Iron incorporation into ferritins: evidence for the transfer of monomeric Fe(III) between ferritin molecules and for the formation of an unusual mineral in the ferritin of *Escherichia coli*

Erika R. BAUMINGER,* Amyra TREFFRY,†§ Aaron J. HUDSON,† Daniel HECHEL,* Nigel W. HODSON,† Simon C. ANDREWS,† Sonia LEVI,‡ Israel NOWIK,* Paolo AROSIO,‡ John R. GUEST‡ and Pauline M. HARRISON‡

*Racah Institute of Physics, The Hebrew University, Jerusalem, Israel, †Krebs Institute, Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2UH, U.K., and ‡DIBIT, Department of Biological and Technological Research, San Raffaele Scientific Institute, Milano, Italy

Iron that has been oxidized by H-chain ferritin can be transferred into other ferritin molecules before it is incorporated into mature ferrihydrite iron cores. Iron(III) dimers are formed at the ferroxidase centres of ferritin H chains at an early stage of Fe(II) oxidation. Mössbauer spectroscopic data now show that the iron is transferred as monomeric species arising from dimer dissociation and that it binds to the iron core of the acceptor ferritin. Human H-chain ferritin variants containing altered threefold channels can act as acceptors, as can the ferritin of *Escherichia coli* (Ec-FTN). A human H-chain ferritin variant with a substituted tyrosine (rHuHF-Y34F) can act as a donor of Fe(III). Since an Fe(III)-tyrosinate (first identified in bullfrog H-chain ferritin) is absent from variant rHuHF-Y34F, the Fe(III) transferred is not derived from this tyrosinate complex. Mössbauer parameters of the small iron cores formed within Ec-FTN are significantly different from those of mammalian ferritins. Analysis of the spectra suggests that they are derived from both ferrihydrite and non-ferrihydrite components. This provides further evidence that the ferritin protein shell can influence the structure of its iron core.

INTRODUCTION

Iron is an essential element for virtually all living organisms, since it participates at the active centres of many proteins involved in catabolic or anabolic pathways. However, owing to its potential toxicity and the lack of mechanisms which precisely couple iron uptake to cellular requirements, a means of iron sequestration is required to provide a temporary depot or a longer-term store for excess iron. The iron-storage protein ferritin therefore plays a central role in cellular iron metabolism, and understanding how it stores iron, both physiologically and mechanistically, is of great interest and importance.

Ferritin comprises a protein shell of M_r 500000, composed of 24 chains, with a central cavity capable of storing a maximum of 4500 Fe(III) atoms as an inorganic complex, usually referred to as the 'iron core' [1]. All ferritins fit this basic description, but, nevertheless, considerable structural variability is found. *Escherichia coli*, for example, possesses two types of ferritin, a haem-containing bacterioferritin (Ec-BFR) [2,3] and a conventional ferritin (Ec-FTN) [4], with only 14% amino acid sequence identity [5]. Snails contain different ferritins in soma and yolk, with only 42% sequence identity between them [5]. Human and other mammalian ferritins have two different protein chains (H and L, 55% amino acid sequence identity), which co-assemble in different proportions within the same 24-mer [6]. Although all ferritins store iron as Fe(III), core composition and crystallinity vary [7–9]. Some ferritin cores are high in phosphate (P_i/Fe from 1:1 to 1:3) and amorphous (bacteria and plants [8]). Others resemble the ferrihydrite mineral in being low in phosphate (P_i/Fe about 1:8 or less) and crystalline (mammals). Yet again, the ferritins isolated from some invertebrates have amorphous iron cores, even though they contain little or no phosphate [9].

Common to all native ferritins so far examined is the ability to catalyse Fe(II) oxidation and to store the hydrolysed Fe(III) inside their protein shells. The catalytic centres reside only in H-type chains [10–14], and in this respect the chains of invertebrates, plants and bacteria are 'H-like' [5]. L-chains (as shown with L-chain homopolymers) can promote Fe(II) oxidation, but at rates that are only slightly greater than those of protein-free controls [14,15]. The function of L-chains may be to facilitate core formation and to increase shell stability [15–17]. When presented with Fe(II) in the absence of P_i *in vitro*, all species of ferritin so far examined store their iron as ferrihydrite, the mineral form of vertebrate ferritins [1,8,16]. Previous kinetic [10,14,15,18–21] or spectroscopic [13,14,21–25] studies of the rates and products of oxidation of Fe(II) by ferritin shells have been made with native heteropolymers, e.g. horse spleen ferritin (85–90% L-chain) and with recombinant H- or L-chain homopolymers. Information on the steps that lead to the build-up of ferritin iron cores has been obtained. Several Fe(III) intermediates have been observed in native or recombinant ferritins: two types of μ-oxo-bridged Fe(III) dimer [13,14,26], a purple-coloured Fe(III)-tyrosinate [14,28], monomeric Fe(III) [13,14,24–27], small non-magnetic Fe(III) clusters [13,14,26] and large magnetic Fe(III) clusters of ferrihydrite [13,14,26]. With the exception of the Fe(III)-tyrosinate, which forms and decays within about 5 s after Fe(II) addition [14], the presence and interconversion of all these species have been observed by Mössbauer spectroscopy. Monomeric Fe(III) and, under certain conditions, Fe(II)-O-Fe(III) species, have been observed in e.p.r. spectra of horse spleen ferritin [25,29] after partial oxidation of added Fe(II). In *E. coli* BFR, Fe(III) monomer and an Fe(II) dimer have been identified (the latter as an NO complex), again by e.p.r. spectroscopy [21,30]. The formation of ferrihydrite iron cores can be

Abbreviations used: Ec-FTN, *Escherichia coli ferritin*; rHuHF-Y34F, a human H-chain ferritin variant with a substituted tyrosine residue; Ec-BFR, *E. coli* haem-containing bacterioferritin; *QS*, quadrupole splitting.

* To whom correspondence should be addressed.

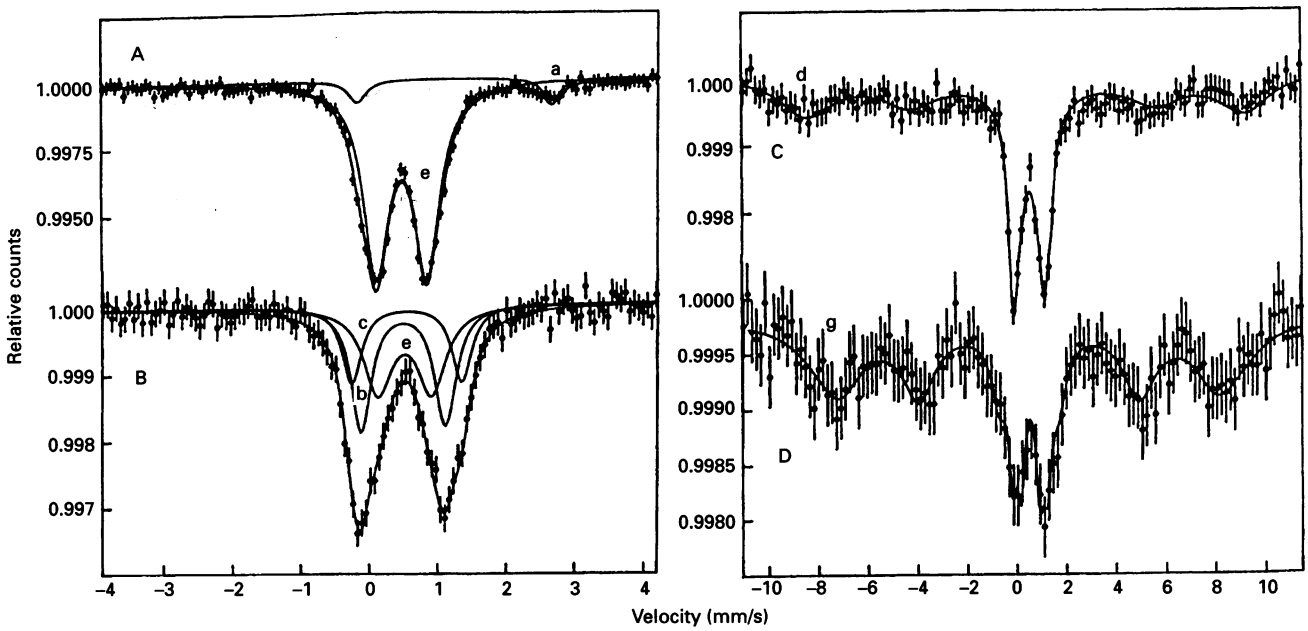

Figure 1 Typical ^{57}Fe Mössbauer spectra showing the different subspectra corresponding to the different Fe(III) subspecies in rHuHF

Spectrum A, 150 ^{56}Fe-rHuHF was added at 3 min after addition of 10 ^{57}Fe(II) atoms/molecule to apo-rHuHF and the solution was frozen 24 h later (sample 3, Table 3); spectra B and C, 10 ^{57}Fe-rHuHF frozen at 1 h after addition of Fe(II) (control sample, Figure 3a); spectrum D, 150 ^{56}Fe-rHuHF was added at 3 min after addition of 10 ^{57}Fe(II) atoms/molecule to apo-rHuHF and the solution frozen 10 h later. Spectra A, B and C were measured at 90 K and D at 4.1 K. The subspectra correspond to Fe(II) (i) and the following Fe(III) species: dimer 1 (subspectrum a), dimer 2 (iii), clusters (e), monomers (d) and large, magnetic clusters (g).

observed optically by the development of their red–brown colour [10,18,19,21,22].

Mössbauer spectroscopic analysis of species formed within the period 30 s to 24 h after Fe(II) addition to horse spleen or recombinant human H-chain ferritin (rHuHF) has established that μ-oxo-bridged Fe(III) dimers are formed early [13,14], and further work with rHuHF variants showed the importance of seven residues situated within the protein shell at a postulated ferroxidase centre [1,5,11–14]. These seven residues (Glu-27, Tyr-34, Glu-61, Glu-62, His-65, Glu-107 and Gln-141) are also required for fast Fe(II) oxidation [13,14], and it has been suggested that the catalytic mechanism may involve the oxidation of a pair of Fe(II) atoms by a single O_2 molecule. Further research has shown that Fe(III) dimers are unstable and established the progression dimer→monomer→small clusters→magnetic clusters [14,23,31]. This progression was assumed to occur within the same molecule. However, it was found that Fe(III), not incorporated into stable cores, could also migrate between molecules [31,32], although the migrating species [e.g. Fe(III) monomers or both monomers and dimers] was not identified. Evidence strongly suggesting that intermolecular migration occurs mainly at the monomer level is now presented. These monomers are not derived from the Fe(III)-tyrosinate formed early in oxidation [14,28], but arise largely, or entirely, from dimer dissociation [14,23,33]. Furthermore, H- and L-chain homopolymers, and heterologous ferritins, can accept Fe(III) monomers produced in another molecule. Transferred monomers are bound to the iron core of the acceptor ferritin. Indeed, detection of migration is based on the presence of the core in the acceptor molecule, because the Mössbauer characteristics of the transferred iron change from those of monomers to those of the core.

EXPERIMENTAL

Site-directed mutagenesis, overexpression of rHuHF and its variants in *E. coli* and purification of the ferritins were performed as in [34]. Similar procedures to those described previously were used for production of recombinant human L-chain ferritin (rHuLF) [32] and Ec-FTN [4]. The rHuHF and its derivatives have a Lys-86→Gln substitution that was made to induce crystallization [12]. Prior to their use in Mössbauer spectroscopy, all ferritins, except Ec-FTN (when acting as acceptor), were treated with sodium dithionite to remove endogenous iron, as detailed in [13]. Iron was not removed from acceptor Ec-FTN, since it contained only 10 Fe atoms/molecule as isolated.

Elemental ^{57}Fe and ^{56}Fe were obtained from the Atomic Energy Establishment (Harwell, Didcot, Oxon., U.K.), and solutions of these isotopes were prepared as in previous experiments [26]. To prepare samples of acceptor ferritin containing an average of 150 ^{56}Fe atoms/molecule the ^{56}Fe(II) was added to the apoferritin solution (1.5–3 mg/ml in 0.1 M Mops buffer, pH 6.5) in ten increments of 15 Fe atoms/molecule, and the reaction allowed to continue to completion before the next addition. This method ensures that the added iron is incorporated into iron cores within ferritin molecules [16], thus avoiding the aggregation and precipitation that can occur when large amounts of iron are added, e.g. 500 or 1000 Fe atoms/molecule, to apo-rHuHF or its variants. After iron additions were complete, the samples were concentrated and dialysed against 10 mM NaCl using an 8MC ultrafiltration cell (Amicon, High Wycombe, Bucks., U.K.). The ^{57}Fe(II) was added to the 0.63 ml donor apoferritin (16 mg/ml, 0.1 M Mops buffer, pH 7.0) to give 12.8 μg of ^{57}Fe and 10 Fe atoms/molecule and the sample left for a short period (usually 3 min) to allow complete oxidation.

Table 1 Mössbauer parameters for the different iron species found in rHuHF and Ec-FTN after addition and oxidation of $^{57}FeSO_4$

The numbers in parentheses are the errors in the last digits. Where errors are not given, the parameters were kept fixed in the fittings. All species are Fe(III) except (a). Abbreviations: *LW*, full linewidth at half maximum of peak height; *IS*, isomer shift; H_{eff}, effective hyperfine field; t_r, relaxation time.

Species	*T* (K)	*LW* (mm/s)	*QS* (mm/s)	*IS* (mm/s)	H_{eff} (T)	t_r (ns)
(a) Fe(II)	90	0.39 (3)	2.76 (2)	1.24 (2)	–	–
(b) Dimer 1	90, 4.11	0.33 (2)	1.23 (1)	0.50 (1)	–	–
(c) Dimer 2	90, 4.1	0.33 (2)	1.62 (2)	0.53 (1)	–	–
(d) Monomer	90, 4.1	0.50	–	0.51 (2)	55.0 (0.5)	7
(e) All ferrihydrite clusters	90	0.50 (3)	0.75 (2)	0.49 (2)	–	–
(f) Small ferrihydrite clusters	4.1	0.50 (3)	0.75 (2)	0.50 (2)	–	–
(g) Large ferrihydrite clusters	4.1	0.44 (2)	0.00	0.51 (2)	49.5 (0.5)	–

Either the acceptor solution containing the ^{56}Fe-ferritin (0.2 ml, 25 mg/ml in Mops buffer, pH 7) or 0.2 ml of 5 mM NaCl solution was then added. The mixtures were transferred to Perspex (Lucite) sample holders and the reaction stopped by freezing at the times shown in Table 2 (below).

To examine samples for their iron donor potential [i.e. ability to produce Fe(III) dimers and monomers by Fe(II) oxidation], $^{57}FeSO_4$ was added to the aerobic apoferritin solution (6.7 mg/ml in 0.1 M Mops buffer, pH 7.0) to give 34 Fe atoms/molecule and solutions were frozen at the times shown in Table 5 (below).

Mössbauer spectra were obtained with a 100 mCi ^{57}Co in a rhodium matrix source at room temperature and a Harwell proportional counter. Velocity calibration was performed with metallic iron foil at room temperature. Measurements were made at 90 K and 4.1 K and in two velocity ranges [13,26,31] and the spectra analysed by computer fitting as described previously [13,31].

RESULTS

Iron species observed by Mössbauer spectroscopy

(a) Human ferritins

Typical spectra, showing subspectra corresponding to the various iron species found in rHuHF, are displayed in Figure 1. The Mössbauer parameters obtained from the computer fits to the experimental spectra are summarized in Table 1. The relative amounts of the different species were calculated from the relative intensities of the corresponding subspectra. In all spectra obtained at 4.1 K, the relative intensities of the subspectra are identical with the values obtained at 90 K, proving that the *f*-factors (the efficiencies for the Mössbauer effect) are similar for all species at these temperatures. The relative intensity of the 90 K spectrum due to clusters (e) was resolved at 4.1 K into two components: a sextet corresponding to large (more than about 70 Fe atoms [31]) magnetic clusters (g) and a doublet corresponding to non-magnetic clusters (f). The parameters for dimers, monomers and non-magnetic clusters at 4.1 K are identical with those at 90 K for all samples. Subspectra of samples containing rHuLF, rHuHF and its variants could all be fitted with the same parameters.

(b) *E. coli* FTN

The spectra obtained at 90 K with Ec-FTN (Figure 2) are distinct from those given by all the human ferritin samples (Figure 1). In Figure 2, spectrum A, as in Figure 1, spectrum A , the main component corresponds to clusters, yet the spectra are very different. Although all the Ec-FTN spectra could be fitted with the same two dimer species, b and c, as in rHuHF (Table 1), the

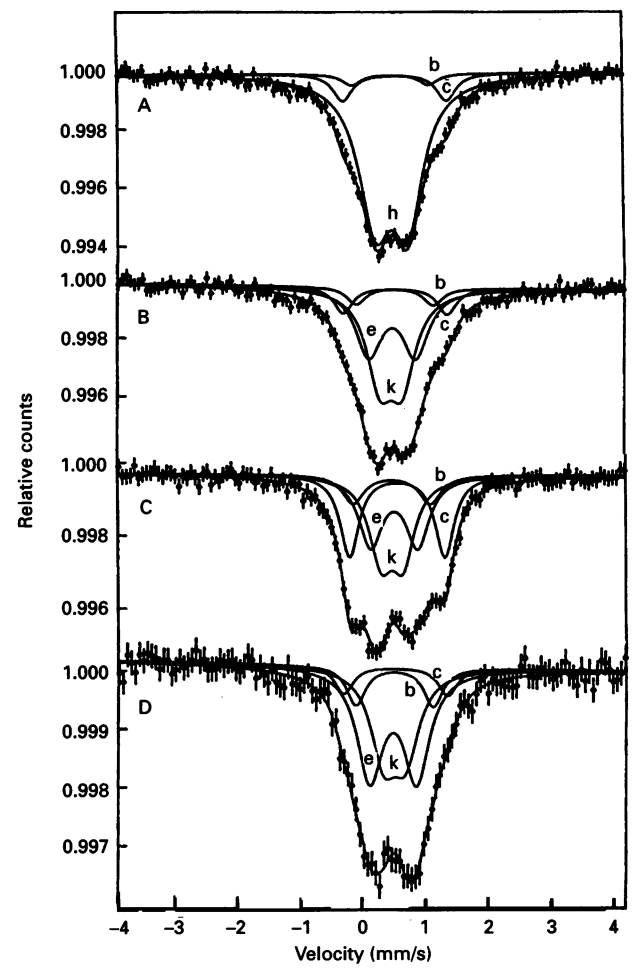

Figure 2 ^{57}Fe-Mössbauer spectra obtained at 90 K with several samples of Ec-FTN

Spectra A and B, 34 ^{57}Fe-Ec-FTN frozen at 40 min after addition of ^{57}Fe(II) (sample 4, Table 5); spectrum C, 34 ^{57}Fe-Ec-FTN frozen at 1 min after addition of ^{57}Fe(II) (sample 3, Table 5); spectrum D, 150 ^{56}Fe-Ec-FTN was added at 3 min after addition of 10 ^{57}Fe(II) atoms/molecule and the solution frozen 10 h later (Figure 3d). Subspectra b and c correspond to Fe(III) dimers 1 and 2 respectively. Subspectrum, h, in spectrum A, corresponds to Fe(III) clusters, and can be decomposed (as shown in spectra B–D) into two doublets, e, with ferrihydrite-like parameters, and k, with smaller *QS*. k is present only in Ec-FTN. In spectrum C, dimers b and c form a major component of the Ec-FTN spectrum. Subspectra corresponding to monomers are not seen in this velocity range.

Table 2 Mössbauer analysis of iron cores in Ec-FTN

The values in parenthesis are the errors in the last digits. Abbreviations: LW, full linewidth at half maximum of peak height; *IS*, isomer shift; H_{eff}, effective magnetic field.

Cluster in Ec-FTN	*T* (K)	*LW* (mm/s)	*QS* (mm/s)	*IS* (mm/s)	H_{eff} (T)
(h) All clusters	90	0.60 (3)	0.54–059	0.49 (1)	–
(k) Non-ferrihydrite clusters	90	0.50 (2)	0.35 (3)	0.48 (2)	–
(e) Ferrihydrite clusters	90	0.50 (2)	0.75 (2)	0.50 (2)	–
(i) All small clusters	4.1	0.40 (2)	0.47 (1)	0.50 (1)	–
(g) Large (ferrihydrite) clusters	4.1	0.44 (2)	0.00	0.50 (2)	49.5 (0.5)

Table 3 Expt. 1: Distribution of different iron species obtained by Mössbauer spectroscopy after addition of 10 ^{57}Fe(II) atoms/molecule to apo-rHuHF and, at a later stage, of either NaCl or another ferritin

Samples all contained 10 Fe(II) atoms/molecule added as $^{57}FeSO_4$ at zero time to the apoferritin (16 mg/ml) in 0.1 M Mops buffer, pH 7.0. Abbreviations: superscript 56 signifies the presence of 150 ^{56}Fe atoms/molecule [added to the apoprotein as Fe(II) and allowed to oxidize to provide molecules containing small cores (as described in the Experimental section)]. rHuHF-175 denotes rHuHF containing two substitutions in the threefold channels: Asp131→His + Glu134→His. rHuHF-Y34F is a variant of rHuHF containing a single amino acid replacement in the ferroxidase centre; t_a, time of addition of substance in previous column, t_f, time of freezing. Errors in percentage values are given in parentheses. Measurements were made at 90 K.

Sample	Substance added	Time (t_a) (min)	t_f	Fe(II) (%)	Fe(III) species (%)			
					Dimer 1	Dimer 2	Monomer	Cluster
1. rHuHF	NaCl	2	3 min	–	27 (3)	18 (3)	38 (11)	17 (5)
2. rHuHF	NaCl	3	24 h	–	15 (3)	14 (2)	26 (9)	45 (5)
3. rHuHF	^{56}rHuHF	3	24 h	7 (1)	4 (1)	–	10 (5)	79 (4)
4. rHuHF	rHuLF	3	24 h	5 (1)	7 (2)	6 (1)	38 (7)	44 (2)
5. rHuHF	^{56}rHuLF	3	24 h	–	–	–	7 (9)	93 (9)
6. rHuHF + rHuLF	–	–	3 min	–	32 (3)	19 (3)	30 (5)	19 (3)
7. rHuHF + rHuLF	–	–	24 h	4 (1)	10 (2)	10 (2)	19 (8)	57 (4)
8. rHuHF	^{56}rHuHF-175	3	24 h	2 (1)	3 (1)	3 (1)	17 (6)	75 (5)
9. rHuHF-Y34F	NaCl	29	30 min	8 (1)	45 (6)	–	23 (7)	24 (6)
10. rHuHF-Y34F	NaCl	30	24 h	7 (1)	29 (9)	4 (3)	28 (8)	32 (6)
11. rHuHF-Y34F	^{56}HuLF	30	24 h	4 (1)	–	–	8 (6)	88 (5)

cluster doublet, h (Figure 2, spectrum A), is broader and has a smaller quadrupole splitting (*QS*) than the corresponding feature, e, of rHuHF (Figure 1, spectra 1 A and B) and other human ferritins. Further analysis (Table 2) showed that doublet, h, could be resolved into two doublets, e and k, as displayed in Figure 2, spectra B, C and D, where e has the same parameters as those found at 90 K for the ferrihydrite clusters of human ferritin shown in Table 1. The rationale for such an analysis is based on the observation that Ec-FTN gives a magnetic subspectrum at 4.1 K with the same ferrihydrite-like parameters as found for magnetic clusters, g, in rHuHF and rHuLF. If it is now assumed that some of the clusters giving the 90 K spectra of Ec-FTN samples are due in part to ferrihydrite, and the parameters are fixed accordingly (e), a second type of cluster with parameters, k, is derived for the remainder. Following this analysis, Ec-FTN samples with 34 ^{57}Fe atoms/molecule give less than 50% ferrihydrite-like clusters, e, (Figure 2, spectra B and C), whereas about 60% of the clusters are ferrihydrite in the 150 ^{56}Fe-Ec-FTN samples containing ^{57}Fe transferred from the donor rHuHF to Ec-FTN molecules containing 150 ^{56}Fe (Figure 2, spectrum D). At 4.1 K the subspectrum, i, due to non-magnetic clusters, showed an appreciably smaller *QS* than that observed at 90 K, h. This can be explained (samples with a 150 ^{56}Fe-core; Table 4 below) as follows: at 90 K, subspectrum, h, is composed of all the ferrihydrite, e, and all the non-ferrihydrite clusters, k, whereas, at 4.1 K the non-magnetic subspectrum consists of all species, k, (which do not split magnetically) and only a part of species, e, corresponding to small, non-magnetic clusters (the remainder giving the magnetic subspectrum with ferrihydrite-like parameters, g).

Transfer of Fe(III) from rHuHF donor to rHuHF or rHuLF acceptor

A previous study [31] demonstrated that iron, recently oxidized by rHuHF, could be transferred to horse spleen ferritin containing a small iron core (average 150 ^{56}Fe atoms/molecule). The acceptor ferritin was composed mainly of L-subunits. Transfer to the cores of H- and L-chain homopolymers has now been investigated (Table 3). The results show that, in the control containing only rHuHF plus NaCl, the percentage of Fe(III) in clusters increased from 17% at 3 min to 45% at 24 h after aerobic Fe(II) addition (rows 1 and 2). When rHuHF containing a Mössbauer-silent core was added at 3 min instead of NaCl, the proportion of ^{57}Fe in clusters at 24 h increased to 79% (row 3). This indicates that ^{57}Fe atoms were now attached to an ^{56}Fe core and hence that transfer of iron between H-chain homopolymers had occurred. The addition of apo-rHuLF instead of NaCl had no effect on the proportion of ^{57}Fe in clusters (compare rows 4 and 2). However, addition of rHuLF containing an average of 150 ^{56}Fe atoms/molecule (150 ^{56}Fe-rHuLF) yielded an increase in the ^{57}Fe-cluster percentage, confirming that iron was transferred from the H- to the L-chain homopolymer (row 5). The proportion of ^{57}Fe in clusters was higher than that found with 150 ^{56}Fe-rHuHF (rows 5 and 3). In another pair of samples (rows 6 and 7) ^{57}Fe was added to a mixture of apo-rHuHF and apo-rHuLF. An increase in ^{57}Fe in clusters was evident (row 7) compared with

that found with apo-rHuHF alone (row 2). Since apo-rHuHF is mainly responsible for the Fe(II) oxidation, transfer of Fe(III) from rHuHF to rHuLF is again implied.

Further experiments were designed to investigate the effects of site-directed changes in donor or acceptor molecules. In the results shown in Table 3 (row 8), the acceptor molecule is a variant in which the threefold intersubunit channels are altered. In rHuHF, the narrowest region of the channel, towards the cavity surface of the shell, is lined by three glutamate and three aspartate residues, where metal ions, including Fe^{3+}, bind [1,12,33]. In the variant used here (rHuHF-175), the aspartate and glutamate residues are replaced by histidine residues. The substitutions have no effect on net transfer over 24 h (rows 8 and 3). Again, replacement of one of the seven conserved ferroxidase centre residues of the donor (tyrosine-34 by phenylalanine) has no effect on iron transfer over 24 h (rows 11 and 5). Because this variant oxidizes Fe(II) more slowly than does the wild-type [14], the acceptor (150 ^{56}Fe-rHuLF) was added at 30 min instead of 3 min after Fe(II).

Time course of Fe(III) transfer from rHuHF donor to rHuHF or Ec-FTN acceptor

In the first experiments, Fe(III) transfer was measured after a period of 24 h. However, it was suspected that the reaction might be completed within a much shorter period. Accordingly, donor/acceptor mixtures were frozen at 1, 3, 6 and 10 h and the corresponding proportions of ^{57}Fe(III) dimers 1 and 2, monomers and clusters formed in rHuHF in the absence of acceptor are shown in Figure 3(a). Within the first hour both types of dimer decreased with a corresponding increase in monomer. Clusters accounted for only 10% of the total at 1 h. This confirms the previous conclusion that monomers increase at the expense of dimers within this time [14]. Between 1 and 3 h monomers decreased and Fe(III) clusters increased, whereas dimers remained unchanged or increased slightly (Figure 3a). There was very little change in the fraction of iron in the various species between 3 and 10 h. Moreover the amounts of Fe(III) in large, magnetic, and small, non-magnetic, clusters remained constant, indicating no further movement within this period (Table 4).

The results of experiments with different acceptors are shown in Figures 3(b)–3(d). In Figure 3(b), 150 ^{56}Fe-rHuHF was added 3 min after Fe(II) was mixed with apo-rHuHF. In Figure 3(b) there was a marked decrease in dimers at 1 h, but the proportion of monomers remained the same, or decreased slightly. By contrast, cluster ^{57}Fe increased sharply. Between 1 and 3 h a further dramatic rise in cluster ^{57}Fe occurred, now accompanied by a parallel decrease in monomer. Between 3 and 10 h little further change in monomer was observed, although dimer 2 decreased slightly. Total ^{57}Fe in clusters increased to a small extent, due to an increase in the large magnetic clusters (Table 4). These results imply that intermolecular transfer is mainly (or entirely) due to movement of Fe(III) monomers, which are then captured by the iron cores of the acceptor molecules, with a corresponding increase in core size. Similar phenomena were seen with the threefold channel variant as acceptor, except that there was now a significant rise in monomer at 1 h and less ^{57}Fe in clusters throughout the 1–10 h period (Figure 3c). The fractions of both small and large clusters increased slightly (Table 4). In this case the variant used as acceptor has two amino acid replacements in the threefold inter-subunit channels: Asp-131→Ala and Glu-134→Ala. The data suggest that the substitutions have decreased the rate of transfer, thereby implicating the threefold channels of the acceptor in the acquisition of ^{57}Fe. No such effect was seen over 24 h in the variant in which histidine

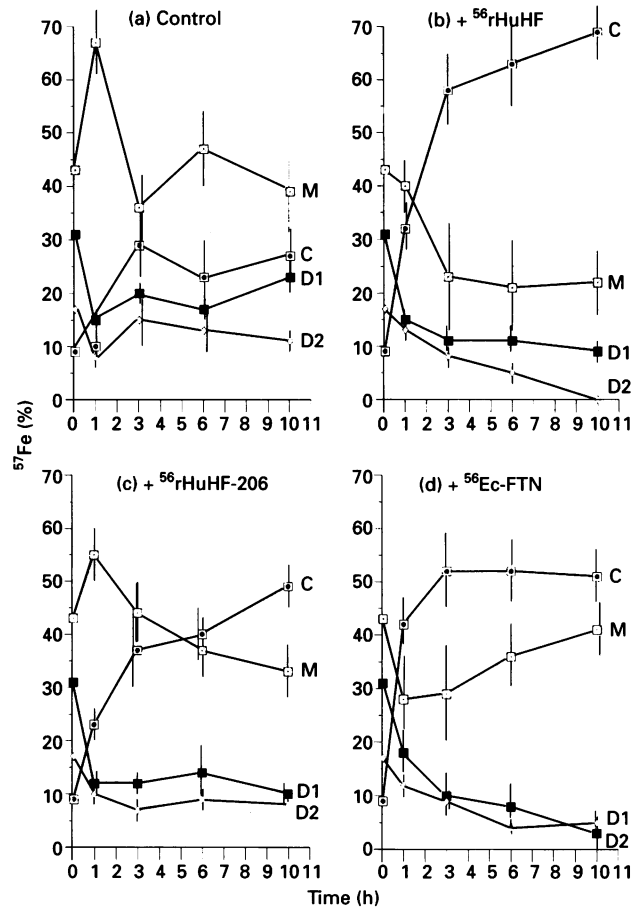

Figure 3 Transfer of Fe(III) from rHuHF to an acceptor ferritin

Ten ^{57}Fe(II) atoms/molecule were added to apo-rHuHF [0.63 ml (16 mg of protein/ml) in 0.1 M Mops buffer, pH 7.0) and, after allowing 3 min for oxidation, either NaCl (control) or an acceptor ferritin (5 mg) was added. Samples were frozen at 1, 3, 6 and 10 h, and Mössbauer spectra were measured at 90 K. Errors of the measurements are shown as vertical lines. (**a**) Control; **b–d**, acceptors were ferritins containing cores with an average of 150 ^{56}Fe atoms/molecule (Mössbauer-silent). rHuHF-206 is a variant of rHuHF with threefold channel substitutions Asp-131→Ala + Glu-134→Ala. Abbreviations: C, cluster; M, monomer; D1, dimer 1; D2, dimer 2.

residues replace carboxylate groups (Table 3, row 8). However, it is possible that a diminished rate would be observed over a shorter time scale (e.g. 1–10 h).

Intermolecular transfer of iron was also observed when a heterologous acceptor, Ec-FTN, was used (Figure 3d). The rise in cluster ^{57}Fe at 1 h was more dramatic than in Figure 3(c). After an initial decrease at 1 h, monomeric Fe(III) appeared to rise slowly over the next 9 h, although this rise is barely significant. Dimers 1 and 2 were much reduced by 6–10 h. There are three points of special interest in the results with this pair of ferritins. First, they confirm the lack of specificity in monomeric iron transfer between ferritin molecules. Secondly, they show that transfer is little affected by differences within the threefold channels (or in other regions where Ec-FTN differs from other ferritins). The three-dimensional structure of Ec-FTN is very similar to that of rHuHF, despite the low sequence identity [5], but its threefold channels are very different: Asp-131 and Glu-134 are replaced by Asn and Gln respectively, and there are several other sequence changes at the outer end of the channel [5]. Thirdly, there appears to be rather less binding of ^{57}Fe to

Table 4 Composition of the iron clusters

Samples were prepared as in the legend to Figure 3. The percentages of ^{57}Fe present as magnetic and non-magnetic clusters were obtained from fits to Mössbauer spectra recorded at 4.1 K. Percentage errors are given in parentheses.

Acceptor	Time after addition of acceptor (h)	Fe species in different clusters (%)		
		Magnetic ferrihydrite (g)	Non-magnetic ferrihydrite (f)	Non-magnetic Ec-FTN (k)
None	3	19 (3)	10 (3)	–
	10	19 (3)	8 (3)	–
^{56}rHuHF	3	46 (7)	12 (2)	–
	10	58 (5)	11 (1)	–
^{56}rHuHF-206	3	31 (5)	8 (4)	–
	10	38 (3)	11 (2)	–
^{56}Ec-FTN	3	20 (3)	11 (5)	21 (5)
	10	18 (3)	11 (3)	22 (3)

Table 5 Mössbauer spectroscopic analysis at 90 K of the products formed by aerobic oxidation of ^{57}Fe(II) in the presence of various apoferritins

Iron has been removed from all samples by sodium dithionite reduction [13] prior to the addition of $^{57}FeSO_4$. Samples 1–7, 34 Fe atoms/molecule, protein, 6.7 mg/ml in 0.1 M Mops buffer, pH 7.0. Sample 8, 10 Fe atoms/molecule, protein, 11 mg/ml in Mes buffer, pH 5.5. apo-rHuHF-175 refers to apo-rHuHF in which two carboxy residues have been changed in the threefold channels (Asp-131→His, Glu-134→His). apo-rHuHF-206 refers to apo-rHuHF in which two carboxy residues have been changed in the threefold channels (Asp-131→Ala, Glu-134→Ala). t_f, time of freezing. Mössbauer spectra were measured at 90 K. Percentage errors are given in parentheses.

Sample	t_f (min)	Fe(II) (%)	Iron(III) species (%)			
			Dimer 1	Dimer 2	Monomer	Cluster
1. apo-rHuLF	3	40	–	–	10 (3)	50 (4)
2. apo-rHuLF	30	–	–	–	–	100
3. apo-Ec-FTN	1	–	9 (2)	21 (2)	22 (4)	48 (4)
4. apo-Ec-FTN	40	–	4 (2)	8 (2)	20 (4)	68 (4)
5. apo-rHuHF-175	1	–	22 (3)	–	–	78 (3)
6. apo-rHuHF-175	9	–	12 (2)	–	–	88 (2)
7. apo-rHuHF	1	–	43 (5)	35 (3)	6 (6)	16 (3)
8. apo-rHuHF-206	30	–	31 (3)	–	–	69 (3)

clusters in Ec-FTN compared with those in rHuHF, possibly owing to the different types of clusters present. However, the data do not distinguish clearly between effects stemming from differences in donor and acceptor protein shells or donor and acceptor iron cores. The distribution of ^{57}Fe between small and large clusters in the acceptor is very different in Ec-FTN compared with that in rHuHF or its channel variant (Table 4). This is likely to reflect differences in iron-core type as well as in the distribution in iron-core sizes, within the acceptors. Thus, in experiments with rHuHF or its variant as acceptor, about two-thirds of the ^{57}Fe appeared in magnetic clusters (Table 4). This is also true of the donor core (control). In contrast, in Ec-FTN, more than half of the cores are non-magnetic and, of these, the majority are of the non-ferrihydrite type.

Other potential ferritin donors

The results given in Table 3 and Figure 3 were all obtained with rHuHF or its variants as donors. It is unlikely that a significant transfer of Fe(III) from rHuLF to rHuHF would occur, since Fe(III) dimers are not formed when Fe(II) is added to apo-rHuLF. This is shown in Table 5 (rows 1 and 2) and can be explained by the lack of specific ferroxidase activity in the L-homopolymer, in which the ferroxidase centre is blocked by a salt bridge [11–14,17]. At 3 min after Fe(II) addition to apo-rHuLF, oxidation is still incomplete (compare with apo-rHuHF, row 7, in which oxidation was complete at 1 min) and by 30 min all the added iron was in clusters. Under these conditions transfer of Fe(III) could not be investigated. In contrast, Ec-FTN (rows 3 and 4) gives both Fe(III) dimers and monomers after Fe(II) oxidation, and oxidation is complete at 1 min. Hence it is likely that Ec-FTN could act as a donor of Fe(III). It would be interesting to investigate the donor ability of the rHuHF threefold channel variants in which the carboxy residues are changed to either histidine or alanine residues. Dimers (type 1 only), but no monomers, and a relatively large percentage of cluster iron, are observed in variants rHuHF-175 (Asp-131→His+Glu-134→His) and rHuHF-206 (Asp-131→Ala +Glu-134→Ala) (Table 5, rows 5, 6 and 8). Since the major binding sites for monomeric Fe(III) (in the channels [33]) are altered, any monomers which are formed may be incorporated into core clusters, but possibly only within the same molecule.

DISCUSSION

The results support the conclusion from previous work [14,23,31,33] that the Fe(III) species produced, when Fe(II) is oxidized in the presence of apo-rHuHF, appear in the sequence: dimer→monomer→cluster. They also show that monomeric Fe(III) can move into the cavities of ferritin molecules other than those in which they were formed. They confirm the existence of a powerful drive towards increasing cluster size [18,26], by

showing that Fe(III) monomer is more likely to bind to pre-existing cores than to form new core nuclei (when only 10 Fe atoms/molecule have been added to apo-rHuHF). Previous evidence has shown that Fe(II) can be bound and oxidized on iron-core surfaces [18,19,21,26]. The present results lend support to the further conclusion [31,32,35] that Fe(III) can bind to the core. They also imply that monomeric Fe(III) formed on ferritin is quite mobile. Transfer of iron to heterologous ferritins suggests that this process does not require specific inter-molecular contacts. However, it is not clear from the experiments whether the Fe(III) is completely dissociated from the donor (i.e. free in solution) prior to entering and binding to the acceptor. The question of whether the threefold channels are used, either as a route through which Fe(III) monomer leaves the donor, or as an uptake route in the acceptor, remains unresolved. Previous evidence [33] indicates that some or all of the Fe(III) monomer formed by dimer dissociation moves to the threefold channels (i.e. those of the donor in the present experiments). In a previous report [31] it was stated that alterations in donor channel residues (Asp-131→Ala + Glu-134→Ala) had no effect on transfer. This conclusion was later proved invalid, because the channel substitutions were not present in the protein used [33] and it would be useful to re-investigate the donor ability of this variant. However, in recent biochemical competition experiments, in which Fe(II) was added to a mixture of rHuHF-206 and rHuLF at pH 5.5 (method of [32]), iron cores were formed in both ferritins (S. Levi and P. Arosio, unpublished work). Since iron cores are not formed in rHuLF alone under these conditions, it was concluded that Fe(III) must have transferred from rHuHF-206. Channel variants rHuHF-206 and rHuHF-175 (Table 5) show production of dimers (type 1 only), but no monomers, on Fe(II) oxidation, and a high proportion of clusters. It is likely that Fe(III) monomers are produced, but that they immediately move into the cavity and, in the absence of competing channel sites, are all retained by the clusters. Although direct binding of Fe(III) has not been examined, X-ray analysis of variant rHuHF-175 in crystals grown from $CaCl_2$ shows that, in contrast with those of rHuHF, no Ca^{2+} ions are bound in the threefold channels [1,12]. The absence of these monomer-binding sites may explain the very large proportion of Fe(III) incorporated into clusters at 1–9 min (Table 5, rows 5, 6 and 8). Unfortunately, the latter finding precludes the use of Mössbauer spectroscopy for the study of iron transfer with these donors. However, Mössbauer spectroscopic studies should be feasible with Ec-FTN, which also has channels that do not bind metal ions (P. D. Hempstead, S. J. Yewdall, A. J. Hudson, P. J. Artymiuk, S. C. Andrews, J. R. Guest and P. M. Harrison, unpublished work).

In the present study no significant difference was found in donor properties of variant rHuHF-Y34F and rHuHF. Since the substitution Y34F prevents the formation of Fe(III)-tyrosinate [14], this species cannot be involved in the transfer process. Further experiments with altered donors would be of interest, but no transfer would be expected with those ferroxidase centre variants, in which little or no dimer or monomer is formed.

The present experiments show that Fe(III) can be transferred from H-chain to L-chain homopolymers. They further suggest that the most important factor for transfer is the presence of a stable iron core. Thus no transfer is observed if apo-rHuLF is added once the oxidation of 10 Fe atoms/molecule by apo-rHuHF is complete (Table 3; compare rows 2 and 4) and only a small amount of transfer occurs when 10 Fe atoms/molecule (with respect to apo-rHuHF) is added to a mixture of apo-rHuHF and apo-rHuLF (Table 3; rows 2 and 7). Efficient transfer is observed only when either 150 ^{56}Fe-rHuLF (row 5) or 150 ^{56}Fe-rHuHF (row 3) is added to rHuHF containing 10 Fe atoms/molecule. Although there is slightly more transfer into 150 ^{56}Fe-rHuLF than into 150 ^{56}Fe-rHuHF, a stronger 'pull' on Fe(III) monomer by the core than by the protein shell is clearly indicated by the combined results.

It is particularly interesting that, according to the computer analysis of Mössbauer spectra, about half of the Ec-FTN iron cores exhibit parameters different from those of rHuHF (Table 2). The few ^{57}Fe atoms transferred to 150 ^{56}Fe-Ec-FTN presumably reflect the structures of the preformed ^{56}Fe cores to which they are bound, and the observation of similar parameters in cores formed when 34 ^{57}Fe(II) atoms/molecule were added to apo-Ec-FTN confirms this. Moreover, approximately two-thirds of the clusters are too small to give magnetic hyperfine spectra, whereas, in 150 ^{56}Fe-rHuHF or its variant, about 80% of the iron is in magnetic clusters (Table 4). Furthermore, most of the small clusters are of the non-ferrihydrite type. This is the first clear evidence for species differences in cores formed in the absence of phosphate. The unique structure of part of the Ec-FTN cores may reflect a specific distribution of residues on the inside surface of the molecule, which is very different from that found in shells of human H- or L-chains. For example, glutamate-64 and -67 and aspartate-42, which lie on the cavity surface of the human ferritins, are replaced in Ec-FTN by threonine, glutamine and serine respectively [5]. On the other hand, carboxy residues not present in human H- or L-chains, occur on the cavity surface of Ec-FTN (P. D. Hempstead, A. J. Hudson, S. J. Yewdall, P. J. Artymiuk, S. C. Andrews, J. R. Guest and P. M. Harrison, unpublished work). Iron-core nucleation may therefore take place at different sites on the *E. coli* and human ferritins. It is of note that the *QS* values derived for the small non-ferrihydrite Fe(III) clusters in Ec-FTN, which are lower than those observed in mammalian ferritins, are at the lower end of the range found for basic iron carboxylates [36]. However, it has been reported previously that Ec-FTN, loaded with 1500 Fe(III) atoms/molecule, gives electron diffraction lines and Mössbauer parameters characteristic of ferrihydrite [4], suggesting that, at this iron loading, ferrihydrite is the major iron-core species. In contrast with the other ferritins, the proportion of Fe(III) in monomers in Ec-FTN, when acting as acceptor, appears to rise between 1 and 10 h after an initial fall at 1 h (Figure 3). Since the channel Fe(III) monomer site of rHuHF [33], is altered in Ec-FTN, it is likely that Ec-FTN contains another site, which binds Fe(III) monomers sufficiently tightly for some of the transferred iron to become attached.

One curious observation is that a small amount of Fe(II) is present in some of the samples held for 24 h before freezing (Table 3, rows 3, 4, 7, 10 and 11 and Figure 1, spectrum A), but not in those frozen at times between 3 min and 10 h (Table 3, rows 1 and 6, and Figure 3). This was noticed previously, but was then attributed to the possible presence of traces of the thioglycollate used for iron-core reduction. Dithionite reduction was employed in the present work. Any remaining dithionite is likely to have been destroyed during purification of the reduced protein and it is not clear what has caused the reappearance of Fe(II). It has been suggested in another connection [37] that redox centres are present on the ferritin shell and it is conceivable that such centres (reduced by the dithionite) account for the slow production of Fe(II) observed here.

It is interesting to consider the possible physiological consequence of the results. Transfer of Fe(III) from another ferritin molecule or, indeed, binding of Fe(III) from an alternative source, may play a role in iron-core building. In animal tissues, ferritins are heterogeneous with respect to both iron content and subunit composition. However, *E. coli* contains two very different

homopolymers, FTN and BFR. It would be of great interest to ascertain whether transfer between these ferritins is possible and, if so, whether it is uni- or bi-directional. It has also been shown that iron oxidized by horse spleen ferritin *in vitro* can be accepted by serum apo-transferrin [38]. This is unlikely to be a physiological process, but it is conceivable that ferritin could be an intracellular donor of iron to different acceptors. A high affinity for iron in the acceptor would be essential for net transfer to take place.

Note added in proof (received 19 July 1994)

Recent X-ray analysis has shown the presence of an Fe(III) site, unique to Ec-FTN, on its inner surface [39]. This could pinpoint the Ec-FTN monomer site or its novel cluster site (or both).

We thank the Wellcome Trust for financial support (to A.T., J.R.G. and P.M.H.) and the Science and Engineering Research Council for a studentship (to A.J.H.) and for an Advanced Fellowship (to S.C.A.).

REFERENCES

1 Harrison, P. M., Andrews, S. C., Artymiuk, P. J., Ford, G. C., Guest, J. R., Hirzmann, J., Lawson, D. M., Livingstone, J. C., Smith, J. M. A., Treffry, A. and Yewdall, S. J. (1991) in Advances in Inorganic Chemistry, vol. 36 (Sykes, A. G., ed.). pp. 449–486, Academic Press, San Diego
2 Yariv, J., Kalb, A. J., Sperling, R., Bauminger, E. R., Cohen, S. G. and Ofer, S. (1981) Biochem. J. **197**, 171–175
3 Cheesman, M. R., Le Brun, N. E., Kadir, F. H. A., Thomson, A. J., Moore, G. R., Andrews, S. C., Guest, J. R., Harrison, P. M., Smith, J. M. A. and Yewdall, S. J. (1993) Biochem. J. **292**, 47–56
4 Hudson, A. J., Andrews, S. C., Hawkins, C., Williams, J. M., Izuhara, M., Meldrum, F. C., Mann, S., Harrison, P. M. and Guest, J. R. (1993) Eur. J. Biochem., **218**, 985–995
5 Andrews, S. C., Arosio, P., Bottke, W., Briat, J.-F-., Von Darl, M., Harrison, P. M., Laulhere, J.-P., Levi, S., Lobreaux, S. and Yewdall, S. J. (1992) J. Inorg. Biochem. **47**, 161–174
6 Arosio, P., Adelman, T. G. and Drysdale, J. W. (1978) J. Biol. Chem. **253**, 4451–4458
7 Treffry, A., Harrison, P. M., Cleton, M. I., De Bruijn, W. C. and Mann, S. (1987) J. Inorg. Biochem. **31**, 1–6
8 Wade, V. J., Treffry, A., Laulhere, J.-P., Bauminger, E. R., Cleton, M. I., Mann, S., Briat, J.-F. and Harrison, P. M. (1993) Biochim. Biophys. Acta **1161**, 91–96
9 St. Pierre, T. G., Kim, K. S., Webb, J., Mann, S. and Dickson, D. P. E. (1990) Inorg. Chem **29**, 1870–1874
10 Levi, S., Luzzago, A., Cesareni, G., Cozzi, A., Franceschinelli, F., Albertini, A. and Arosio, P. (1988) J. Biol. Chem. **263**, 18086–18092
11 Lawson, D. M., Treffry, A., Artymiuk, P. J., Harrison, P. M., Yewdall, S. J., Luzzago, A., Cesareni, G., Levi, S. and Arosio, P. (1989) FEBS Lett. **254**, 207–210
12 Lawson, D. M., Artymiuk, P. J., Yewdall, S. J., Smith, J. M., Livingstone, J. C., Treffry, A., Luzzago, A., Levi, S., Arosio, P., Cesareni, G., Thomas, C. D., Shaw, W. V. and Harrison, P. M. (1991) Nature (London) **349**, 541–544
13 Bauminger, E. R., Harrison, P. M., Hechel, D., Nowik, I. and Treffry, A. (1991) Biochim. Biophys. Acta **1118**, 48–58
14 Bauminger, E. R., Harrison, P. M., Hechel, D., Hodson, N. W., Nowik, I., Treffry, A. and Yewdall, S. J. (1993) Biochem. J. **296**, 709–719
15 Levi, S., Salfeld, J., Franceschinelli, F., Cozzi, A., Dorner, M. H. and Arosio, P. (1989) Biochemistry **28**, 5179–5184
16 Wade, V. J., Levi, S., Arosio, P., Treffry, A., Harrison, P. M. and Mann, S. (1991) J. Mol. Biol. **221**, 1443–1452
17 Santambrogio, P., Levi, S., Arosio, P., Palagi, L., Vecchio, G., Lawson, D. M., Yewdall, S. J., Artymiuk, P. J., Harrison, P. M., Jappelli, R. and Cesareni, G. (1992) J. Biol. Chem. **267**, 14077–14083
18 Macara, I. G., Hoy, T. G. and Harrison, P. M. (1972) Biochem. J. **133**, 151–162
19 Sun, S. and Chasteen, N. D. (1993) J. Biol. Chem. **267**, 25160–25166
20 Sun, S., Arosio, P., Levi, S. and Chasteen, N. D. (1993) Biochemistry **32**, 9362–9369
21 Le Brun, N. E., Wilson, M. T., Andrews, S. C., Guest, J. R., Harrison, P. M., Thompson, A. J. and Moore, G. R. (1993) FEBS Lett. **333**, 197–202
22 Treffry, A. and Harrison, P. M. (1984) J. Inorg. Biochem. **21**, 9–20
23 Treffry, A., Hirzmann, J., Yewdall, S. J. and Harrison, P. M. (1992) FEBS Lett. **302**, 108–112
24 Cheng, Y. G. and Chasteen, N. D. (1991) Biochemistry **30**, 2947–2953
25 Hanna, P. M., Chen, Y. and Chasteen, N. D. (1991) J. Biol. Chem. **266**, 886–893
26 Bauminger, E. R., Harrison, P. M., Nowik, I. and Treffry, A. (1989) Biochem **28**, 5486–5493
27 Rosenberg, L. P. and Chasteen, N. D. (1982) in The Biochemistry and Physiology of Iron (Saltman, P. and Hegenauer, J., eds.), pp. 405–407, Elsevier Biomedical, New York
28 Waldo, G. S., Ling, J., Sanders-Loehr, J. and Theil, E. C. (1993) Science. **259**, 796–798
29 Chasteen, N. D., Antanaitis, B. C. and Aisen, P. (1985) J. Biol. Chem. **260**, 2926–2929
30 Le Brun, N. E., Cheesman, M. R., Thomson, A. J., Moore, G. R., Andrews, S. C., Guest, J. R. and Harrison, P. M. (1993) FEBS Lett. **323**, 261–266
31 Bauminger, E. R., Harrison, P. M., Hechel, D., Nowik, I. and Treffry, A. (1991) Proc. R. Soc. London B **244**, 211–217
32 Levi, S., Yewdall, S. J., Harrison, P. M., Santambrogio, P., Cozzi, A., Rovida, E., Albertini, A. and Arosio, P. (1992) Biochem. J. **288**, 591–596
33 Treffry, A., Bauminger, E. R., Hechel, D., Hodson, N. W., Nowik, I., Yewdall, S. J. and Harrison, P. M. (1993) Biochem. J. **296**, 721–728
34 Treffry, A., Harrison, P., Luzzago, A. and Cesareni, G. (1989) FEBS Lett. **247**, 268–372
35 Treffry, A. and Harrison, P. M. (1979) Biochem. J. **181**, 709–716
36 Long,G. J., Robinson, W. T., Tappmeyer, W. P. and Bridges, D.L (1973) J. Chem. Soc. Dalton Trans. 573–579
37 Watt, R. K., Frankel, R. B. and Watt, G. D. (1992) Biochemistry **31**, 9673–9679
38 Bakker, G. R. and Boyer, R. F. (1986) J. Biol. Chem. **261**, 13181–13182
39 Hempstead, P. D., Hudson, A. J., Artymiuk, P. J., Andrews, S. C., Banfield, M. J., Guest, J. R. and Harrison, P. M. (1994) FEBS Lett. in the press

Received 17 February 1994/25 April 1994; accepted 28 April 1994