denatured at very low ionic strength and diminish greatly in intensity on salt-induced refolding of the protein.

H1 dissolved directly in water to a final concentration of 0.1 mM has a pH of 4 (due to the method of preparation). Under these conditions the protein is extensively unfolded (17). Fig. 2 shows excitation and emission spectra for H1 in water at pH 4. (Solvent background was subtracted to obtain these results.) The corresponding spectra of tyrosine in water (dotted curves) are included for comparison; they are not affected by pH in the range 5 to 8 nor by the monitoring wavelengths. For emission at 305 nm the excitation spectrum of H1 in water (solid line, Fig. 2A) closely resembles that of tyrosine. With emission at 340 nm

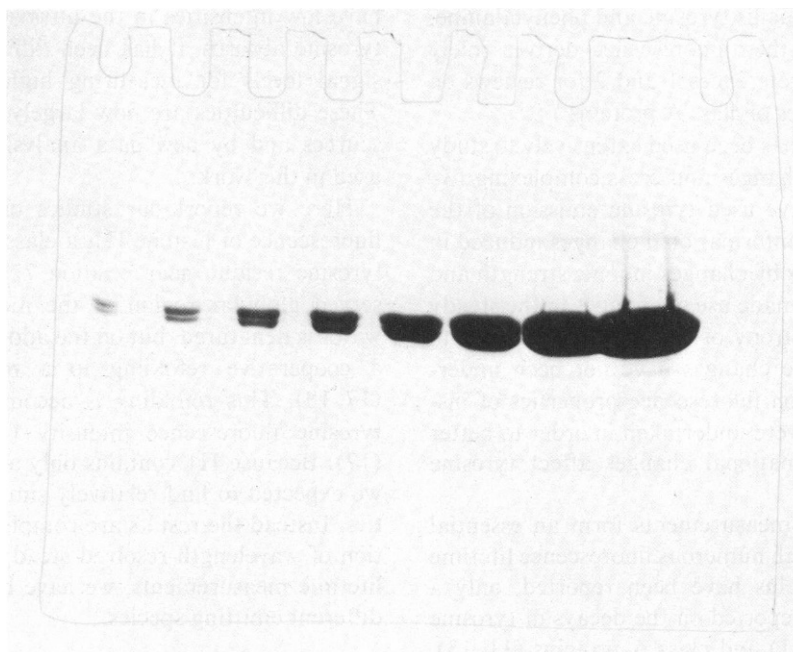


FIGURE 1 SDS polyacrylamide gel electrophoresis of histone H1. The gel was run as described in reference 6 except that the ratio of acrylamide to bisacrylamide was 37.5:1. The maximum amount of H1 loaded was  $48 \mu\text{g}$  (right lane) with progressive twofold decreases in the other lanes. The double band seen at the left is typical of unfractionated H1.

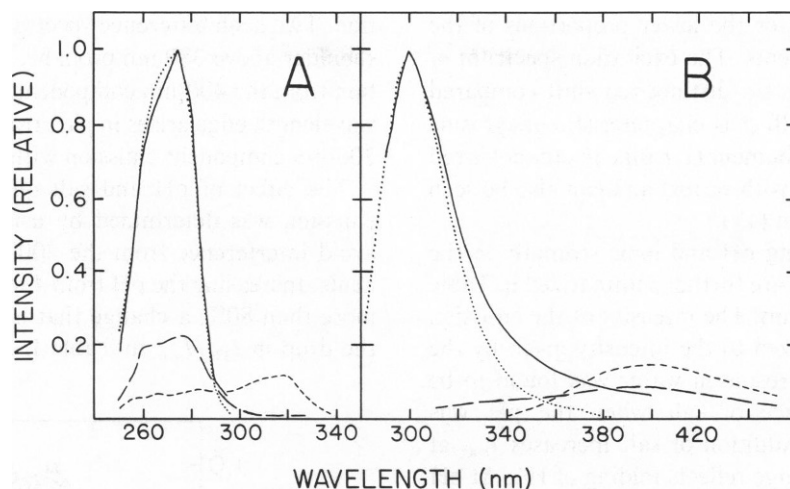


FIGURE 2 Fluorescence excitation and emission spectra for calf thymus H1 in water at pH 4. (A) Excitation spectra with emission collected at 305 nm (solid curve), 340 nm (long dashed curve), and 380 nm (short dashed curve). (B) Emission spectra with excitation at 280 nm (solid curve), 295 nm (long dashed curve), and 320 nm (short dashed curve). The dotted curves are those for tyrosine in water using 305-nm emission in A and 280-nm excitation in B. For each spectrum the solvent background (primarily Raman scattering from water) has been subtracted, corrections for variation of the xenon lamp intensity with excitation wavelength have been applied, and corrections for wavelength dependence of the emission sensitivity have been made. The spectra for tyrosine, for H1 emitting at 305 nm (A), and for H1 excited at 280 nm (B) have been arbitrarily normalized to a maximum value of 1. The other spectra are given relative to the normalized H1 results.

the excitation spectrum is somewhat broadened (long dashes) but is otherwise similar to that for 305 nm. In contrast, monitoring the emission at 380 nm results in a much broader excitation profile with a peak near 320 nm (short dashes), which clearly indicates a distinct fluorescent species. This species is the 400-nm component as is shown by the emission spectrum obtained when the sample is excited at 320 nm (Fig. 2 B, short dashes).

When we reduce the excitation wavelength to 295 nm (long dashes), we observe mainly the 400-nm component. However, the emission curve is broadened at shorter wavelengths in a manner that cannot be attributed to normal tyrosine emission. This broadening is mainly due to a fluorescent component that emits at 340 nm. (Difference spectra can be used to approximate the shape of the

emission for this component as shown below.) When the H1 is excited at 280 nm, the spectrum is clearly dominated by normal tyrosine emission; however, above 310 nm large contributions by the 340-nm and 400-nm components are evident.

The proportions of the three components are strongly dependent on the pH and salt concentration, as is illustrated by the excitation and emission spectra of H1 in 0.01 M sodium phosphate, pH 7.5, in Fig. 3 A. The 400-nm component has virtually disappeared while the contribution of the 340-nm component has decreased to a relatively minor perturbation of the emission with 280-nm excitation. The emission intensity of the 300-nm component increases about twofold with increased pH and ionic strength as a result of partial refolding of the H1; however, this change is

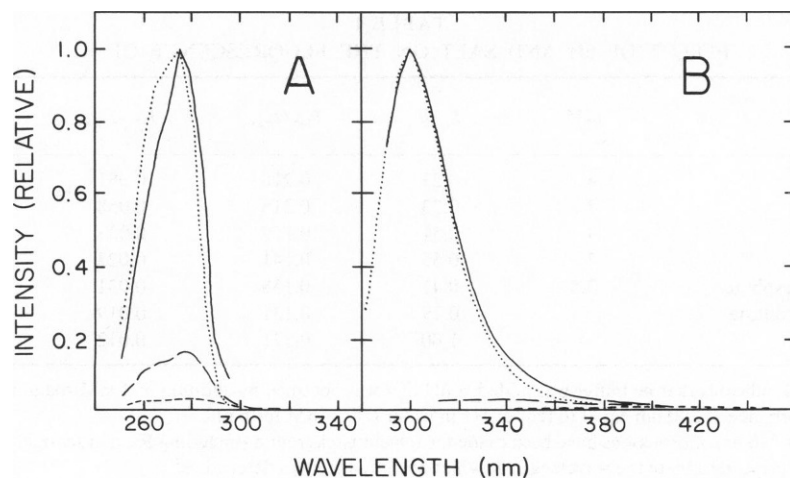


FIGURE 3 Fluorescence excitation and emission spectra for calf thymus H1 in 0.01 M sodium phosphate, pH 7.5. Further information is as for Fig. 2.

not sufficient to account for the lower proportions of the 340 and 400-nm components. The excitation spectrum of the 305-nm emission shows a distinct red shift compared with H1 in water. This effect is characteristic of tyrosine entering a less polar environment (tyrosine in ethanol is red shifted  $\sim 4$  nm compared with water) and can also be seen in the absorption spectrum (17).

The effects of increasing pH and ionic strength on the emission properties of H1 are further summarized in Table I. Excitation was at 280 nm. The intensity at the emission maximum ( $I_{\max}$ ) normalized to the intensity given by the same concentration of tyrosine in water was found to be unaffected in the absence of salt when the pH was increased from 4 to 7. Addition of salt increases  $I_{\max}$  at either pH 4 or 7; this change reflects folding of H1. At pH 7 and 0.1 M NaCl the degree of folding can be estimated from the data of Smerdon and Isenberg (17) to be  $\sim 50\%$ . Very high ionic strength is required for complete refolding as is indicated by the result in 0.5 M  $(\text{NH}_4)_2\text{SO}_4$  that, by extrapolation, results in  $\sim 95\%$  folded H1.

The 400-nm component makes little contribution to the emission intensity at 340 nm (Fig. 2 B). Thus the ratio  $I_{340}/I_{\max}$  listed in the table provides an indication of the relative contribution of the 340-nm component. For fully folded H1 this ratio should fall between 0.121, the value for tyrosine in water, and 0.131, the value for H1 in  $(\text{NH}_4)_2\text{SO}_4$ . Assuming a value of 0.126 a more useful parameter, the absolute intensity of the 340-nm component, can be estimated; values are listed in the last column of the table. It is apparent that changing the pH from 4 to 7 in the absence of salt has little or no effect on the 340-nm component. Addition of salt at pH 7 reduces the 340-nm contribution dramatically.

An approximate shape for the 340-nm component emission can be obtained as a difference spectrum for H1 in water minus H1 in salt (both at pH 7). Since the 300-nm component changes intensity under these conditions, it was necessary to normalize the spectra to  $I_{\max}$  before subtraction.

Two such difference spectra are shown in Fig. 4. The shoulder above 380 nm probably reflects a small contribution from the 400-nm component; also the rather steep low wavelength edge arises in part from a small red shift of the 300-nm component emission with increasing salt.

The effect of pH and salt on the 400-nm component emission was determined by using 312-nm excitation to avoid interference from the 300-nm and 340-nm components. Increasing the pH from 4 to 7 results in a decrease of more than 80%, a change that is also clearly reflected by the drop in  $I_{380}/I_{\max}$  in Table I. Addition of salt at pH 7

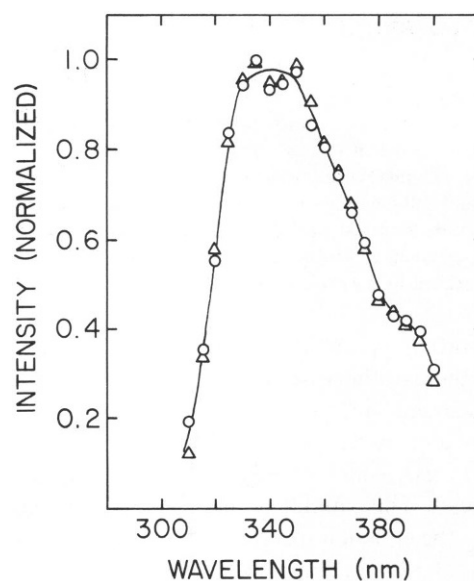


FIGURE 4 Fluorescence emission spectrum of the 340-nm component of H1. These results were obtained as difference spectra when the emission of H1 in 2 mM sodium cacodylate, pH 7, containing either 0.1 M NaCl (open circles) or 0.5 M  $(\text{NH}_4)_2\text{SO}_4$  (triangles) was subtracted from that of H1 in 2 mM sodium cacodylate, pH 7. Before subtraction, the individual spectra were normalized to the result at 300 nm in order to eliminate effects of changes in the intensity of the 300-nm component emission.

TABLE I  
EFFECT OF pH AND SALT ON THE FLUORESCENCE OF H1

Sample	pH*	$I_{\max}$ §	$I_{340}/I_{\max}$	$I_{380}/I_{\max}$	Intensity of the 340-nm component
H1 in water	4	0.23	0.218	0.081	1.00
	7	0.23	0.215	0.058	$0.97 \pm 0.01$
H1 in 0.1 M NaCl	4	0.51	0.159	0.038	$0.79 \pm 0.08$
	7	0.55	0.141	0.023	$0.38 \pm 0.10$
H1 in 0.01 M sodium phosphate	7.5	0.41	0.158	0.031	$0.62 \pm 0.06$
H1 in 0.5 M ammonium sulfate	7	0.79	0.131	0.019	$0.18 \pm 0.18$
Tyrosine in water‡	—	1.00	0.121	0.012	—

\*For the H1 solutions a pH of 4 indicates that no buffer was added; a pH of 7 was obtained by addition of 2 mM sodium cacodylate.

‡Tyrosine in water had an absorbance at 280 nm equal to that of H1 in water ( $\sim 0.1$  mM for both).

§All results are for excitation at 280 nm. Corrections have been made for solvent background (including Raman scatter) and for wavelength dependence of the emission sensitivity. Subscripts designate the wavelength at which the intensity was determined.

||The intensity of the 340-nm component at 340 nm was estimated as  $I_{340} = 0.126 I_{\max}$ . The resulting values were then normalized to the result for H1 in water at pH 4. The number 0.126 is an assumed ratio of  $I_{340}/I_{\max}$  for the 300-nm component alone (see text). The uncertainties given were obtained by varying this ratio between 0.121 and 0.131.

further quenches the 400-nm emission to only 3% of that in the absence of salt at pH 4. A large decrease in 400-nm component emission was also observed when H1 was folded at pH 4 by the addition of salt.

In addition to increasing the emission intensity, folding of H1 increases the tyrosine fluorescence anisotropy. Initially intending to see what effect the 340-nm component has on the anisotropy, the data in Fig. 5 were obtained. The rather steep slopes of the plots for folded H1 (curves 3 and 4) were unexpected. The results for unfolded H1 (curves 1 and 2) are also shown in the figure. The 340-nm component appears to have little effect on the anisotropy of the unfolded protein, even though it can be estimated to contribute nearly half of the total intensity at 340 nm. This suggests that it has an anisotropy similar to that for the tyrosine. If the 340-nm emission has little effect on the anisotropy of the unfolded protein, then it is very unlikely that it could cause the slope of the anisotropy plot for the folded protein where it constitutes a much smaller fraction of the emission.

Measurements on tyrosine in viscous solution (95% glycerol using 280-nm excitation, labeled 5 in the figure) clearly suggest that this slope is an inherent property of the tyrosine emission. That the slope is not due to impurities in the tyrosine was indicated by a number of observations. First, the same slope was observed when 289-nm excitation was used. Second, the excitation spectrum was not dependent on the emission wavelength monitored. And third, with 280-nm excitation, a good single component decay with a lifetime of 4.3–4.4 ns was obtained when emission was measured at 300 or 340 nm. Since the results in Fig. 5 were corrected for solvent background, an impurity in the glycerol was not responsible. Also, tyrosinate emission, either by excitation of ground-state tyrosinate or by excited-state proton transfer cannot be responsible since such emissions, induced by adding either KOH or  $K_2HPO_4$  to the glycerol solution, were found to have anisotropy

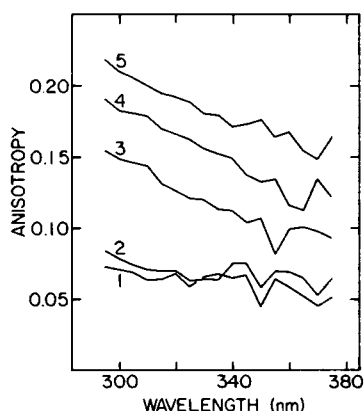


FIGURE 5 Emission anisotropy spectra for H1 and tyrosine. Results are shown for H1 in: (1) water at pH 4; (2) 2 mM sodium cacodylate at pH 7; (3) 0.1 M NaCl at pH 7; and (4) 0.5 M  $(NH_4)_2SO_4$  at pH 7. The curve 5 was obtained from tyrosine in 95% glycerol, 5% water. All results were at 20°C.

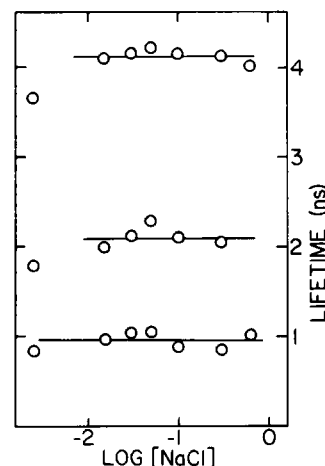


FIGURE 6 Decay lifetimes for the 300-nm component of H1 at pH 7 as a function of NaCl concentration. H1 in 2 mM sodium cacodylate was titrated with increasing concentrations of NaCl. Lambda invariance plots for each of the three component analyses showed good flatness, MD agreement, and component incrementation (requirements for acceptance of an analysis using the Method of Moments) (28). The result shown at lowest [NaCl] is that obtained in the buffer alone. Temperature was 20°C.

values and slopes with emission wavelengths comparable with those for tyrosine.

The results of fluorescence decay analyses for the 300-nm component of H1 at pH 7 and various ionic strengths are shown in Fig. 6. Excitation was at 280 nm and emission was collected through a 300-nm narrow band-pass filter. MD agreement and component incrementation were very good in these analyses. Again the result is surprisingly complex, indicating three decay components with lifetimes of ~1, 2, and 4 ns. At the highest NaCl concentration, so little of the 1 and 2-ns decays were present that they were not resolved. The relative contributions ( $\alpha\tau$  percentages) of the different components to the decay are plotted in Fig. 7. The proportion of the 4-ns decay increases progressively from ~10% at 0 added salt to over 90% at 0.5 M NaCl. It can be assigned to tyrosine in folded H1. The 1 and 2-ns contributions show a coordi-

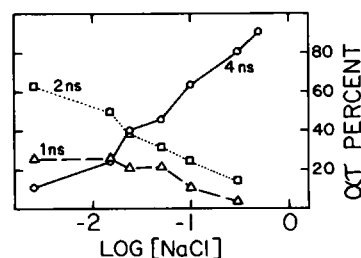


FIGURE 7 Effect of salt on the percentages of the three decay components that constitute the 300-nm emission of H1 at pH 7. In a multiexponential decay  $\sum \alpha_i \exp(-t/\tau_i)$ , the product of the pre-exponential factor ( $\alpha_i$ ) and the decay lifetime ( $\tau_i$ ) gives the relative contribution of the  $i$ th component to the total fluorescence intensity. The  $\alpha\tau$  percentages plotted here are for the decay results in Fig. 6.

nated decrease with increasing salt and thus appear to arise from tyrosine in denatured H1.

Attempts were made to resolve the decays of H1 in water excited at 280 nm and measured at wavelengths >300 nm, but  $\lambda$  invariance tests indicated lack of resolution. We were, therefore, unable to obtain information on the decay of the 340-nm emission. However, when the H1 in water was excited at 315 nm and the emission observed at wavelengths >380 nm, 95% of the emission decayed with a lifetime of between 9 and 10 ns. This indicates that the 400-nm component decays with a relatively long lifetime.

## DISCUSSION

One of the main reasons we undertook these detailed studies of the intrinsic tyrosine fluorescence was to find out why the steady state fluorescence is such a good indicator of the conformational state of the class A proteins. We chose H1 for our initial studies because it has only a single tyrosine residue and therefore was expected to give relatively simple fluorescence properties. To our surprise we found at least five different emitting species. In steady state excitation and emission spectra we see three different emission components. Two can be excited maximally at ~280 nm and show peak emission near 300 and 340 nm, respectively, while the third is maximally excited at ~320 nm and shows peak emission much further to the red at ~400 nm. The 300-nm emission, which would be considered typical for tyrosine fluorescence from a class A protein, can be further split by lifetime measurements into three species with lifetimes of ~1, 2, and 4 ns.

When sodium chloride is added to an H1 solution at neutral pH, both long wavelength species diminish. At progressively higher salt concentrations the fraction of the 300-nm fluorescence attributable to the 1- and 2-ns components decreases and is replaced by a single species with a lifetime of ~4 ns. The three tyrosine lifetimes themselves do not change as a function of salt concentration; they change only in relative amounts. If we assume that the 1 and 2-ns decays derive from denatured H1 while the 4-ns component arises from folded H1, this result supports the conclusion of Smerdon and Isenberg that H1 refolding is a cooperative two-state process (17).

The 300-nm fluorescence from denatured H1 consists mainly of two components with lifetimes of ~1 and 2 ns. This might be explained by the fact that the H1 used here consists of at least four subfractions. Considering our result, which indicates that folded H1 has a single decay lifetime of ~4 ns, one would expect a subfraction with a lifetime of 1 ns in the denatured form to show a distinctly larger increase in fluorescence intensity on folding than one with a lifetime of 2 ns. Steady state results (17), however, show that three of four major subfractions give the same increase in emission intensity on folding and the

fourth is <10% lower. This suggests that each subfraction has about the same lifetime values in the denatured state.

It is not surprising that the fluorescence of denatured H1 could decay as a sum of two exponentials. In their work on the decays of tyrosyl peptides, Gauduchon and Wahl found double exponential decays for peptides that are substituted on the carboxyl side of the tyrosine (9). Those workers proposed that the different decays result from different rotameric forms of the tyrosine.

We believe that the 340-nm emission derives from tyrosinate, resulting from excited-state proton transfer. In the ground state the hydroxyl group of the H1 tyrosine has a  $pK$  of ~11.6 (29), but the  $pK$  of the excited state of tyrosine is much lower at ~4.2 (30). Normally, fluorescence from ionized tyrosine is not observed at pH values much lower than the ground state  $pK$  because excited-state proton transfer is not rate competitive with emission. The rate of tyrosine proton transfer, however, can be increased by the presence of weak bases (30–33). Presumably, groups near the tyrosine residues of proteins can have a similar effect. There have been a number of recent reports of anomalous emission in this long wavelength region from class A proteins. These include H1 from *Ceratitis capitata* (34), parsley plastocyanin (35), adrenodoxin (36), bovine brain S-100b protein (37), human serum albumin (38), and two cytotoxins isolated from Indian cobra venom (39). Most of these results are probably due to the presence of tyrosinate fluorescence, although in the case of the bovine brain S-100b protein the emission was subsequently shown to derive from a tryptophan-containing impurity protein (40).

The 340-nm emission that we observe from denatured H1 may, of course, also result from an impurity fluorescence. The H1 preparation, however, is highly purified and is not likely to be contaminated by tryptophan-containing proteins. Also, the 340-nm emission is not diminished by dialysis in urea and therefore does not derive from a low molecular weight impurity. If the results at pH 7 in the last column of Table I (emission of the 340-nm component) are plotted against the values of  $I_{\max}$ , an inverse correlation is apparent. This would be expected if folding of H1 eliminates the normally highly quenched tyrosinate, allowing those residues to emit as normal tyrosine. The lifetime results further support this suggestion. The average lifetime for denatured H1 is ~1.5 ns  $[(\alpha_1\tau_1 + \alpha_2\tau_2)/(\alpha_1 + \alpha_2)]$ . Increasing the lifetime to 4 ns should give only a 2.7-fold increase in steady state emission with folding of the H1. However, the actual increase observed varies from 3.6 to 4.0 for the various subfractions (17). The remaining increase may arise from the shift from tyrosinate to tyrosine emission on folding.

The 400-nm emission is more uncertain. It diminishes greatly on increasing the pH from 4 to 7 without a change in emission intensity at 300 nm. For all practical purposes it disappears on salt-induced refolding (even at pH 4).

Also, like the 340-nm emission, it is not diminished by dialysis in urea; it must result from a chromophore that is covalently linked to high molecular weight material, presumably the H1. Bityrosine, which can form when tyrosine is excited at higher pHs, has excitation and emission properties similar to the 400-nm component (41). Perhaps the latter represents a photoproduct of H1 tyrosine with another group such as a phenylalanine, a tyrosine from another protein, a nucleotide, or an adenine from residual poly-ADP-ribose. It is also possible that it represents luminescence from an unusual tyrosine species. For example, a charge transfer complex between the tyrosine and an unionized carboxylate on the glutamic acid three residues away might be expected to increase intersystem crossing (42). The 400-nm emission could be phosphorescence from the decay of the lowest triplet. Similar emissions have been observed at room temperature from tyrosine charge transfer complexes in concentrated calcium chloride solutions (43). This kind of mechanism would be consistent with the observed slow decay for this component. It will be of interest in future work to look for such long wavelength emissions from other class A proteins. Such emissions could easily be missed because their absorbance and emission occur outside of the normal range of wavelengths used to study tyrosine fluorescence.

In Fig. 5 we have made the observation that the anisotropy varies over the emission band both for tyrosine residues in H1 and for tyrosine itself in glycerol. A number of artifactual sources for this variation have been eliminated in the latter case. These include impurity in the solvent and presence of ground-state tyrosinate or of excited-state proton transfer. Furthermore, no evidence of an impurity in the tyrosine sample could be obtained. Effects of solvent relaxation also seem unlikely to be responsible since the decay lifetime for tyrosine in 95% glycerol was essentially the same for emission at 300 or at 340 nm. It appears that the effect is an inherent property of tyrosine. This dependence of anisotropy on the emission wavelength may actually be a common occurrence since, as pointed out by Steinberg (44), "the measurement of polarization across emission bands does not seem to be popular." Steinberg points out that such effects may arise from the vibrational structure of electronic transitions since changes in the direction of the emission moment may result from the different vibrational levels involved in the transition.

The salt-induced folding of H1 is accompanied by an increase in the steady state fluorescence intensity and an increase in the anisotropy. From the work reported here we are now in a position to understand these events more fully. As the salt is increased, four emitting species are replaced by the single emitter of the refolded histone. We can safely neglect the 400-nm emission because it is not efficiently excited at 280 nm and does not emit significantly at 325 nm where the steady state fluorescence properties are normally monitored. If we assume that the intrinsic life-

time of the tyrosyl chromophore remains approximately the same in the denatured and refolded forms of the protein, then the species corresponding to the two shorter lifetimes in the denatured H1 have lower quantum yields. Tyrosinate also has a much lower quantum yield than tyrosine. (This would explain the sudden large drop in fluorescence intensity observed for H1 above pH 10 [17].) Therefore, we conclude that the steady state fluorescence intensity increases on folding because the three highly quenched emitters of the denatured protein are replaced by the less quenched fluorescence of the tyrosine in the folded protein.

The lifetime of tyrosine is sensitive to its environment, becoming greater under more hydrophobic conditions. For example, the 3.4-ns lifetime of tyrosine in water increased to 4.0 ns in ethanol and 4.3 ns in 95% glycerol. Since the decay time of the tyrosine in the refolded H1 is ~4.2 ns, we conclude that it is folded into a moderately hydrophobic environment.

The two tyrosine species of denatured H1 and also the presumed highly quenched tyrosinate all have lifetimes shorter than that of the refolded tyrosine. If all of the different species had the same degree of mobility, then the shorter lifetime components should contribute higher anisotropies, since there would be less time in the excited state during which depolarization could occur. Since the anisotropy increases on the addition of salt, we conclude that this increase results from a significant decrease in the mobility of the tyrosine residue in the refolded H1.

Irvin Isenberg died on September 6, 1984, ending a long and distinguished career in science. Of his many important contributions, he is probably best known for his work on the physical properties and interactions of the histones. The work presented here grew from his work. We deeply regret the loss of our friend and colleague. The H1 sample, prepared by Roswitha Blohm Hopkins, was provided by Isenberg.

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