How the presence of three iron binding sites affects the iron storage function of the ferritin (EcFtnA) of *Escherichia coli*

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Abstract The iron storage proteins, ferritins, are found in all organisms which use iron. Here iron storage processes in the *Escherichia coli* ferritin (EcFtnA) are compared with those in human H-type ferritin (HuHF). Both proteins contain dinuclear iron centres that enable the rapid oxidation of 2 Fe(II) by O_2. The presence of a third iron binding site in EcFtnA, although not essential for fast oxidation, causes the O_2/Fe ratio to increase from 2 to 3–4. In EcFtnA the rate of iron oxidation falls markedly after the oxidation of 48 Fe(II) atoms/molecule probably because some of it remains at the oxidation site. However a compensatory physiological advantage is conferred because this iron is more readily available to meet the cell's needs.

Key words: Ferritin; Dinuclear centre; Iron oxidation; Iron metabolism

1. Introduction

The widespread occurrence of the iron storage proteins, ferritins, emphasises their importance in physiological processes. Although conservation at the primary structural level is relatively low, subunit folding, packing and iron storage processes are remarkably conserved for all ferritins across the living kingdom.

In vitro studies with animal and bacterial ferritins have shown that the first steps leading to iron storage are the uptake and catalytic oxidation of Fe(II) by the protein shell [1]. The resulting ferric iron is stored either as the mineral ferrihydrite (in animals) or as ferric phosphate (in bacteria).

Kinetic data obtained with recombinant human H chain ferritin (HuHF) have indicated that the Fe(II) oxidation step occurs at a dinuclear site within the protein shell (there are 24 such sites per molecule, one for each of the 24 subunits [2]). The dinuclear metal sites have been located at the centre of the subunit 4-helix bundle by X-ray crystallographic analysis of Tb(III) derivatives and their function in iron uptake confirmed with the aid of site-directed mutagenesis [2,3]. In the ferritin of *Escherichia coli* (EcFtnA, the product of the *ftnA* gene) such sites (A and B, Fig. 1) have been defined by direct observation of iron binding to the crystalline protein [4].

The diiron centres of ferritins are similar in structure to those found in a number of other proteins where they are associated with reactions involving protein bound iron and oxygen, e.g. the activation of oxygen in methane monooxygenase [5], tyrosyl radical formation in ribonucleotide reductase (RNR) [6], and desaturation of fatty acids in stearoyl-ACP desaturase [7]. The different functionality of these proteins is achieved by changes in the ligands and environment of the dinuclear centre. Even when the immediate ligands of the dinuclear centres are conserved, differences in the rates of oxidation and the reaction intermediates are observed with different members of the ferritin family [8].

X-ray analysis has shown that each EcFtnA subunit binds three iron atoms [4]. The two atoms of the dinuclear sites (A and B) are separated by about 3.8 Å and the third iron site, C, lies on the inner surface of the protein shell at 7–8 Å from the A, B pair (Fig. 1). Four of the iron ligands at the dinuclear centres of EcFtnA and HuHF are at equivalent positions: E17/27, E50/62, H53/65, E94/107, the fifth ligands E130/E61 being non-equivalent (Fig. 2). Site C is unique to EcFtnA, its ligands are E49, E126 and E130 (which alternates as a site B or C ligand). These three glutamate residues (and also E129) are conserved in all known Ftn sequences in which dinuclear site ligands occur. The sequence of a new *E. coli* ferritin, EcFtnB, has recently been defined (accession number D90832), only three of the dinuclear centre ligands are conserved in this sequence (Fig. 2) and no function has been identified for this ferritin. Based on an analysis of inter-subunit interactions in EcFtnA and the aligned sequence of EcFtnB, the formation of stable copolymers is unlikely. A third metal (Tb^{3+}) binding site has also been located on the inner surface of HuHF, but in a different position relative to EcFtnA, its ligands are E61 and E64 [3]. Binding of iron at this site has not been clearly established.

Compared to HuHF [1,2,9–13] few studies on the iron uptake processes have been made with EcFtnA [11,14]. The stoichiometry and kinetics of Fe(II) oxidation are here investigated with the special reference to defining the role of the third iron binding site, C. The iron ligands of site A have been substituted (E17A and E50A), as have those of site B (E50A, the A–B bridging ligand; E94A and E130A) and those of site C (E49A, E126A and E130A) (see Fig. 1). Two aromatic side chains near the dinuclear site have also been replaced (Y24F and W122F) as well as a glutamine residue (Q127E) which, although not a direct ligand, is conserved in the dinuclear sites of EcFtnA and HuHF.

2. Materials and methods

2.1. Site-directed mutagenesis, protein purification and iron removal

Overexpression of the *ftnA* gene and the production and overexpression of site-directed FtnA derivatives were carried out with the pALTER-Ex1 system, as supplied by Promega UK (Southampton, England). Overproduction of HuHF was performed as described in [12]. Both, HuHF and EcFtnA ferritins were purified [15] with minor modifications [11]. Wild-type HuHF was treated with sodium dithionite to remove endogenous iron prior to use [9]. Such treatment was

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not necessary for EcFtnA and its variants since they contained no iron. Iron-free (apo) ferritins were used in all experiments.

Protein concentrations were determined with the Bio-Rad reagent. The colour responses of all proteins were standardised using samples whose concentration had been determined by amino acid analysis.

2.2. Oxygen consumption

Oxygen consumption during Fe(II) oxidation was measured with an oxygen electrode (Rank Brothers, Cambridge) [2] using 1.5 ml of 3 μM protein solution in 0.1 M MES buffer pH 6.5 and adding 48 Fe(II) atoms/molecule as 5 μl of ferrous ammonium sulphate in 1 mM H_2SO_4.

2.3. Rapid kinetic experiments

Rapid kinetic experiments were carried out according to [11] using an SX.17MV stopped-flow instrument (Applied Photophysics, Leatherhead, UK) fitted with a sequential mixing option. Unless otherwise stated, all quoted concentrations are the final concentrations after 1:1 mixing. The source of Fe(II) was ammonium ferrous sulphate (99.997% pure, Aldrich Chemical Company, Dorset, England). Stock solutions of Fe(II) were prepared in 5 mM H_2SO_4 and diluted to 50 μM H_2SO_4 immediately prior to use. In a typical experiment Fe(II), at 48 Fe atoms/molecule (96 μM), was mixed with 2 μM solution of protein in 0.2 M MES buffer, pH 6.5. For measurements of progress curves at 600 nm an additional cut-off filter (<370 nm) was placed between the monochrometer and the light pipe. All stopped-flow experiments were carried out at 25°C.

The half time of Fe(II) oxidation was determined by the sequential stopped-flow assay described previously [11] using 0.25 μM protein in 0.1 M MES buffer pH 6.5 and 48 Fe(II) atoms/molecule. This assay measures residual Fe(II) available for chelation with 1,10-phenanthroline. Only half times ($t_{1/2}$) shorter than 900 s can be measured in this assay.

For the sequential additions of 48 Fe(II) atoms/molecule the protein solution, 4 μM protein in 0.4 M MES buffer pH 6.5, was first mixed (1:1), with either 50 μM H_2SO_4 or 192 μM Fe(II) in 50 μM H_2SO_4. Between additions the protein/Fe solution was held in the ageing loop for 1, 10 or 15 min before being mixed (1:1) with 96 μM Fe(II) in 50 μM H_2SO_4. Thus the protein solution to which Fe(II) was added only in the second mixing gave the progress curve for the first 48 Fe(II) atoms/molecule and that to which Fe(II) was added twice gave the progress curve for the second addition. The timing of the second addition of Fe(II) was chosen to ensure that the first 48 Fe(II) atoms/molecule had been oxidised.

2.4. Discontinuous assay of Fe(II) oxidation

Residual Fe(II) was measured using ferrozine as an Fe(II) chelator according to the method described in [13].

Table 1
Effects of site-directed substitutions in EcFtnA on the stoichiometries and half times of the oxidation of 48 Fe(II) atoms/molecule

	Fe(II) oxidation		
	Fe oxidised/O_2	$t_{1/2}$ (s)[a]	Position of substituted residue
EcFtnA	3.5	0.43	
W122F	3.9	0.38	near the dinuclear centre
Q127E	n.d.[b]	0.42	at the dinuclear centre
E130A	1.8	1.0	site B or C ligand
E126A	1.8	1.27	site C ligand
Y24F	2.4	1.89	hydrogen bonded to a site B ligand
E49A	n.d.	2.4	site C ligand
E17A	3.6	281	site A ligand
E50A	n.d.	>900	ligand for sites A and B
E94A	n.d.	>900	site B ligand
HuHF	2.3	0.4	

[a]Half time of Fe(II) oxidation was measured using sequential stopped-flow with phenanthroline as Fe(II) chelator. 0.25 μM protein in 0.1 M MES buffer pH 6.5 (only $t_{1/2}$ less than $\approx$900 s can be measured).
[b]n.d., not done.

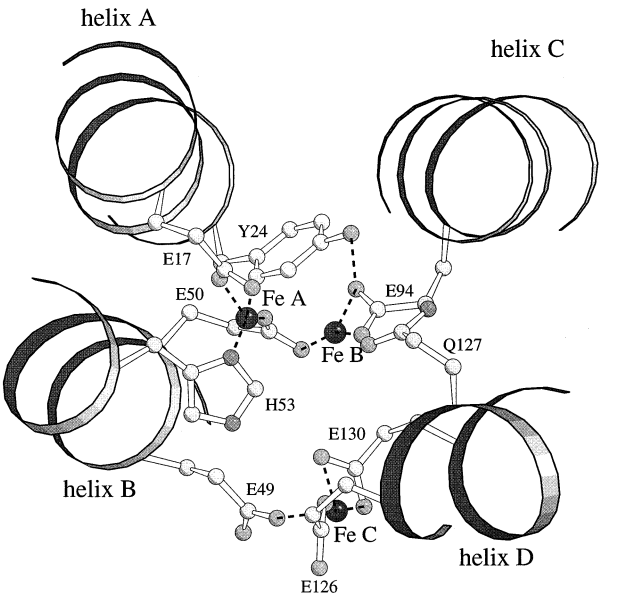

Fig. 1. The dinuclear centre of EcFtnA showing the iron binding sites A and B and the neighbouring third iron site, C. Note that E130 shown here as a ligand for Fe at site C, can also serve as a ligand of Fe at site B.

3. Results

3.1. Oxygen and Fe(II) stoichiometry

The stoichiometries of Fe(II) oxidation by dioxygen are shown in Table 1 for EcFtnA and some of its variants. Compared to the Fe(II)/O_2 ratio of 2.3 obtained with HuHF, more than three Fe(II) atoms were oxidised for each molecule of O_2 consumed by EcFtnA. Site-directed substitutions of ligands at site C in EcFtnA (E126A and E130A) reduced the Fe/O_2 ratio to that of HuHF whereas the stoichiometry was unaffected by changing a site A ligand (E17A) or residue W122 which is situated close to the centre (W122F). These results suggest that an intact site C is required to give the observed oxidation stoichiometry of EcFtnA and that the proposed third site in HuHF does not participate in the oxidation of iron at the dinuclear centre.

The reason why the Fe/O_2 ratio is lowered by the Y24F substitution is unclear. This residue may be involved in the transfer of electrons from Fe(II) at site C to the Fe-O_2 complex formed at the dinuclear centre.

3.2. Effects of site C modification on the rate of Fe(II) oxidation

The half times of Fe(II) oxidation by EcFtnA and its variants, as well as that of HuHF, are also shown in Table 1. Replacing three of the dinuclear site ligands by alanine (E17A, E50A or E94A) caused a dramatic increase in $t_{1/2}$, whereas replacing the site C ligands (E49A or E126A) produced only a small reduction in the oxidation rate. Thus although site C is involved in the oxidation of a third iron atom, binding of Fe(II) at site C is not essential for fast Fe(II) oxidation at the dinuclear centre. Because an E130A substitution had only a small effect on the half time of Fe(II) oxidation compared to E50A or E94A, it is concluded that E130 is not an essential ligand and that Fe(II) can be anchored at site B by E50 and E94 alone.

The increase in $t_{1/2}$ of oxidation observed with variant

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HuHF numbering  27                  34                    61 62      65
Ftn numbering   17                  24                    49 50      54
                ↓                   ↓                     ↓  ↓       ↓
HuHF     I N L E L Y A S Y V Y L S    Q S H E E R E H A E K L M
EcFtnA   M N L E L Y S S L L Y Q Q    H A Q E E M T H M Q R L F
EcFtnB   M N R E F Y A S N L Y L H    Q A Q S N V T Q M M R M F

HuHF numbering      107                       141     144
Ftn numbering        94                       127     130
                     ↓                        ↓       ↓
HuHF     C A L H L E K N V N Q      L N E Q V K A I K E L
EcFtnA   E T Y K H E Q L I T Q.     V S E Q H E E E K L F
EcFtnB   K T M E E Y E Q R S S      E K E Q Q H D G L L L
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Fig. 2. Amino acid sequences around the diiron centre residues in HuHF, EcFtnA and EcFtnB.

Y24F resembles the effects of similar modifications in HuHF [2] and frog H ferritin [8]. Although Y24 is not a direct ligand in either site A or B, it is hydrogen bonded to E94, which is an essential ligand at site B.

3.3. Effects of site C modification on oxidation intermediates

With the exception of the dinuclear site variants E17A, E50A and E94A which exhibited slow rates of Fe(II) oxidation, all of the EcFtnA variants examined showed a transient coloured oxidation intermediate which peaked at 0.5–2 s and disappeared at 10–100 s (with 1 μM protein). Thus it would appear that the intermediates are formed at or near the diiron centres and their properties depend on the detailed structural features of these centres. In EcFtnA (Fig. 3) and also in the variants Q127E and Y24F [12], the transient intermediate had a relatively high absorbance at 370 nm and a low absorbance at 600 nm. However, in variants E130A, E49A and E126A, the absorbance at 600 nm increased and that at 370 nm decreased so that they were nearly equal and the intermediate was blue rather than yellow as in EcFtnA. The results also showed that under the condition studied here, modification of site C ligands alters the rates of both formation and decay of the transient blue species (Fig. 3).

3.4. Post-oxidation effects

In EcFtnA when 48 Fe(II) atoms/molecule are added, oxidation was complete within 1 min yet spectral changes in the UV and near-UV continued over a 20 min period (Fig. 4a). These changes were greatly reduced or absent in site C variants (Fig. 4b and c). The well-defined isosbestic points observed with EcFtnA suggest that one iron species is converted into another. The absence of such changes in the variants could mean that they occur before the first spectrum is recorded (about 20–40 s) or that the alterations in metal binding that are responsible for the spectral changes do not occur in these variants.

The spectral changes observed in EcFtnA were paralleled by changes in the ability of EcFtnA to oxidise Fe(II). It had been observed previously with HuHF [2] and BfHF [16] that whereas the oxidation of the first 48 Fe(II) atoms/molecule was fast, a subsequent aliquot was oxidised at a much slower rate, and that it took several hours for the initial activity to be restored. In EcFtnA the loss of activity, following the oxidation of the first 48 Fe(II) atoms/molecule, was very marked (Fig. 5a). Furthermore, the rate of Fe(II) oxidation became progressively slower as the time between the first and second addition was increased (Fig. 5a, d). Indeed there was no sign

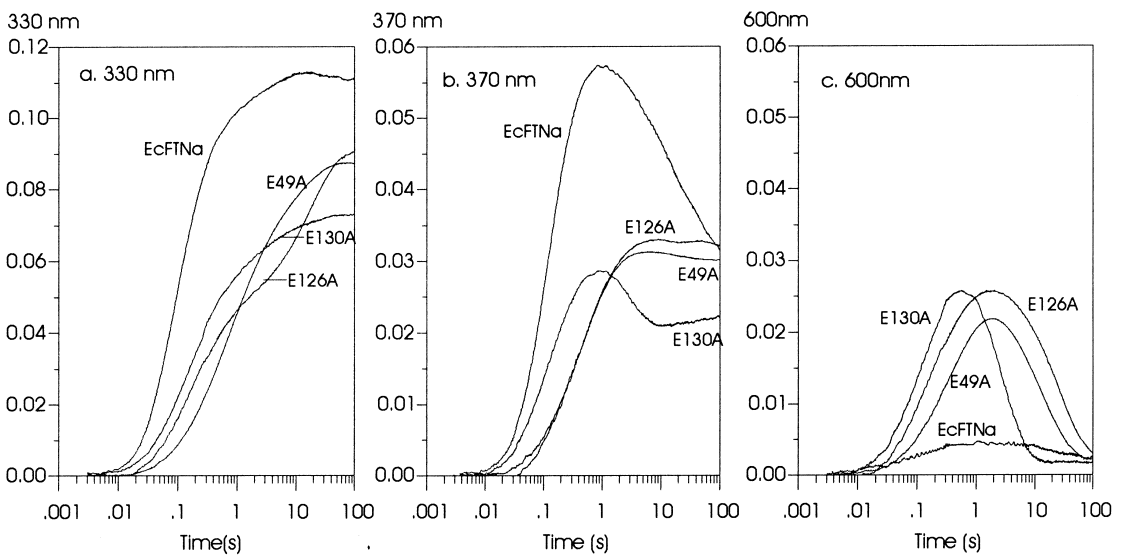

Fig. 3. Stopped-flow traces for the oxidation of 48 Fe(II) atoms/molecule by EcFtnA and variants. Reactions were followed at: a: 330 nm, b: 370 nm and c: 600 nm with 1 μM protein (final) in 0.1 M MES buffer pH 6.5.

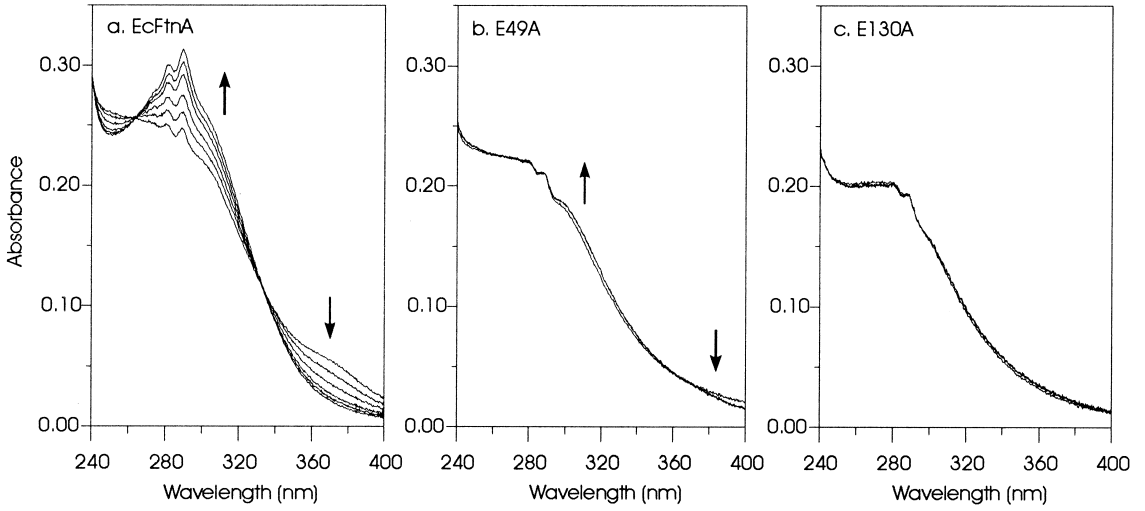

Fig. 4. Post-oxidation spectral changes in EcFtnA and variants. UV difference spectra were recorded after the addition of 48 Fe(II) atoms/molecule to 1 μM protein solution in 0.1 M MES buffer pH 6.5. a: EcFtnA, spectra are at 1, 2, 4, 8, 12 and 19 min after Fe(II) addition. b: E49A, spectra are at 1, 3 and 5 min after Fe(II) addition. c: E130A, spectra are at 1, 5 and 8 min after Fe(II) addition.

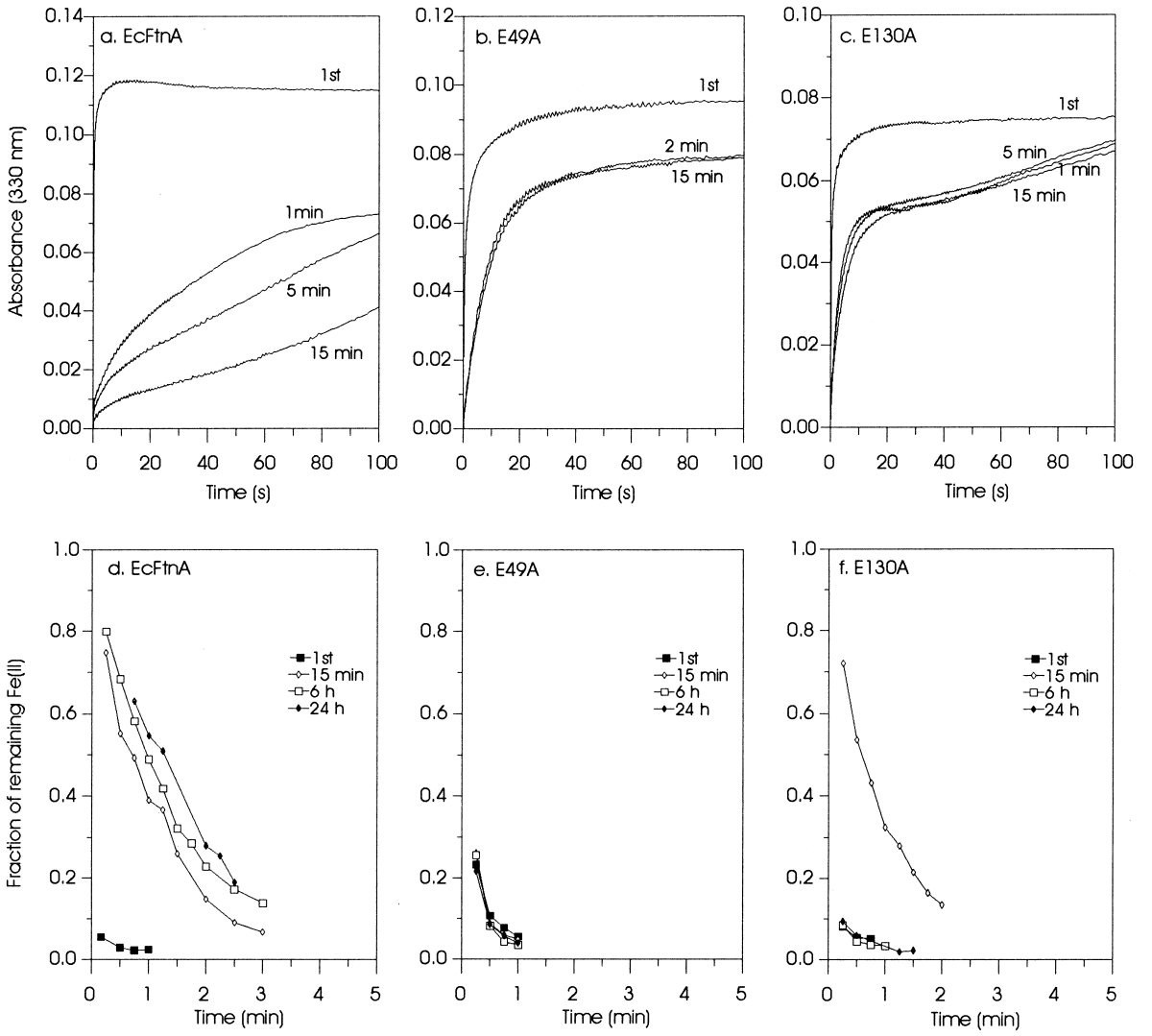

Fig. 5. Comparison of the oxidation of the first 48 Fe(II) atoms/molecule with a second aliquot of 48 Fe(II) atoms/molecule added at different times after the first addition. a–c: Sequential stopped-flow progress curves recorded at 330 nm following the sequential mixing of the ferritin solution with either one (1st) or two solutions of Fe(II). The second additions were made at the times specified for each trace. d–f: Discontinuous assay using microtitre plates. In both assays the final protein concentration was 1 μM in 0.1 M MES buffer.

of recovery even after a 24 h interval. In contrast, with variants E130A and E49A (which did not show the time-dependent spectral changes, Fig. 4) there was an initial loss of activity after the first addition of 48 Fe(II) atoms/molecule but this loss did not increase with time (Fig. 5b and c). Moreover, with E130A the activity was restored 6 h after the first addition (Fig. 5f) whereas with E49A the activity was essentially the same for all additions (Fig. 5e). Thus it seems that modification of site C ligands has a very marked effect on the rate of regeneration of oxidative activity.

4. Discussion

The present results confirm that the initial event in the sequestration of iron by EcFtnA is the oxidation of Fe(II) at a dinuclear centre, as in HuHF. However, the two ferritins show marked differences which are seemingly due to the involvement of a third iron atom bound at site C close to the dinuclear centre of EcFtnA.

What then is the role of site C in EcFtnA? The increase in oxidation stoichiometry from 2 in HuHF to 3–4 Fe(II)/O_2 in EcFtnA is due, at least in part, to oxidation of the third iron at site C. It is uncertain whether a fourth Fe(II), in solution or at a non-specific protein site, is also oxidised to enable the full reduction of dioxygen to water. There is an interesting parallel with the R2 subunit of *E. coli* ribonucleotide reductase, where the oxidation of two Fe(II) takes place at a dinuclear centre together with the conversion of a nearby tyrosine to a tyrosyl radical and the oxidation of a third Fe(II) at an unknown site [17].

In EcFtnA even though site C participates in iron oxidation, removal of site C ligands causes only a small reduction in the primary oxidation rate compared with the effects of altering ligands of site A (E17) or site B (E94) or both (E50). The role of site C appears to be in influencing oxidation intermediates and the fate of the oxidised iron. The observed changes in the transient intermediate could be directly due to the loss of iron binding at site C or to changes in charge distribution near the dinuclear centre. Loss of the blue absorbance observed in HuHF variants Q127E and A144E [2] suggests that changes in the absorbance spectrum of the intermediate may be caused by the introduction of negative charges in the vicinity of the dinuclear centre. The interpretation of the spectral changes is uncertain but the nature of the intermediates is clearly affected by their environment.

Of more significance is the effect of site C on the rate of regeneration of activity. It is apparent (Fig. 5) that its presence leads to a prolonged reduction in ferroxidase activity as a consequence of oxidising 48 Fe(II) atoms/molecule. Because a high rate of oxidation depends on the availability of the dinuclear centres, the early recovery by site C variants suggests that the absence of iron in site C increases the turnover rate of these centres. Preliminary Mössbauer spectroscopic studies on the distribution of Fe(III) species in EcFtnA following the oxidation 48 Fe(II) atoms/molecule have shown that the movement of Fe(III) from dimers to core is very slow compared with that in HuHF and that even at 24 h none of the iron is found in clusters compared to 100% in HuHF [18]. This suggests that in EcFtnA most of the dinuclear centres are still occupied 24 h after the initial oxidation. Mössbauer studies have shown that variant E130A is more similar to HuHF than to EcFtnA, with most of the iron present in the core at 6 h (Bauminger, Treffry and Harrison, unpublished work). Thus the regeneration of activity correlates with the movement of iron into the protein cavity and the simultaneous recovery of Fe-free dinuclear centres.

The presence of the third iron site in EcFtnA therefore confers two main advantages. The first involves the change of iron oxidation stoichiometry from 2 Fe(II)/O_2 in HuHF to 3–4 in EcFtnA. This avoids the production of reactive oxygen species resulting instead in the complete reduction of dioxygen to water and is of obvious benefit in vivo. The second benefit is associated with the distribution of the oxidised iron in EcFtnA. Thus although the prolonged loss of 98–99% of the ferroxidase activity after the initial oxidation of 48 Fe(II) atoms/molecule may appear as a negative consequence of the presence of site C, EcFtnA can still accumulate iron at a significant rate (Fig. 5) and act as an iron storage protein. Indeed it has been found by Mössbauer spectroscopy that when 144 Fe(II) atoms/molecule are added, 80% of this iron is stored in the iron core at 24 h and only 20% (29 Fe atoms) remains attached to the protein (Bauminger, Treffry and Harrison, unpublished results). The retention of iron at the site of oxidation may confer a physiological advantage. Thus in EcFtnA the iron has been shown to be more readily available to small chelators than iron in HuHF [19]. This observation can now be explained by the difference in the distribution of iron: in EcFtnA it is retained at the binding sites in the protein shell under conditions in which in HuHF it more readily moves to core. Hence EcFtnA may be well adapted to satisfy the cellular demand for iron.

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