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Time-resolved Fluorescence of Apoferritin and Its Subunits*

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The decay of the intrinsic fluorescence of the apoferritin polymer and its subunits has been studied by pulse and phase shift techniques. Both techniques show that the fluorescence decay of all the samples tested cannot be described by a single exponential function. The fluorescence decay data of the apoferritin subunits obtained with either technique can be fitted satisfactorily with a function resulting from the sum of two exponential components. However, the polymer data obtained with the high resolution phase shift technique operated either by synchrotron radiation or by a mode-locked argon ion laser can be fitted better using a bimodal gaussian continuous distribution of lifetime components. The molecular basis for this distribution of lifetime values may lie in the heterogeneity of the tryptophan environment generated by the assembly of the subunits into the polymer.

The binding of the first 100 irons to apoferritin quenches the intrinsic fluorescence without affecting the lifetimes in a proportional way. This finding may be taken as an indication that the quenching of the tryptophan fluorescence induced by the binding of iron has both static and dynamic components.

Fluorescence spectroscopy has been widely used to probe the environment of aromatic amino acids in proteins. In single tryptophan-containing proteins the position of emission spectrum and the values of fluorescence lifetimes give useful information concerning the environment of the tryptophan residue. Most of these proteins have been shown to decay in a complex way due to conformational heterogeneity (1, 2).

An interesting case is provided by the iron storage protein ferritin. The protein moiety, apoferritin, is a hollow shell that can harbor up to 4,500 Fe(III) in the form of ferrihydrite crystallites (3) and is formed by 24 subunits arranged in 4:3:2 symmetry. The subunits are of two kinds, L ($M_r = 19,000$) and H ($M_r = 21,000$), each derived from different gene families and expressed in various proportions in different tissues. The L and H subunits show extensive sequence homology, in particular at the level of intersubunit contacts. Hence the two subunit types are structurally equivalent in the assembled shell, and ferritins from different tissues are made up by characteristic populations of heteropolymers or isoferritins (4).

The protein from horse spleen used in the present study for example has a subunit ratio of 9L:1H. The two types of subunit contain a single tryptophan residue at the same

position along the polypeptide chain (5-7). In the assembled shell, the tryptophan residue is embedded at the contact region of its dimeric building block (3). This location renders the spectroscopic characteristics of tryptophan extremely sensitive probes of changes that occur at the intersubunit contact. Thus, differences in the static fluorescence and in the CD spectrum have been observed not only upon dissociation but also among apoferritins characterized by a different subunit composition. In particular the emission wavelength is red-shifted in the H-rich polymers with respect to the L-rich ones (8). The presence of iron quenches the tryptophan fluorescence. The degree of quenching depends on the iron content and on the oxidation state of the metal, being much higher for Fe(III) (9).

In this paper the fluorescence characteristics of horse spleen apoferritin and its subunits have been studied by different time-resolved techniques to establish how the quaternary structure of the protein affects the decay pattern of the tryptophan emission. Moreover, the effect of Fe(III) has been examined in order to gain information on the mechanism of the fluorescence quenching brought about by iron.

MATERIALS AND METHODS

Sample Preparation, Iron Binding, and CD Experiments—Horse spleen ferritin was prepared as described previously; apoferritin was obtained by reduction of iron with sodium dithionite and chelation with α, α' -bipyridyl (10). Apoferritin concentration was calculated from the absorbance at 280 nm by using the extinction coefficient $E_{1\text{cm}}^{1\%} = 9.0$ (11) and a polymer molecular weight of 450,000. The fluorescence measurements were performed on samples dialyzed against 10 mM Tris-HCl buffer at pH 7.0 or 40 mM glycine acetate at pH 3.5. Apoferritin subunits were prepared by overnight dialysis in the cold of apoferritin solutions against 200 mM glycine HCl at pH 1.8. Subsequently the subunits were dialyzed against 40 mM glycine HCl or glycine-acetate buffers for the experiments to be carried out at pH 1.8 and 3.5, respectively. In the experiments designed to study the effect of iron binding a degassed solution of ferrous ammonium sulfate in water was used as the source of iron (8). After addition of the required amount of iron to apoferritin, in 20 mM imidazole HCl at pH 7.0, the solution was kept in air for at least 1 h to allow for the complete oxidation of the metal.

Circular dichroism experiments were carried out in the UV region with a Jasco J500 spectropolarimeter equipped with a DP500 data processor.

Time-resolved Fluorescence Measurements—The decay of ferritin fluorescence has been studied by both pulse and phase shift techniques. Measurements with the correlated single photon counting technique were performed using an apparatus assembled with commercial components (12). The apoferritin samples ($A = 0.4$ at 280 nm) were excited at 280 nm (bandwidth = 10 nm). The fluorescence was collected after passing through a solution of Triton X-100 in water ($A = 2$ at 285 nm) to remove scattered light. The multifrequency phase shift and demodulation measurements (13) were performed either using a synchrotron radiation or a mode-locked laser as light sources. In the first case the Adone storage ring of the Progretto Utilizzazione Luce di Simcrotro-ne laboratory (Frascati, Italy) was

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used in connection with a commercially available electronic setup (Industria Strumentazioni Scientifiche, La Spezia, Italy) in the configuration previously described (14). The fluorescence experiments were performed as described above. The argon ion laser fluorometer has been described elsewhere (15). In this case the samples were excited at 298 nm, and the fluorescence was collected after passing through a Corning O-54 filter. For the sake of comparison some experiments were conducted under the same conditions used with the single photon counting technique. The fluorescence experiments were always carried out at 20 °C.

The decay profiles obtained with the single photon counting technique were analyzed using a least-squares iterative reconvolution procedure to correct for the excitation pulse profile (16); the calculations were made on a PDP 11-23 Digital computer. The data were fitted to a sum of discrete exponential functions.

$$F(t) = \sum_{i=1}^n a_i \exp(-t/\tau_i)$$

The adequacy of the fits was evaluated on the basis of the χ^2 values and of the distribution of the weighted residuals, i.e. a fit was considered adequate when no significant decrease in χ^2 occurred upon addition of a further component and when the weighted residuals were randomly distributed around zero. Adequate fits of the fluorescence decays of all the samples were obtained using a double-exponential function.

The data obtained with the phase shift technique were analyzed either in terms of a double and a triple exponential decay (17) or with a new model involving a continuous distribution of lifetimes (18). In this second model the fluorescence decay function can be written as

$$F(t) = \int_0^{\infty} a(\tau) \exp(-t/\tau) d\tau$$

where $a(\tau)$ is a function representing the fraction of molecules (at time = zero) which decay with a lifetime between τ and $\tau + d\tau$. By introducing $f(\tau) = a(\tau) \cdot \tau$ which represents the contribution to the total fluorescence of the molecules with lifetime between τ and $\tau + d\tau$ one can write

$$F(t) = \int_0^{\infty} f(\tau)/\tau \exp(-t/\tau) d\tau$$

with the following normalization condition.

$$\int_0^{\infty} f(\tau) d\tau = 1$$

It is important to remark that if the interconversion between fluorescent species occurs during the fluorescence lifetime, the function $a(\tau)$ no longer represents the fraction of molecules decaying with a lifetime between τ and $\tau + d\tau$ but is also related to the interconversion rates between the different species (19). The arbitrary functions used for $f(\tau)$ have been reported in Table I. In each case the centers, C , and the widths, W , were obtained by minimizing the χ^2 values with a routine based on the simplex method running on an IBM personal computer equipped with a 8087 math coprocessor. For the sum of two lorentzians and of two gaussians the normalization condition $f_1 + f_2 = 1$ has been used to allow a straightforward comparison with the values obtained by means of the two exponentials model.

The relative significance of the χ^2 values obtained by the three methods of analysis has been further assessed by the Snedecor's F-test (20).

TABLE I

Arbitrary continuous distributions of lifetime used to fit the fluorescence decay data

$f(\tau)$, fractional intensity versus τ ; f_0 , normalization factor; f_1, f_2 , fractional contributions to the total fluorescence; C, C_1, C_2 , centers of distributions; W, W_1, W_2 , widths of distributions.

Lorentzian distribution	$f(\tau) = f_0 / \{1 + [(\tau - C)/(W/2)]^2\}$
Gaussian distribution	$f(\tau) = f_0 \exp[-(\tau - C)^2/2W^2]$
Bimodal lorentzian distribution	$f(\tau) = f_1 / \{1 + [(\tau - C_1)/(W_1/2)]^2\} + f_2 / \{1 + [(\tau - C_2)/(W_2/2)]^2\}$
Bimodal gaussian distribution	$f(\tau) = f_1 \exp[-(\tau - C_1)^2/2W_1^2] + f_2 \exp[-(\tau - C_2)^2/2W_2^2]$

RESULTS

The fluorescence decay parameters of the apoferritin polymer and of its monomeric subunits obtained using the single photon counting technique are reported in Table II. In both cases a very good fit was obtained using a double-exponential function. In particular in the case of the apoferritin polymer the value of χ^2 decreased from 30 to 2 upon addition of the second exponential term, and the weighted residuals became randomly distributed around zero. The average lifetime was shorter in the polymer than in the subunits in agreement with the lower quantum yield.

Table III shows data obtained with the phase shift technique under the same experimental conditions. In addition it shows the results of measurements carried out on apoferritin and its subunits at pH 3.5. At this pH value, apoferritin is undissociated while the subunits are still dissociated due to the marked hysteresis in the dissociation and reassembly processes (9, 21). Actually, at pH 3.5 the subunits dimerize with the concomitant embedding of the tryptophan residue in the hydrophobic dimeric contact. However, their CD spectrum in the tryptophan region resembles that measured at lower pH values (21). As far as the polymer is concerned, all its physicochemical properties, including the near-UV CD spectrum, are unchanged over the pH range 7 to 3.5. In line with these findings the data given in Table III do not show significant pH effects on the fluorescence decay parameters of the subunits and the polymer although differences in the quantum yield were observed.

As to the adequacy of the fit provided by the double-exponential analysis of Table III the χ^2 values were unsatisfactorily high in particular in the case of the polymer. The use of a three exponential function did not improve the fit

TABLE II

Fluorescence decay parameters (single photon counting)

$a_1 + a_2 = 1$; ($\Delta a_1 \sim \Delta a_2 \sim 0.05$); $\langle \tau \rangle = a_1 \tau_1 + a_2 \tau_2$, average lifetime in ns; τ_1, τ_2 , lifetime in ns; ($\Delta \tau_1 \sim \Delta \tau_2 \sim 0.2$ ns); χ^2 , reduced chi squared; q , relative quantum yield from steady state measurements.

Sample	pH	τ_1	τ_2	a_1	χ^2	$\langle \tau \rangle$	q
Apoferritin polymer	7	0.9	5.3	0.85	2.0	1.6	50
Apoferritin subunits	1.8	2.3	7.1	0.80	2.7	3.3	100

TABLE III

Fluorescence decay parameters (phase shift technique)

Analysis with a two-exponential function. $\tau_1, \tau_2, a_1, \langle \tau \rangle, q, \chi^2$, as in Table I; $f_1 = a_1 \tau_1 / (a_1 \tau_1 + a_2 \tau_2)$, fractional intensity relative to τ_1 ; ($f_1 + f_2 = 1$); ($\Delta f_1 \sim \Delta f_2 \sim 0.05$). $F_{2\tau/3\tau}$, significance level of F-test between a two- and a three-exponential analysis (see "Materials and Methods"). Apof. (1), apoferritin polymer, pH = 7, measured with synchrotron radiation; Apof. (2), apoferritin polymer, pH = 3.5, measured with synchrotron radiation; Apof. (3), apoferritin polymer, pH = 7, measured with argon-laser; Subun. (4), apoferritin subunits, pH = 1.8, measured with synchrotron radiation; Subun. (5), apoferritin subunits, pH = 3.5, measured with synchrotron radiation; Apof. (6), apoferritin polymer, pH = 7, in imidazole buffer (see text), measured with argon-laser; (7), same as above with iron ions, Fe/molecule = 50; (8), same as above, Fe/molecule = 100.

Sample	τ_1	τ_2	f_1	a_1	$\langle \tau \rangle$	q	χ^2	$F_{2\tau/3\tau}$
Apof. (1)	0.9	4.0	0.48	0.80	1.5	50	9	0.05
Apof. (2)	0.5	3.7	0.46	0.86	1.0	63	12	0.5
Apof. (3)	0.4	3.3	0.29	0.78	1.0	50	11	0.025
Subun. (4)	1.9	7.2	0.61	0.86	3.9	100	5	0.10
Subun. (5)	1.6	6.4	0.58	0.84	2.4	86	6	0.5
Apof. (6)	0.3	5.7	0.25	0.87	1.0	48	27	0.5
+Fe (7)	0.3	6.3	0.27	0.90	0.9	38	30	0.5
+Fe (8)	0.3	6.3	0.27	0.90	0.8	24	27	0.5

significantly (Table IV). Thus, a further analysis of the data in terms of continuous distributions of lifetime values was attempted (Table V). In the case of the polymer (Fig. 1) a significantly improved fit is given with a bimodal gaussian

TABLE IV

Fluorescence decay parameters (phase shift technique)

Analysis with a three-exponential function. τ_1, τ_2, τ_3 , lifetimes in ns. The standard deviations of the lifetimes are about 0.2 ns. f_1, f_2 , fractional intensity relative to τ_1 and τ_2 , $f_1 = a_1\tau_1/(a_1\tau_1 + a_2\tau_2 + a_3\tau_3)$, $f_2 = a_2\tau_2/(a_1\tau_1 + a_2\tau_2 + a_3\tau_3)$ ($f_1 + f_2 + f_3 = 1$); the standard deviations of the fractional intensity are about 0.05. a_1, a_2 , pre-exponential terms, ($a_1 + a_2 + a_3 = 1$). χ^2 , reduced chi squared. $F_{2G/3r}$, significance levels of F-test between two gaussian distribution and three-exponential analysis (see "Materials and Methods"). The sample description is the same as in Table III.

Sample	τ_1	τ_2	τ_3	f_1	f_2	a_1	a_2	χ^2	$F_{2G/3r}$
Apof. (1)	-0.2 ^a	0.9	4.1	0.01 ^b	0.49	-0.10	0.89	10.0	0.05
Apof. (2)	0.3 ^a	0.6 ^a	3.8	0.15	0.31	0.40	0.46	13.0	0.05
Apof. (3)	0.1	0.9	4.6	0.09	0.39	0.69	0.24	2.4	0.5
Subun. (4)	0.6 ^a	1.8	6.9	-0.01 ^b	0.59	-0.06	0.90	8.0	0.10
Subun. (5)	>100 ^a	1.3	4.4	0.05 ^b	0.39	0.01	0.69	6.0	0.5
Apof. (6)	0.2 ^a	0.3 ^a	5.7	0.16 ^b	0.09 ^b	0.64	0.24	31.0	0.005
+Fe (7)	0.2 ^a	0.3 ^a	6.3	0.17 ^b	0.11 ^b	0.63	0.26	35.0	0.005
+Fe (8)	0.2 ^a	0.3 ^a	6.3	0.15 ^b	0.13 ^b	0.54	0.36	32.0	0.005

^a S.D. larger than 10 ns.

^b S.D. larger than 0.5.

TABLE V

Fluorescence decay parameters (phase shift technique)

Analysis with two-gaussian continuous distribution. f_1 , fractional intensity relative to C_1 ; ($f_1 + f_2 = 1$) (see "Materials and Methods"), ($\Delta f_1 \sim \Delta f_2 \sim 0.05$); C_1, C_2 , centers of gaussian distributions in ns; ($\Delta C_1 \sim \Delta C_2 \sim 0.2$ ns); W_1, W_2 , widths of gaussian distributions in ns; ($\Delta W_1 \sim \Delta W_2 \sim 0.2$ ns); χ^2 , reduced chi squared; $F_{2G/2r}$, significance level of F-test between two-gaussian distribution and two-exponential analysis (see "Materials and Methods"). The sample description is the same as in Table III.

Sample	C_1	W_1	C_2	W_2	f_1	χ^2	$F_{2G/2r}$
Apof. (1)	1.2	0.4	4.8	1.0	0.63	3.8	0.25
Apof. (2)	0.7	0.3	3.9	2.5	0.48	3.8	0.05
Apof. (3)	0.8	0.4	4.3	1.7	0.45	1.8	0.01
Subun. (4)	1.8	0.1	7.5	0.2	0.62	3.6	0.5
Subun. (5)	1.8	0.4	6.8	0.1	0.65	6	0.5
Apof. (6)	1.6	2.7	8.5	8.2	0.48	5.1	0.01
+Fe (7)	0.1	35	6	10	0.10	1.5	0.005
+Fe (8)	0.1	25	6	10	0.15	1.8	0.005

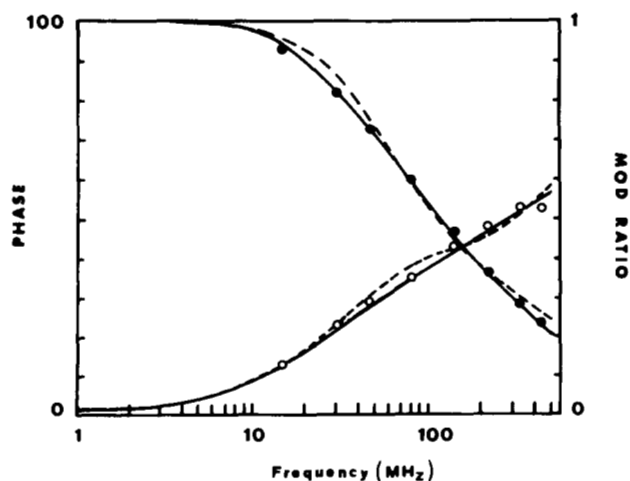


FIG. 1. Phase (○) and modulation (●) data as a function of light modulation frequency in apoferritin at pH 7.0. Best fits obtained using two lifetimes (---) or a bimodal gaussian distribution of lifetimes (—) are shown. For experimental details see "Materials and Methods."

distribution. Fittings with one or two lorentzian functions or with a single gaussian were not as good as those obtained with two gaussians. In contrast, the fluorescence decays of all the other single-tryptophan proteins studied so far and analyzed within a continuous distribution model were always better described by two lorentzian than by two gaussian functions (see "Discussion"). Fig. 2B shows the two gaussians used for the distribution of lifetimes in the fluorescence decay of apoferritin with their centers at 0.81 and 4.33 ns, respectively. Superimposed (heavy lines) are the values of f_1 and f_2 obtained with the analysis in terms of two exponentials. Fig. 2A shows a similar analysis for the apoferritin subunits at pH 1.8. It should be stressed that for the subunits there was no significant improvement of the fit using two lorentzian or two gaussian distributions with respect to the simple two exponentials function since the width of the distribution was <0.2 ns (19). As a matter of fact the phase and modulation data of apoferritin subunits at pH 1.8 can be fitted using a double-exponential function (Fig. 3).

The effect of iron binding to apoferritin is also shown in Table III. Apoferritin samples loaded with 50 and 100 iron ions/polymer molecule display differences in the fluorescence lifetimes in comparison with iron-free apoferritin that are nonproportional to the change in quantum yield which is 18–20% and 36–40%, respectively (9). The near and far UV CD spectra of these samples (data not shown) were indistinguishable from those of the iron-free protein demonstrating that incorporation of 50–100 iron ions does not induce significant conformational changes nor perturbations of the aromatic residues.

DISCUSSION

The fluorescence lifetime data of apoferritin and its derivatives show several features that deserve comment. The good overall correspondence between the results obtained with the single photon counting technique and with the phase shift technique should be emphasized. The greater resolution achieved with the latter method showed that, at least in the

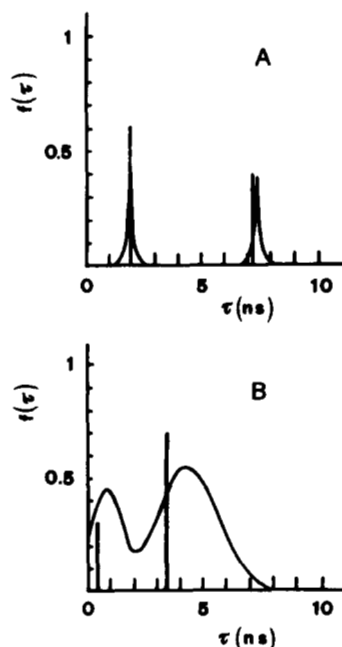


FIG. 2. Continuous distributions of lifetimes of apoferritin subunits at pH 1.8 (A) and apoferritin at pH 7.0 (B). The vertical bars (heavy lines) correspond to the lifetimes and fractional intensity recovered using an analysis with two lifetimes.

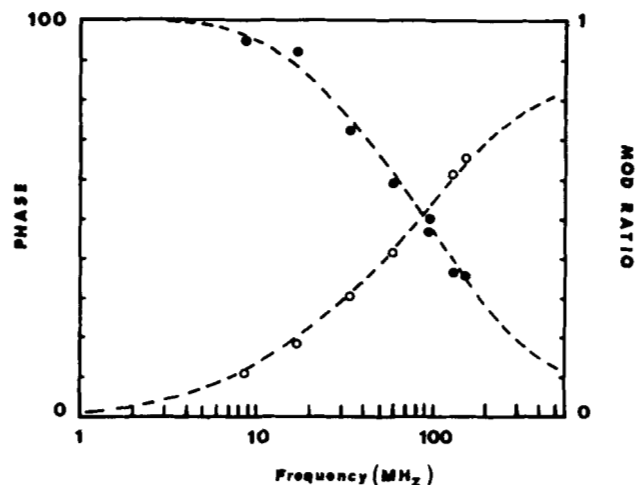


FIG. 3. Phase (○) and modulation (●) data as a function of light modulation frequency in apoferritin subunits at pH 1.8. Best fit obtained using two lifetimes (---) is shown. For experimental details see "Materials and Methods."

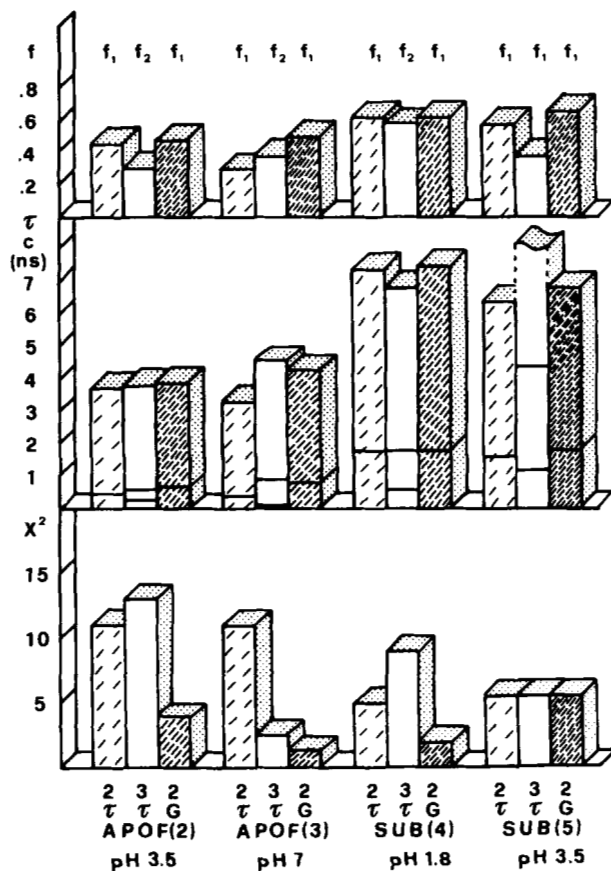


FIG. 4. Graphical comparison of the fluorescence decay parameters from Tables III, IV and V. Top, fractional intensities; Middle, lifetime values and centers of gaussian distributions in ns; Bottom, reduced chi squared.

polymer, the fittings obtained using functions formed by a sum of two or three exponentials were often unsatisfactory as indicated by the high χ^2 values. The only systematic reduction in the χ^2 values is obtained by means of a continuous distribution of lifetimes (Table V, Fig. 4). As shown in Fig. 4, if one disregards the very fast lifetime component of the three-exponential analysis, the mean values of the lifetimes and fractional intensities are very similar in the three methods of analysis used.

The presence of complex decays in the intrinsic fluorescence of proteins is becoming more and more documented (2, 19, 23). Even single tryptophan-containing proteins show at least two lifetimes. Interestingly, one of the two lifetimes is always shorter than that of *N*-acetyltryptophanamide in water. It should be pointed out that tryptophan itself may show a complex decay (22). This finding has been interpreted as due to the presence of two main conformations of this amino acid in solution. One of these conformations would bring the indole ring nearer to the ionized group, thus quenching the lifetime. By analogy, the presence of two conformations in proteins like azurin has been postulated as being responsible for the observed double-exponential decay of the intrinsic fluorescence (1).

The fluorescence decay of apoferritin subunits is in line with the behavior of single tryptophan-containing proteins and can be described by a two exponentials function or by two narrow lorentzian distributions. The decay pattern does not change significantly upon dimerization of the subunits as indicated by the data obtained at pH 1.8 and 3.5. In the assembled apoferritin shell the fluorescence decay pattern is more complex and can be described by two gaussian distributions rather than by three lifetimes if one considers the statistical analysis of all the experimental data obtained. The existence of distribution of lifetimes seems to be a likely possibility on the basis of biochemical considerations. The polymerization process can give rise to structural inequivalence of the environment of the tryptophan residues due to their position in the molecule endowed with 4:3:2 symmetry. Moreover, the assembly of the L and H subunits into polymers brings about slight differences in the surface charge of the protein, which results in the well known spectrum of isoferitins of different isoelectric point and salting out properties (4). These factors are not likely to be generating just a third lifetime component with respect to the dissociated subunits. Therefore, even disregarding the statistical evidence, the presence of a discrete number of different tryptophans, each decaying with narrow lorentzian distributions of slightly shifted centers resulting in a gaussian envelope, may better fit the structural data.

A final remark on the intrinsic fluorescence decay of apoferritin concerns the effect of iron binding. A comparison between the effect of the metal ion on the lifetime values and on the relative quantum yield (Table III) indicates that the first 50 metal ions decrease the lifetime of tryptophan, and that upon a further increase of the iron content up to 100 atoms/molecule no additional changes in the lifetime values are observed. These findings, taken together with the decrease in the relative quantum yield, suggest that at least part of the quenching occurs via a static mechanism. The static contribution may be due to contact quenching or to a conformational effect. Under the experimental conditions used CD experiments, however, have ruled out large conformational rearrangements upon iron binding. Moreover, since precise structural information on the relative position of the tryptophan and the iron binding and/or nucleation sites is still lacking (3, 24, 25) it is difficult to assess the relative weight of the two factors.

In conclusion, the present data on the decay characteristics of the tryptophan fluorescence in apoferritin and its subunits bring out heterogeneity among the tryptophan residues in the polymer due to subunit assembly in the same line as previously suggested by isoelectric focusing and salting out experiments (26). Moreover the initial stages of iron incorporation lead to tryptophan fluorescence quenching by both static and kinetic mechanism.

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