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Formation and movement of Fe(III) in horse spleen, H- and L-recombinant ferritins. A fluorescence study

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Abstract

Iron oxidation and incorporation into apoferritins of different subunit composition, namely the recombinant H and L homopolymers and the natural horse spleen heteropolymer (10–15% H), have been followed by steady-state and time-resolved fluorescence. After aerobic addition of 100 Fe(II) atoms/polymer, markedly different kinetic profiles are observed. In the rL-homopolymer a slow monotonic fluorescence quenching is observed which reflects binding, slow oxidation at the threefold apoferritin channels, and diffusion into the protein cavity. In the rH-homopolymer a fast fluorescence quenching is followed by a partial, slow recovery. The two processes have been attributed to Fe(II) binding and oxidation at the ferroxidase centers and to Fe(III) released into the cavity, respectively. The fluorescence kinetics of horse spleen apoferritin is dominated by the H chain contribution and resembles that of the H homopolymer. It brings out clearly that the rate of the overall process is limited by the rate at which Fe(III) leaves the ferroxidase centers of the H chains where binding of incoming Fe(II) and its oxidation take place. The data obtained upon stepwise addition of iron and the results of optical absorption measurements confirm this picture. The correspondence between steady-state and time-resolved data is remarkably good; this is manifest when the latter are used to calculate the change in fluorescence intensity as apparent in the steady-state measurements.

Keywords: ferritin, iron incorporation, steady-state and time-resolved fluorescence

Ferritin is a ubiquitous iron storage protein composed of 24 polypeptide chains arranged to form a hollow protein shell where the metal is stored as inorganic ferrihydrite micelles in a readily mobilizable form (Rice et al., 1983; Ford et al., 1984). Mammalian ferritins are copolymers of two distinct subunits, known as H and L, which share the same tertiary fold consisting of a bundle of four long α-helices, a fifth short helix, and a long extended loop (Arosio et al., 1978; Andrews et al., 1992). In the assembled molecule, characterized by 432 symmetry, the H and L subunits occupy equivalent positions and the loop forms part of the dimer interface (Ford et al., 1984; Harrison et al., 1986).

Ferritin function consists in the catalysis of Fe(II) oxidation and in the subsequent clustering of Fe(III) to generate ferrihydrite micelles. In these processes the H and L subunits play different roles that have been brought out in recent studies on recombinant ferritins containing a single subunit type (Levi et al., 1988; Santambrogio et al., 1993; Levi et al., 1994). The H subunits catalyze Fe(II) oxidation due to the presence of a ferroxidase center localized within the four helix bundle (Lawson et al., 1989). In the L subunits the iron nucleation and incorporation capacity has been attributed to Glu-57, Glu-60, and other carboxylate groups that protrude into the internal cavity of the apoferritin shell (Levi et al., 1994; Santambrogio et al., 1996). In fact, clusters of carboxylates are known to be endowed with ferroxidase activity (Harris & Aisen, 1973). In rH-ferritin Mössbauer spectroscopy shows that oxidation of iron leads to formation, at the ferroxidase center itself, of μ-oxo-bridged Fe(III) dimers which, in turn, slowly give rise to polymeric clusters (Bauminger et al., 1989, 1991, 1993). In horse spleen ferritin (85–90% L chains), a significant proportion of polymeric clusters is formed immediately after iron oxidation consistent with the absence of the ferroxidase center (Treffry et al., 1996).

The route(s) used for the entry of Fe(II) and the movement of Fe(III) have not been fully clarified. In horse spleen apoferritin various studies indicate that Fe(II) enters through the hydrophilic channels at the threefold symmetry axes, where it is bound by the Asp 131 and Glu 134 carboxylate side chains and oxidized (Stefanini et al., 1989; Desideri et al., 1991). The hydrophilic channels appear to be involved in Fe(II) entry also in the H homopolymer,

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since substitution of Asp 131 and Glu 134 results in a slower rate of iron incorporation (Treffry et al., 1993). At variance with Fe(II), Fe(III) most likely follows different routes in the L and H homopolymers. In the L homopolymer, Fe(III) can enter the cavity directly from the threefold channels. In the H homopolymers, it could follow the same route since the threefold channels have been shown to contain monomeric Fe(III) species (Treffry et al., 1993) or, alternatively, could utilize a small intrasubunit channel (Lawson et al., 1991).

In the present paper, Fe(III) formation and its movement toward the apoferritin cavity in horse spleen apoferritin (85–90% L) and in the rL and rH homopolymers have been monitored by means of steady-state and time-resolved fluorescence experiments after aerobic addition of iron. Advantage has been taken of the fact that the loop region of the L and H subunits contains a single, conserved tryptophan residue whose intrinsic fluorescence is quenched upon aerobic and anaerobic addition of iron (Stefanini et al., 1976, 1982; Treffry et al., 1995). In the different ferritins studied, the change in intrinsic fluorescence follows a markedly different kinetics that has been correlated to the specific characteristics imparted to the iron oxidation and incorporation processes by the two chain types. In particular, the behavior of horse spleen apoferritin clearly brings out that release of Fe(III) from the ferroxidase centers limits the rate at which further Fe(II) can be oxidized. The present study, therefore, shows that the intrinsic tryptophan fluorescence provides not only a useful structural probe in the study of ferritins (Rosato et al., 1987), but also a sensitive means to single out the functional contributions of the two chain types.

Results

Aerobic fluorescence measurements

In a first series of experiments, the fluorescence emission spectrum of the different apoferritins was determined. In agreement with previous data (Stefanini et al., 1976; Rosato et al., 1987; Santambrogio et al., 1993), the fluorescence emission maximum of the polymers peaks at different wavelengths, namely at 316 nm in horse spleen apoferritin and at 325 nm in the rH and rL homopolymers (Fig. 1).

In a second series, the intensity change attendant the aerobic addition of iron (100 atoms/polymer, at pH 6.5 and 20 °C) was followed at the wavelength of maximum emission. The fluorescence change follows a different time course in the three apoferritins investigated (Fig. 2A). In the rL homopolymer, the intensity decreases monotonically and reaches about 80% of the initial value after about 1,500 s. In the rH homopolymer, fluorescence is first quenched rapidly, reaches a constant value of about a third of the original signal after 70–80 s, and increases slowly thereafter; it attains about 50% of the initial intensity at around 1,500 s. In the horse spleen protein (85–90% L), the kinetics of the fluorescence change resembles that of the rH homopolymer indicating that H chains dominate the experimental situation. The extent of the fluorescence intensity changes for the three proteins and the corresponding half times are summarized in Table 1. The extent of quenching at the end of the reaction differs in the different apoferritins, being largest in the rH homopolymer; this is brought out also by the emission spectra included in Figure 1.

In order to correlate the kinetics of the fluorescence changes with iron oxidation and incorporation, the reaction was followed in parallel by measuring the absorbance at 310 nm. In the rL homopolymer, no absorbance changes were observed in line with literature data (Levi et al., 1994). In the rH homopolymer and in horse spleen apoferritin, the absorbance changes were complete in about 400 and 1,500 s, respectively, and the corresponding half times were 30 s and 180 s (Table 1).

Finally, the same amount of iron (100 atoms/polymer) was added in three steps (25 atoms in the first steps and 50 atoms in the last)

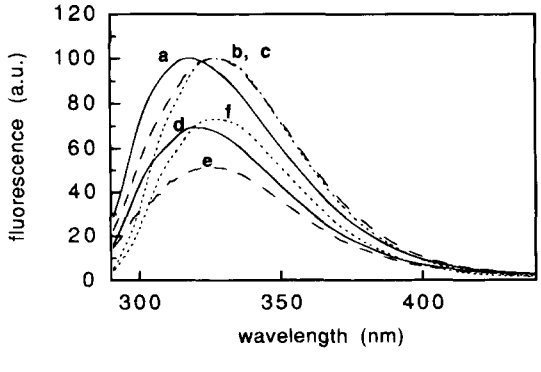

Fig. 1. Steady-state fluorescence emission spectra of horse spleen (a), rH (b), rL apoferritin (c), and quenching effect after incorporation of 100 iron atoms/polymer (d, e, f, respectively). Protein concentration is 2.2 × 10^{-7} M in 20 mM MOPS-NaOH at pH 6.5. Temperature is 20 °C.

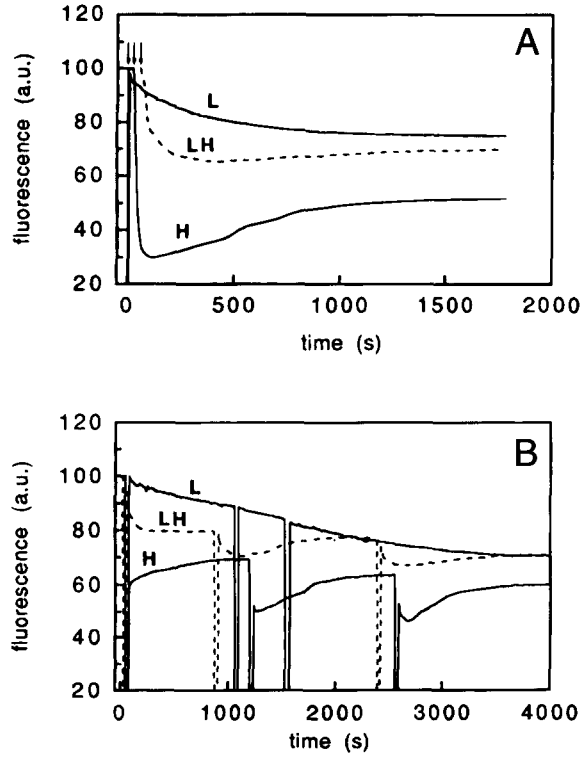

Fig. 2. Fluorescence of rL (L), rH (H), and horse spleen apoferritin (LH) after aerobic addition of Fe(II). The same amount of 100 Fe(II) atoms/polymer was added in (**A**) a single step, or in (**B**) three steps corresponding to 25, 25, and 50 atoms. Arrows indicate the time of addition. Protein concentration is 2.2 × 10^{-7} M in 20 mM MOPS-NaOH at pH 6.5. Temperature is 20 °C.

Table 1. *Fluorescence intensity and absorbance changes attendant aerobic addition of iron to horse spleen, rH and rL apoferritin*[a]

	F		A
Apoferritin	ΔF (%)	$t_{1/2}$ (s)	$t_{1/2}$ (s)
rL	22	180	—
rH	71[b]	<15[b]	30
	47[c]	450[c]	
Horse spleen	35[b]	<40[b]	180
	30[c]	500[c]	

[a]Conditions: protein concentration 2.3×10^{-7} M in 20 mM MOPS-NaOH, pH 6.5 plus 100 iron atoms/polymer; temperature 20 °C. Absorbance was measured at 310 nm, fluorescence at the wavelength of maximum emission: 325 nm for rL and rH apoferritin, 316 nm for horse spleen apoferritin.
[b]The values refer to the initial fluorescence quenching phase.
[c]The values refer to the fluorescence recovery phase.

and the fluorescence change was monitored after each addition (Fig. 2B). In the rL homopolymers, the fluorescence intensity shows a slow monotonic decrease after each addition, similarly to what observed when iron is added in a single step (Fig. 2A). In the rH homopolymer, a fast fluorescence quenching phase and a subsequent slow recovery phase are observed after each addition, albeit with significant differences in amplitude and time course. Thus, the initial fast quenching after the addition of the first 25 iron atoms is about twice as large as that characterizing the subsequent additions; in turn, the slow recovery phase is characterized by an abrupt onset and is less marked than after the later additions. The absorbance change monitored in parallel displays the same rate after the first two iron additions ($t_{1/2}$ = 12 s) and a slower one after the third addition ($t_{1/2}$ = 30 s) in accordance with the observations of Treffry et al. (1995). In the case of horse spleen apoferritin, the first 25 iron atoms quench the intrinsic fluorescence, but the recovery phase is not observed, whereas in the subsequent additions both phases are present and are of similar amplitude. When absorbance is followed, the observed rate decreases significantly after the first iron addition ($t_{1/2}$ is 85 s after the first addition and 300 s after the second). Figures 1 and 2B also show that very similar fluorescence intensities are attained at the end of the reaction for all apoferritins independent of the method of iron administration.

The availability of incorporated iron to chelating agents was tested by monitoring the fluorescence intensity after adding citrate at the end of the reaction (i.e., after 4,000 s). Only in the case of the rL homopolymer the addition of citrate resulted in a slow, partial fluorescence recovery. Essentially no changes of the fluorescence signal were observed with rH and horse spleen apoferritins (data not shown).

Time-resolved fluorescence measurements

In horse spleen apoferritin, iron was shown to affect the fluorescence decay of both the short and long lifetime components (Rosato et al., 1987). Time-resolved experiments have been performed with the rH homopolymer and horse spleen apoferritin; rL apoferritin has not been studied due to the paucity of the available material.

The fluorescence decay of the rH homopolymer displays two lifetime components, although the polypeptide chain contains a single tryptophan. Other single-tryptophan protein systems are characterized by a complex emission decay that has been described in terms of a sum of discrete exponentials (as in azurin, Mei et al., 1996), or a continuous distribution of lifetimes (as in human superoxide dismutase (Rosato et al., 1990) or apomyoglobin (Bismuto et al., 1988)) or even a bimodal distribution (as in the S-100a protein (Zolese et al., 1996)).

The parameters which characterize the fluorescence decay as a function of time after the aerobic addition of iron (100 atoms/polymer at pH 6.5) are shown in Figures 3A and 4A for the two apoferritins. The width and the center of the distribution associated to the shorter lifetimes (w_1, τ_1) change with different trends in the two proteins. In particular, the center of the distribution increases from 0.6 to 0.7 ns in the rH homopolymer but is essentially constant at 0.9 ns in horse spleen apoferritin. On the other hand, the long lifetime component of the two samples (τ_2) decreases albeit to a different extent in rH and horse spleen apoferritin (9% and 20%, respectively). This decrease is probably associated to the iron-induced quenching effect observed in the steady-state measurements. It should be stressed that the time-resolved data, when used to calculate the fluorescence intensity change as apparent in the steady-state measurements, are in remarkably good correspondence with the latter set of data (Figs. 3B, 4B).

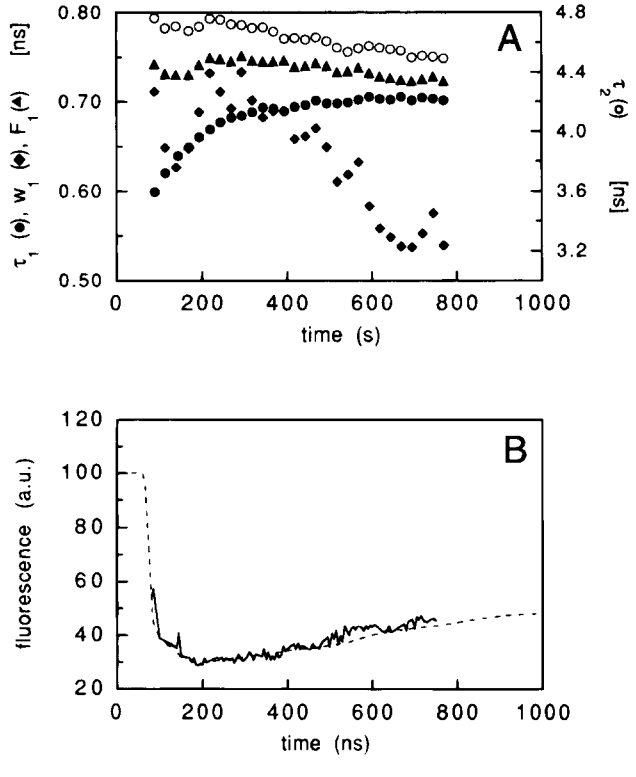

Fig. 3. Fluorescence decay parameters of rH apoferritin after aerobic addition of 100 Fe(II) atoms/polymer. **A:** Center (●), width (◆), and fraction (△) of the fast, Lorentzian-distributed lifetime component and center (○) of the slow lifetime component. Experimental conditions are identical to those of Figure 2A. **B:** The time-resolved data of panel **A** are presented as fluorescence intensity and compared to the data of Figure 2A (dotted line); for details of the calculation see Materials and methods. The experimental conditions are as in Figure 2.

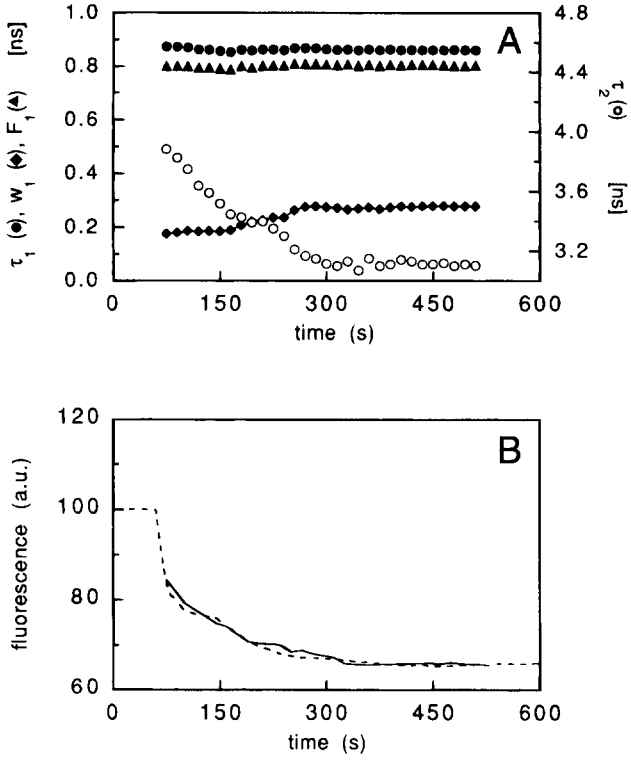

Fig. 4. Fluorescence decay parameters of horse spleen apoferritin after aerobic addition of 100 Fe(II) atoms/polymer. **A:** Center (●), width (♦), and fraction (△) of the fast Lorentzian-distributed lifetime component and center (○) of the slow lifetime component. Experimental conditions are identical to those of Figure 2A. **B:** The time-resolved data of panel **A** are presented as fluorescence intensity and compared to the data of Figure 2A (dotted line); for details of the calculation see Materials and methods. The experimental conditions are as in Figure 2.

Discussion

The emission properties and the excited state decay of the single, conserved tryptophanyl residue of the apoferritin L and H chains were known to represent a sensitive probe of the state and mode of assembly of the apoferritin polymer. Thus, the decay patterns differ in horse spleen apoferritin (85–90% L) and its subunits (Rosato et al., 1987) and the emission maximum occurs at significantly different wavelengths in the horse spleen and in the rH and rL proteins (Stefanini et al., 1982; Santambrogio et al., 1993; Fig. 1). The latter observation, in particular, indicates that the local conformation around the tryptophan residue differs when the L and H chains are assembled in a homo- or heteropolymer, a feature that is not revealed by other techniques. The present work shows that tryptophan fluorescence is sensitive also to the different features of iron oxidation and incorporation processes in ferritins of different subunit composition.

After aerobic addition of iron (100 atoms/polymer at pH 6.5), the steady-state emission spectra of the three apoferritins are not altered significantly in shape but are quenched to varying extents (Fig. 1 and Table 1). This finding is in accordance with the suggestion that in horse spleen apoferritin quenching is due to a Förster-like energy transfer between tryptophan and iron (Stefanini et al., 1976). The different mechanisms that lead to iron oxidation and incorporation in the three proteins are clearly apparent in the different kinetic features of the fluorescence intensity change. Thus, in the rH-homopolymer fluorescence is quenched rapidly ($t_{1/2} \leq$ 15 s) after aerobic administration of iron. Iron oxidation monitored by optical absorption has a $t_{1/2}$ value of about 30 s (Table 1). On this basis, the initial quenching phase must reflect both Fe(II) binding to the ferroxidase center, which is at 10–12 Å from the tryptophan residue of the same chain, and iron oxidation at this very same site, a fast process which gives rise to μ-oxo-bridged Fe(III) dimers (Treffry et al., 1995). Moreover, the subsequent increase of the fluorescence signal ($t_{1/2} \sim 450$ s) must be related to factors other than iron oxidation, namely to the migration of Fe(III) from the ferroxidase center to the nucleation sites in the internal cavity, a process that cannot be singled out by optical absorption which monitors Fe(III) formation. This interpretation is in good agreement with the model of iron incorporation proposed on the basis of other spectroscopic experiments (Bauminger et al., 1989; Treffry et al., 1996) and of crystallographic data (Hempstead et al., 1994). It is also consistent with the fluorescence changes observed upon stepwise addition of iron (Fig. 2B). Thus, the first 25 iron atoms produce a relatively stronger quenching phase and a smaller Fe(III) release phase if compared to subsequent iron additions, when incoming Fe(II) has to displace any Fe(III) that may still occupy the ferroxidase centers. It is as if after the addition of the first 25 atoms a sort of steady-state situation is established at the ferroxidase centers between Fe(II) and Fe(III); accordingly, the amplitudes of the fluorescence quenching and recovery phases are very similar upon further additions of iron.

In the rL homopolymer, the Fe(III) formation and nucleation processes give rise to a monotonic decrease of the fluorescence signal. This finding is consistent with the knowledge that Fe(II) atoms diffuse into the threefold channels, bind to specific, exposed carboxylate groups where a slow oxidation takes place, and thereafter move into the internal cavity (Levi et al., 1989, 1994). Since the threefold channels are at a greater average distance from the tryptophan residues than the ferroxidase centers, the extent of quenching in rL apoferritin is less than in the rH homopolymer. Moreover, there is no recovery of the fluorescence signal consistent with the proposed correlation of this phase with the release of Fe(III) from the H chain ferroxidase center. In addition, again consistently with the proposed picture, upon stepwise addition of iron the fluorescence change occurs at the same slow rate. The partial recovery of intrinsic fluorescence observed after the addition of sodium citrate at the end of the iron incorporation reaction is of interest; it indicates that micellar iron in rL ferritin is stored in a more labile fashion than in the horse spleen and rH apoferritins.

The kinetics of the fluorescence change in horse spleen apoferritin is dominated, in particular during the initial quenching phase, by the contribution of the H chains although they represent only 10–15% of the subunit content (Fig. 2A). In fact, the intrinsic tryptophan fluorescence is expected to be quenched more when iron is bound at the ferroxidase centers than at the threefold channels, since the average distance of the former from the tryptophan residues is smaller. In the later phases of the iron incorporation process, which reflect the progressive localization of polymeric Fe(III) clusters in the apoferritin cavity, the relative contribution of the H chains becomes progressively smaller. Thus, at the end of the reaction (Figs. 1, 2A), a linear combination of the L and the H contribution roughly accounts for the fluorescence intensity of the horse spleen heteropolymer. Of interest is the comparison between the $t_{1/2}$ values for the iron oxidation/incorporation process obtained by fluorescence and optical absorption (Table 1) as it pro-

vides a further proof of the usefulness of tryptophan fluorescence in probing ferritin function. Thus, by fluorescence Fe(II) binding and oxidation at the ferroxidase center of the few H chains can be clearly distinguished from the subsequent Fe(III) migration step, whereas by optical absorption the overall reaction appears as a single process. Consequently, the halftime of the overall reaction determined by optical absorption (180 s) is intermediate between the values of the two distinct phases monitored by fluorescence ($t_{1/2} < 40$ s and 500 s, respectively). Moreover, the $t_{1/2}$ value of the fluorescence change, which reflects quenching of tryptophan fluorescence by Fe(II) and/or Fe(III) in both chain types, is similar in horse spleen and rH apoferritin ($t_{1/2} < 15$ and < 40 s, respectively). In line with the proposed picture, stepwise addition of iron yields different kinetic profiles in rH and horse spleen apoferritin (Fig. 2B). In particular, the first 25 iron atoms produce a quenching and a recovery phase in the rH protein, but only quenching in the horse spleen heteropolymer, indicating that the H chain ferroxidase centers do not release iron under these conditions. It is only after the addition of further Fe(II), which displaces the Fe(III) from the ferroxidase centers, that both processes occur. The amplitude and kinetics of the two phases remain constant after the second addition of iron pointing again to the establishment of a sort of steady-state situation between Fe(II) and Fe(III) at the ferroxidase centers. To our knowledge, the rate-limiting effect that Fe(III) bound at the ferroxidase centers has on the overall rate of oxidation has never been brought out so clearly by other techniques.

In the discussion of the steady-state fluorescence data, the possible contribution of local conformational changes has not been taken into account. Fe(II) binding and Fe(III) release could indeed require small structural changes involving, for example, part of the four helix bundle in the H chains. Since such changes can be expected to take place in a faster time regime than the iron oxidation and incorporation processes, their possible occurrence has been verified in dynamic fluorescence experiments carried out on the rH homopolymer and on horse spleen apoferritin. The fluorescence lifetimes of both components change in a distinctly different manner in the two proteins (Figs. 3A, 4A) most likely due to the existence of different local drifts in the tryptophan environment leading to different arrangements of the protein matrix. Whatever is the physical reason of the observed effects, it should be stressed that the time-resolved data when used to calculate the fluorescence intensity change during iron incorporation are in remarkably good correspondence with the steady-state experiments (Figs. 3B, 4B). The changes in the width of the Lorentzian distribution are of interest as this parameter reflects the number of conformational microstates which influence tryptophan emission during the excited state (Figs. 3A, 4A). The width of the distribution increases slightly in the horse spleen heteropolymer pointing to an enhanced heterogeneity of the system. In contrast, in the rH homopolymer the width of the distribution shows a fast decrease which parallels the Fe(II) binding and oxidation phase at the ferroxidase centers. These processes render the local tryptophan environment more homogeneous and/or more rigid. Subsequently, the width of the distribution displays an abrupt increase followed by a slow decrease. These two phases can be correlated respectively to the movement of Fe(III) toward the cavity, a process entailing an increased heterogeneity in the system, and to formation of polymeric Fe(III) clusters which, in turn, renders the tryptophan environment more homogeneous.

In conclusion, the present work shows that the steady-state and time-resolved fluorescence parameters in apoferritins of different subunit composition respond differently to the addition, oxidation, and movement of iron. Therefore, fluorescence techniques provide a simple means to single out specific differences due to changes in subunit composition or resulting from site-specific mutations.

Materials and methods

Sample preparation

Horse spleen ferritin (85–90% L) was prepared as described previously (Stefanini et al., 1982). Human recombinant H-chain ferritin (100% H subunit, rH) was overexpressed in *Escherichia coli* and purified with the procedure described by Levi et al. (1988). The recombinant human L-homopolymer (rL), overexpressed in *E. coli*, was a generous gift from Drs. P. Arosio and S. Levi; it did not contain significant amounts of iron and was used as such. Iron was removed from horse spleen ferritin by using thioglycollic acid and chelation with 2,2′-bipyridyl and from rH-ferritin by incubation for 24 h in 0.3% sodium dithionite in 0.1 M MES-NaOH buffer at pH 6.0 and chelation with 2,2′-bipyridyl. For all apoferritins the concentration was calculated from the absorbance at 280 nm using the extinction coefficient $E_{1\%,1cm} = 9.0$.

Fluorescence measurements

Steady-state fluorescence measurements were performed using a photon-counting spectrofluorometer (Fluoromax, I. S. A. Paris, France). The optical absorbance of all samples at 280 nm was always around 0.10. In the experiments designed to follow iron incorporation, the sample was excited at 280 nm and the fluorescence signal was recorded at a wavelength corresponding to the maximum of the emission spectrum (i.e., 316 nm for horse spleen apoferritin and 325 nm for the L and H homopolymers). The bandwidths of the excitation and emission monochromators were between 2 and 4 nm. The sample holder was thermostatted at 20 °C using an external circulating water bath (Haake, CHF3, Germany) and the samples were continuously stirred during measurements. The fluorescence signal was corrected for the absorbance at 280 nm due to iron oxidation which was determined in parallel optical absorption measurements. The inner-filter effect along the emission pathway was taken into account according to Kubista et al. (1994). However, this correction was always negligible (less than 3%).

The fluorescence decay of rH and horse spleen apoferritin was measured as a function of time after aerobic addition of iron at 20 °C. The phase shift and demodulation technique have been used to collect the data at frequencies corresponding to the harmonic content of a Nd-Yag mode-locked laser (Gratton & Limkeman, 1983). A frequency range between 20 and 300 MHz was selected for the measurements. The excitation (through a polarizer at the "magic angle") was at 292 nm, while emission was observed through a WG 305 cutoff filter to remove scattered light.

A Marquardt-algorithm routine was used to analyze the data (Beechem & Gratton, 1988). The best fit of the data was always complex and required at least two distinct components, one of which was distributed according to a Lorentzian function. The parameters characterizing the fluorescence decay (i.e., lifetime and relative fluorescence fractional intensity) have been used to evaluate the steady-state signal as a function of time according to the expression

$$\langle I(t)\rangle = \int_0^\infty [F_1 L(\theta) + F_2 e^{-\theta/\tau 2}]\, d\theta,$$

where $\langle I(t)\rangle$ represents the fluorescence intensity at time t after iron addition, $L(\theta)$ is the Lorentzian-distributed lifetime function, τ_1 and τ_2 are the lifetimes associated to the shorter and longer lifetime component, respectively, and F_1 and F_2 are the relative fractions.

Iron incorporation experiments

A freshly degassed solution of ferrous ammonium sulfate in water ($1–5 \times 10^{-2}$ M) was used as source of iron. The kinetics of iron uptake was followed in air at 20 °C after mixing apoferritin at concentrations around 0.2 mM (apoferritin polymer) in 20 mM MOPS-NaOH buffer at pH 6.5 with the appropriate volume of ferrous ammonium sulfate and by monitoring the increase of optical absorbance at 310 nm, where the extinction coefficient of ferritin iron, $E_{1\%,1cm}$, is 450 (Macara et al., 1972). The optical absorption measurements were recorded on a Cary 210 (Varian) or a Hewlett Packard 8452 A spectrophotometer.

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