Incorporation of iron by the unusual dodecameric ferritin from *Listeria innocua*

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The polypeptide chain that assembles into the unusual dodecameric shell of *Listeria innocua* apoferritin lacks the ferroxidase centre characteristic of H-type mammalian chains, but is able to catalyse both Fe(II) oxidation and nucleation of the iron core. A cluster of five carboxylate residues, which correspond in part to the site of iron core nucleation typical of L-type mammalian ferritins, has been proposed to be involved in both functions. The features of the iron uptake kinetics and of Fe(II) autoxidation in the presence of citrate followed spectrophotometrically confirm this assignment. In *Listeria* the kinetics of iron uptake is hyperbolic at low Fe(II)-to-dodecamer ratios and becomes sigmoidal when iron exceeds 150 Fe(II) atoms per dodecamer, namely when a fast crystal growth phase follows a slow initial nucleation step. Iron autoxidation in the presence of

citrate displays a similar behaviour. Thus the time course is sigmoidal at low citrate-to-Fe ratios at which Fe(III) polymerization is predominant, but is hyperbolic at ligand concentrations high enough to prevent polymerization. The marked inhibitory effect of Tb(III) on the kinetics of iron incorporation confirms that carboxylates provide the iron ligands in *L. innocua* apoferritin. Iron uptake followed in steady-state fluorescence experiments allows one to distinguish Fe(II) binding and oxidation from the subsequent movement of Fe(III) into the apoferritin cavity as in mammalian ferritins despite the different localization of the tryptophan residues.

Key words: Dps proteins, iron–citrate complexes, iron oxidation.

INTRODUCTION

Ferritin extracted from the Gram-positive bacterium *Listeria innocua* is unusual in many respects [1]. The protein shell is constructed from one subunit type with a molecular mass of 18 kDa. The subunit bears a great similarity in sequence to the DNA-binding proteins of the Dps family, rather than to members of the ferritin family, with the exception of some similarities to the specific region of mammalian L-chains corresponding to the carboxylate-rich iron core nucleation site. Interestingly, in *Listeria* this cluster is enriched by an additional aspartic residue relative to L-type chains. The ferroxidase centre residues characteristic of mammalian H-chains are lacking. The structural similarity of *Listeria* ferritin and Dps proteins carries over to the quaternary structure, an assembly of 12 subunits and not 24 as in all other known ferritins [1–3]. From a functional viewpoint, *L. innocua* ferritin incorporates iron efficiently within its protein shell, but does not bind DNA. In particular, it can store a maximum of approx. 500 Fe atoms per dodecamer, an amount consistent with a protein cage significantly smaller than that of mammalian apoferritin, which can harbour up to 4500 Fe atoms per dodecamer.

The high iron-accumulation capacity of *L. innocua* ferritin, a property characteristic of L-type mammalian ferritins, and the sequence similarity with the iron nucleation site of this chain type, suggested that the corresponding cluster of carboxylate residues in the *Listeria* subunit could form the sites of iron oxidation and nucleation of the ferritin core. This hypothesis has been tested in the present study. The kinetics of iron uptake by *L. innocua* apoferritin has been followed by optical and fluorescence spectroscopy after the aerobic addition of Fe(II) at various iron-to-dodecamer ratios and pH values. In parallel, the kinetics of iron oxidation has been measured in the presence of citrate because it is known that strong Fe(III)-complexing agents

facilitate iron oxidation [4]. Again, the reaction has been followed at different citrate-to-iron ratios and pH values. The results obtained with this model system indicate that both the catalysis of Fe(II) oxidation and the subsequent clustering of Fe(III) to generate ferrihydrite micelles can be performed by appropriately placed carboxylate residues.

EXPERIMENTAL

Ferritins

L. innocua ferritin was isolated and purified from packed bacterial cells by following the method described previously [1]. Iron was removed from native ferritin, which contains 50–100 iron atoms per dodecamer, by incubation for 24 h in 0.1 M Mes/NaOH buffer, pH 6.0, containing 0.3% sodium dithionite and by subsequent chelation with 2,2'-bipyridyl. Apoferritin concentration was determined spectrophotometrically on the basis of the molar (molecular mass 216 kDa) absorption coefficient, ϵ , of $2.59 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 280 nm [1]; it is expressed on a dodecamer basis unless otherwise stated. The iron content in native and/or reconstituted ferritin was measured as the 2,2'-bipyridyl complex at 520 nm [5] and/or by atomic absorption; the values obtained with the two methods were in very good agreement. The recombinant human L-homopolymer (rL) was a gift from Dr. P. Arosio and Dr. P. Santambrogio; it did not contain significant amounts of iron and was used as supplied.

Iron incorporation

Solutions of ferrous ammonium sulphate were prepared before each experiment by dissolving weighed amounts of the salt in AnalaR water deoxygenated by bubbling with nitrogen. The iron incorporation kinetics was monitored spectrophotometrically at

Abbreviation used: r, recombinant.

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310 nm and 25 °C after the addition of different amounts of iron to protein solutions equilibrated in air. The solutions were kept under continuous stirring during the course of the reaction. The absorption coefficient of micellar iron was taken as $A_{1\text{cm}}^{1\%} = 450$ [6]. The kinetics of iron oxidation in the presence of sodium citrate was measured by adding to the buffer an appropriate amount of citrate to yield a 10-fold or 50-fold molar excess over iron. As a control the rate of Fe(II) autoxidation was measured in parallel.

In competition experiments between two metals, *L. innocua* apoferritin was incubated with different concentrations of TbCl_3 , ZnSO_4 or CaCl_2 for at least 5–10 min at 25 °C before the addition of iron. All the solutions were prepared freshly before each experiment by dissolving weighed amounts of the salts in AnalaR water. The optical absorption measurements were performed on a Hewlett Packard 8452 A spectrophotometer.

After the incorporation of iron, the samples were subjected to gel electrophoresis under non-denaturing conditions in 6% (w/v) polyacrylamide gels. The gels were stained for iron with potassium ferrocyanide and for protein with Coomassie Blue.

Fluorescence measurements

Steady-state fluorescence measurements were performed with a photon-counting spectrofluorimeter (Fluoromax; I.S.A., Paris, France). The optical absorbance of all samples at 280 nm was always approx. 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. 330 nm) after approx. 10 s from the addition of iron. The bandwidths of the excitation and emission monochromators were between 2 and 4 nm. The sample holder was thermostatically controlled at 25 °C by using an external circulating-water bath, CHF3 (Haake, Karlsruhe, Germany) and the samples were stirred continuously during measurements.

Fluorimetric titrations in the presence of TbCl_3 were performed by adding to 0.16 μM *L. innocua* apoferritin, in 50 mM Mops/NaOH buffer, pH 7.0, increments of 0.5 μM TbCl_3 at 25 °C. The fluorescence emission spectra were measured between 520 and 560 nm with excitation at 295 nm. When the effects of iron, calcium or zinc were studied, the experiments were performed by adding the metal to apoferritin containing 2 Tb(III) per subunit. The fluorescence emission spectrum of the lanthanide was monitored as a function of time. The intensity of the tryptophan fluorescence emission was also measured in parallel.

RESULTS

Iron incorporation experiments

Iron uptake by *L. innocua* apoferritin was studied in 50 mM Mops/NaOH at pH 7.0 after the aerobic addition of Fe(II) to the protein. A maximum of 400–500 iron atoms per apoferritin molecule was added because at higher iron-to-dodecamer molar ratios precipitation of ferric ions outside the protein is observed [1]. In the experiment shown in Figure 1(A) and whenever Fe(II) was less than 150 atoms per dodecamer, the time course of Fe(II) oxidation by *Listeria* was hyperbolic. The significant facilitation of Fe(II) oxidation relative to buffer alone is presented in Figure 1(B) in terms of initial rates. At Fe(II)-to-dodecamer ratios of 150 or higher, the time course of iron oxidation became sigmoidal (Figure 1A), an indication that oxidation of the mineral core itself became important. In the absence of protein, as expected, massive precipitation of ferric ions was observed. An experiment was performed with the rL homopolymer in the

Figure 1 Progress curves (A) and corresponding initial rates (B) of iron uptake by *L. innocua* apoferritin as a function of Fe(II) concentration

Conditions: apoferritin, 1.75 μM in 50 mM Mops/NaOH, pH 7.0; temperature, 25 °C. In (A) the number of Fe(II) atoms/dodecamer is indicated against each trace. Symbols in (B): ■, *Listeria*; ●, the corresponding autoxidation in buffer.

presence of 30 atoms of Fe(II) per dodecamer; the rate of Fe(II) oxidation was about twice that measured in buffer alone, in accordance with the data of Sun et al. [7].

In another series of experiments the iron uptake kinetics was followed after the addition of 100 iron atoms per dodecamer at pH 8.0 and 5.5. At pH 8.0 in 50 mM Hepes/NaOH, the reaction had a hyperbolic time course and was significantly faster than at pH 7.0 (initial rate approx. 550 μM Fe/min); the optical absorbance at the end of the reaction was the same at the two pH values, pointing to the incorporation of similar amounts of iron. At pH 5.5 in 50 mM Mes/NaOH, iron oxidation took place at a very low rate that was not altered by the presence of *L. innocua* apoferritin. All the spectrophotometric findings were confirmed by Prussian Blue staining of the samples subjected to electrophoresis 24 h after the kinetic experiments.

The presence of an iron core influences the iron uptake kinetics in the same way as that described for the L-rich horse spleen protein [6]. Thus when 200 Fe(II) per dodecamer were added at pH 7.0 to native *L. innocua* ferritin containing 100 Fe(III) per dodecamer, the progress curves were hyperbolic and significantly faster than observed in the presence of the apoprotein ($t_{1/2} \approx 100$ s as compared with 300 ± 100 s).

In mammalian ferritins, steady-state fluorescence has been shown to provide a simple means of distinguishing between the formation and movement of Fe(III). Fe(II) binding and oxidation

Figure 2 Fluorescence intensity of *L. innocua* apoferritin after aerobic addition of 100 Fe(II)/dodecamer

Conditions: apoferritin, 0.52 μ M in 50 mM Mops/NaOH, pH 7.0; temperature, 25 $^{\circ}$ C. Excitation wavelength, 280 nm; fluorescence emission recorded at 330 nm. Inset: comparison of the fluorescence and absorbance data measured in parallel. Arrows indicate the addition of iron.

Figure 3 Effect of citrate and pH on the progress curves of Fe(II) autoxidation

The molar ratio of citrate to iron was 10 in (A) and 50 in (B); iron concentration: 300 μ M. Buffers: trace a, 50 mM Hepes/NaOH, pH 8.0; trace b, 50 mM Mops/NaOH, pH 7.5; trace c, 50 mM Mops/NaOH, pH 7.2; trace d, 50 mM Mops/NaOH, pH 7.0; trace e, 50 mM Mops/NaOH, pH 6.5. Temperature, 25 $^{\circ}$ C. Arrows indicate the common absorbance values at the end of the reaction.

result in quenching of the intrinsic tryptophan fluorescence irrespective of the subunit composition of the apoferritin shell, whereas Fe(III) movement has a different effect in the various apoferritins [8]. In *Listeria* apoferritin the addition of iron quenches the intrinsic fluorescence, although the two tryptophan

Figure 4 Effect of Tb(III) (upper panel) and Zn(II) (lower panel) on the iron uptake kinetics of *L. innocua* apoferritin

Conditions: 1.76 μ M apoferritin and 352 μ M iron (upper panel); 1.50 μ M apoferritin and 300 μ M iron (lower panel); buffer, 50 mM Mops/NaOH, pH 7.0; temperature, 25 $^{\circ}$ C. Numbers against traces indicate the ratio of Tb(III) or Zn(II) to apoferritin. Arrows indicate the common absorbance values at the end of the reactions. Abbreviation: sub, subunit.

residues are both in different positions from the conserved tryptophan of mammalian ferritin subunits [1].

The fluorescence change attendant on the aerobic addition of 100 Fe(II) per dodecamer to 0.5 μ M *L. innocua* apoferritin at pH 7.0 comprised several phases (Figure 2). After a rapid quenching, a fast phase with $t_{1/2} \approx 170$ s was observed, followed by a significantly slower quenching phase leading in 3000–4000 s to a constant fluorescence intensity corresponding to approx. 50% of the initial signal. Thereafter a slight recovery was observed. To correlate the fluorescence changes with the iron uptake process monitored by optical absorbance at 310 nm, the reaction was followed in parallel in the spectrophotometer. The time course of the reaction was hyperbolic and the half-time was approx. 700 s (initial velocity 3.2 μ M Fe/min), consistent with the results in Figure 1(A) and with the lower protein concentration used (0.52 μ M).

Kinetics of iron autoxidation in the presence of citrate

To establish whether the features of the iron uptake curve by *L. innocua* apoferritin can be ascribed solely to the carboxylate residues corresponding to the Fe(III) nucleation site of mammalian L-chains, the oxidation of Fe(II) was monitored in the presence of sodium citrate. In fact, strong Fe(III)-chelating agents such as citrate or EDTA are known to accelerate the rate of Fe(II) autoxidation as compared with that measured in buffer alone [4].

Figure 3 shows that at neutral pH the shape of the Fe(II) autoxidation curves changed from sigmoidal to hyperbolic when the molar ratio of citrate to Fe(II) changed from 10:1 to 50:1 at a constant iron concentration of 300 μ M. This finding is consistent with the observation that citrate in 20–30-fold molar excess over iron is sufficient to prevent the Fe(III) polymerization of Fe(NO₃)₃ [9].

The kinetics of Fe(II) autoxidation, monitored as a function of pH at various molar ratios of citrate to Fe(II) and at a constant

Figure 5 Fluorimetric titration with Tb(III) of *L. innocua* apoferritin (A) and fluorescence intensity change after Fe(II) addition to the Tb(III)-apoferritin complex (B)

(A) Aliquots (0.5 μ M) of a Tb(III) solution were added to apoferritin (2 μ M in subunit) in 50 mM Mops/NaOH, pH 7.0. Excitation was at 295 nm. The inset shows fluorescence emission spectra. (B) A solution containing 135 μ M Fe(II) was added to a Tb(III)-apoferritin complex [6.6 μ M in apoferritin subunits, 13.2 μ M in Tb(III)]. The fluorescence intensity of Tb(III) at 545 nm (■) and the intrinsic fluorescence of the Tb(III)-apoferritin complex at 330 nm (●) were monitored in parallel. Tb(III) emission spectra (excitation at 295 nm) and Tb(III)-apoferritin complex emission spectra (excitation at 280 nm) are shown in insets (a) and (b) respectively.

iron concentration of 300 μ M, displayed the same features described for *Listeria* apoferritin (Figure 3). When the molar ratio of citrate to Fe(II) was 50:1, the progress curves were hyperbolic over the whole pH range investigated (5.5–8.0). In contrast, at a 10:1 molar ratio, the curves were sigmoidal up to pH 7.2 and become hyperbolic at higher pH. The rate of reaction displayed the expected pH-dependence, being faster at alkaline pH. At all pH values, $t_{1/2}$ values were significantly shorter than those measured in the presence of *L. innocua* apoferritin.

Effect of Tb(III) and Zn(II) on iron uptake kinetics

The effect of metal ions known to inhibit iron incorporation into mammalian ferritins, namely Tb(III) and Zn(II) [10–12], was studied at pH 7.0 (Figure 4). The addition of 12 Tb(III) per dodecamer [i.e. 1 Tb(III) per subunit] before the addition of 200 Fe per dodecamer resulted in a significant decrease in the rate of iron uptake by *L. innocua* apoferritin ($t_{1/2}$ = 600 s compared

with $t_{1/2}$ = 250 s in the absence of the competing metal). When 3 Tb(III) per subunit were added, very little iron was incorporated in the first 1200 s. Zn(II) had a smaller effect than Tb(III) under the same experimental conditions. The same end point was attained in all the experiments.

The effect of Tb(III) on the rate of iron uptake by *L. innocua* apoferritin just reported points to a competition between the two metals for the same protein binding sites. To confirm this finding, we took advantage of the well-known enhancement of Tb(III) luminescence attendant on binding of the lanthanide to proteins [13]. Fluorescence titrations were performed in 50 mM Mops/NaOH, pH 7.0, by adding Tb(III) to *L. innocua* apoferritin and monitoring the fluorescence emission spectrum (excitation at 295 nm) after each addition of the lanthanide. The saturation point was reached at 2 Tb(III) per subunit (Figure 5A). The intrinsic fluorescence of the apoprotein did not change after the addition of Tb(III).

In a second series of experiments the competition between iron and Tb(III) was followed at pH 7.0 by measuring the fluorescence of the Tb(III)-apoferritin complex at 545 nm. As expected, after the aerobic addition of 200 Fe(II) per dodecamer [16.7 Fe(II) per subunit] the fluorescence of apoferritin containing 2 Tb(III) per subunit decreased slowly and was almost zero at approx. 2 h after the addition of iron. The intrinsic fluorescence of the protein monitored in parallel at 330 nm was quenched with similar kinetics (Figure 5B). Thereafter, the possible competition between Tb(III) and Zn(II) was assessed in a similar fashion. The addition of 200 Zn(II) per dodecamer did not change the fluorescence of the Tb(III)-apoferritin complex (results not shown).

Lastly, the effect of Ca(II) on Tb(III) luminescence was studied, because these two metals share binding sites on several proteins [14]. A 10-fold excess of Ca(II) over Tb(III) did not affect the luminescence of the Tb(III)-apoferritin complex. Consistently with this observation, the addition of 6 Ca(II) per subunit [72 Ca(II) per dodecamer] did not affect the rate of the iron incorporation reaction (results not shown).

DISCUSSION

L. innocua apoferritin has unique structure-function relationships among ferritins. It is a 12-mer constructed with a single subunit type that accomplishes with high efficiency both the oxidation of Fe(II) and nucleation of the iron core. In the mammalian proteins these two functions are performed on different chain types: Fe(II) is bound and oxidized at the so-called ferroxidase centre localized within the four-helix bundle of the H chains, whereas formation of the Fe(III) core takes place on the L chains at a cluster of four carboxylate residues that protrude into the cavity of the apoferritin molecule from the B helix. The ferroxidase centre residues are conserved in plant ferritin and haem-containing bacterioferritin homopolymers and confer a high Fe(II) oxidation capacity to the constituent subunit [3]. In *L. innocua* the ferroxidase centre residues are lacking. However, the cluster of glutamic residues typical of L-type chains (Glu-44, Glu-52, Glu-55, Glu-59 and Glu-62, with the *Listeria* chain numbering) comprises an additional carboxylate (Asp-48) that faces the same side of the B helix [1]. The involvement of this site in iron oxidation and core nucleation, proposed by Bozzi et al. [1] also in view of the Fe(II) autooxidation facilitated by Fe(III)-complexing agents such as EDTA or citrate [4], is consistent with the present results.

The time course of iron oxidation and incorporation by *L. innocua* ferritin at neutral pH depends on the Fe(II)-to-dodecamer ratio and changes from hyperbolic to sigmoidal when

Fe(II) is in 150-fold molar excess over dodecamer. A similar change in shape of the iron uptake kinetics is displayed by the L-rich spleen protein when large amounts of iron are provided, because oxidation on the iron core itself becomes significant with respect to oxidation at the ferroxidase sites [6]. In contrast, in H-rich ferritins, in which the Fe(II) oxidation function dominates, iron uptake follows a hyperbolic time course irrespective of the amount of iron furnished [15]. In *Listeria*, in which the same subunit favours both iron oxidation and core growth, the prevalence of the former or the latter process is dictated by the relative amounts of iron and protein. The behaviour of the citrate/Fe(II) model system can be explained along the same lines. The sigmoidal or hyperbolic kinetics of Fe(II) autoxidation measured at various molar ratios of citrate to Fe(II) (Figure 3) can be taken to reflect, respectively, the predominant formation of polymeric species at relatively high concentrations of iron or of low-molecular-mass complexes at relatively high ligand concentrations [9].

The similarity of the Fe(II) oxidation process in the presence of *Listeria* and citrate lends support to the proposal of Bozzi et al. [1] that carboxylates constitute the iron oxidation and nucleation sites in this unusual ferritin. The pH dependence of the Fe(II) oxidation kinetics further substantiates this contention. In both systems the initial rate of Fe(II) oxidation increases with increasing pH over the range 5.5–8.0. A similar behaviour has been reported for the human liver protein (96% L), whereas a bell-shaped pH profile characterizes the horse spleen protein (approx. 85% L) and the rH homopolymer [7,17]. These differences emphasize the complex interplay between the major factors involved, namely the deprotonation of co-ordinating functional groups and Fe(II) oxidation. As with horse liver ferritin [7], the behaviour of *Listeria* can be ascribed to the relatively low activity of the protein, which is obscured by the high rate of Fe(II) oxidation at pH > 7.0.

The effects of Tb(III) are likewise consistent with the presence of carboxylates as the metal ligands in *L. innocua* apoferritin. The fluorescence titration experiments demonstrate that 2 Tb(III) ions are bound per *L. innocua* subunit with similar affinity (Figure 5). Moreover the Tb(III)–apoferritin luminescence is fully quenched on the addition and incorporation of iron, indicating that the lanthanide and the iron compete for the same sites on the protein. In accordance with these results, Tb(III) decreases the rate of incorporation of iron significantly (Figure 4). Zn(II) has a small effect on the iron uptake kinetics (Figure 4); moreover, in line with this observation, it does not compete with Tb(III), suggesting the presence of different binding sites on the protein. Ca(II) has no effect on the luminescence of the Tb(III)–apoferritin complex nor on the rate of incorporation of iron.

Insight into the rates at which the different steps of the iron incorporation process take place can be obtained by correlating the optical absorbance changes with those in intrinsic tryptophan fluorescence. On the addition of Fe(II), a quenching phase is observed that comprises several processes and is followed by a small recovery phase after 3000–4000 s, whereas optical absorption monitored in parallel increases hyperbolically with a half time of approx. 700 s. On this basis, the fast initial quenching must reflect the binding and oxidation of Fe(II), whereas the slow quenching and recovery phases must reflect the movement of Fe(III) into the internal apoferritin cavity and the formation of the iron core. In mammalian ferritins the same type of kinetics, characterized by quenching and a subsequent recovery

phase, is observed when both Fe(II) oxidation and movement of Fe(III) contribute to the experimental picture, namely in the horse spleen and in the rH proteins. In the rL homopolymer, in which Fe(II) binding and a slow oxidation at the cluster of carboxylate residues take place without significant core formation, only a monotonic fluorescence quenching is observed [8]. In terms of structure–function relationships, it is of interest that the fast initial fluorescence quenching phase is of comparable magnitudes in *Listeria* and in mammalian ferritins, indicating that the distance between the tryptophan residue(s) and the iron binding/oxidation sites is similar in the two types of molecule.

The structure of the *Listeria* ferritin iron cores has not been investigated. The increased rate of iron uptake displayed by native *Listeria* ferritin containing about 100 iron atoms per molecule relative to the apoprotein points to the occurrence of iron oxidation on preformed cores as in mammalian ferritins [6].

Lastly, it is of interest to compare the carboxylate-based catalytic sites of *Listeria* and of the rL homopolymer, which have elements of both ‘ferroxidase’ and ‘nucleation’ activities. Whereas the latter is strong in each protein, the ferroxidase activity is weaker in the rL homopolymer than in *Listeria*. The only difference in the carboxylate clusters of the two proteins is the additional carboxy function carried in *Listeria* by Asp-48. This residue seems to be in a strategic position, as it not only provides additional iron-co-ordinating ligands but links the glutamic residues forming the L-chain nucleation site (Glu-52, Glu-55, Glu-59 and Glu-62; *Listeria* chain numbering) to Glu-44, thereby endowing the cluster with a greater flexibility in its iron-binding potential.

In conclusion, *Listeria* demonstrates that efficient Fe(II) oxidation in ferritins is not necessarily associated with the presence of the ‘ferroxidase residues’ characteristic of H-type chains, but can be performed by appropriately placed carboxylate residues.

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REFERENCES

- Bozzi, M., Mignogna, G., Stefanini, S., Barra, D., Longhi, C., Valenti, P. and Chiancone, E. (1997) *J. Biol. Chem.* **272**, 3259–3265
- Grant, R. A., Filman, D. J., Fink, S. E., Kolter, R. and Hogle, J. M. (1998) *Nature Struct. Biol.* **5**, 294–303
- Harrison, P. M. and Arosio, P. (1996) *Biochim. Biophys. Acta* **1275**, 161–203
- Harris, D. C. and Aisen, P. (1973) *Biochim. Biophys. Acta* **329**, 156–158
- Bothwell, T. H. and Mallet, B. (1955) *Biochem. J.* **59**, 599–602
- Macara, I. G., Hoy, T. G. and Harrison, P. M. (1972) *Biochem. J.* **126**, 151–162
- Sun, S., Arosio, P., Levi, S. and Chasteen, N. D. (1993) *Biochemistry* **32**, 9362–9369
- Cavallo, S., Mei, G. P., Stefanini, S., Rosato, N., Finazzi-Agrò, A. and Chiancone, E. (1998) *Protein Sci.* **7**, 427–432
- Spiro, T. G., Bates, G. and Saltman, P. (1967) *J. Am. Chem. Soc.* **89**, 5559–5562
- Stefanini, S., Chiancone, E. and Antonini, E. (1983) *Arch. Biochem. Biophys.* **222**, 430–434
- Treffry, A. and Harrison, P. M. (1984) *J. Inorg. Biochem.* **21**, 9–20
- Levi, S., Santambrogio, P., Corsi, B., Cozzi, A. and Arosio, P. (1996) *Biochem. J.* **317**, 467–473
- Saviotti, M. L. and Galley, W. C. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4154–4158
- Bruce, R. M. and Richardson, F. S. (1979) *Q. Rev. Biophys.* **12**, 181–209
- Levi, S., Luzzago, A., Cesareni, G., Cozzi, A., Franceschini, F., Albertini, A., Arosio, P. and Harrison, P. (1988) *J. Biol. Chem.* **263**, 18086–18092
- Reference deleted
- Sun, S. and Chasteen, N. D. (1992) *J. Biol. Chem.* **267**, 25160–25166