DALTON COMMUNICATION

Catalytic iron(II) oxidation in the non-haem ferritin of *Escherichia coli*: the early intermediate is not an iron tyrosinate *

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The non-haem ferritin of *Escherichia coli* (EcFTN) has been shown to give a blue complex during iron(II) oxidation, the colour being enhanced by amino acid substitutions near the diiron site; this intermediate is not a tyrosine→iron charge-transfer complex.

Ferritin stores iron as iron(III) hydroxide oxide phosphate inside its tetracosameric protein shell.[1] Studies of ferritin iron biomineralisation, carried out *in vitro*, usually in the absence of phosphate, have shown the initial step to be the binding and catalytic oxidation of the first Fe^{II} entering the iron-free apoferritin molecule.[1–5] The mechanism of oxidation is as yet unresolved although an active site has been identified by means of a variety of experimental techniques in three different ferritins human H chain ferritin[2,3] (HuHF), and the non-haem[3,4] and haem-containing[5] ferritins of *Escherichia coli* (EcFTN and EcBFR, respectively). The site of catalysis is considered to be a dinuclear metal centre situated within a four-helix bundle and closely resembling those of ribonucleotide reductase, methane monooxygenase, stearoylacyl carrier protein Δ^9-desaturase and rubrerythrin.[6,7] In EcFTN this site has been shown by X-ray analysis to bind a pair of iron atoms and a third Fe atom was found at 7–8 Å from the diiron centre[4] (Fig. 1).

During the course of previous stopped-flow kinetic investigations with HuHF a blue intermediate absorbing in the range 400–800 nm (maximum at 650 nm) was observed when Fe^{II} was added to apoferritin at a ratio of two Fe^{II} per subunit.[2] The absorbance was maximal at 0.2–0.3 s, but declined over the following 10 s as the intermediate was replaced by products absorbing at shorter wavelengths. A blue-purple intermediate had previously been described for bullfrog H chain ferritin (BfHF) and its colour attributed to an iron–tyrosinate complex.[8,9] In contrast, EcBFR[5] and EcFTN[2] were reported not to produce this intermediate, suggesting that the mechanism of iron(II) oxidation may be different in the prokaryotic ferritins.

It has now been shown that wild-type EcFTN does indeed give the blue complex during iron(II) oxidation, although the observed absorbance was low.† However, the intensity of the blue colour was enhanced at least five-fold by specific site-directed amino acid substitutions at, or in the vicinity of, the dinuclear centre, and was diminished by other substitutions (Fig. 2). Successive spectra were collected over the range 350 to 750 nm for variant E129K + E130A with a diode-array attachment to the stopped-flow instrument (data not shown; notation explained in the legend to Fig. 2). The intermediate had an absorption maximum at 600 nm and a shoulder at 370 nm. Stopped-flow single-wavelength measurements showed that the absorbance at 330 nm increased in parallel with those at 600 and 370 nm but continued to increase as the blue species declined (Fig. 2). Thus, the transitory nature and lifetimes of the 370 and 600 nm absorbances (Fig. 2) suggest that the blue complexes formed by EcFTN and HuHF are analogous.

The data in Fig. 2 indicate that the absorbance at 600 nm is enhanced by removing a negatively charged side chain in the vicinity of the dinuclear centre (variants E49A, E126A, E130A, E129K + E130A). Conversely, colour formation is diminished by introducing a negative charge (Q127E). These results are consistent with the loss of blue colour observed with two HuHF variants, Q127E and A130E (residues numbered according to the EcFTN sequence; note that K129 is extant in HuHF). Of some significance is the observed 600 nm absorbance in variant Y24F (Fig. 2) and the transitory absorbance at 370 nm. The ultimate intensity at 600 nm is the same as that of the wild type but it is generated more slowly with the variant. The equivalent variant of HuHF was reported to give no blue colour,[2] but further experiments have shown a weak absorption. It is now clear that, with EcFTN at least, the intermediate is not a Y24→Fe charge-transfer complex. Replacing the other tyrosines and the only tryptophan in HuHF had no effect,[2] whereas substituting a tryptophan residue (W122F) near the diiron centre of EcFTN caused loss of absorbance at 600 nm (Fig. 2) but not its elimination and with no change at 370 nm.

The appearance of the 600 nm absorbance parallels that at 330 nm (Fig. 2) and the latter has been shown to coincide with an increase in Fe^{III} and not with the binding of Fe^{II}.[3] Thus the production of the blue complex appears to be due to an early intermediate in protein-catalysed iron(II) oxidation. This is further indicated by the observation that no blue colour is formed by EcFTN variants E17A, E50A and E94A that exhibit exceedingly low rates of iron(II) oxidation[3] (data not shown). Taken together, past[2,3] and present data strongly indicate that the initial stage of iron(II) oxidation, including the production of the blue complex, occurs at the diiron centre (Fig. 1) and that the

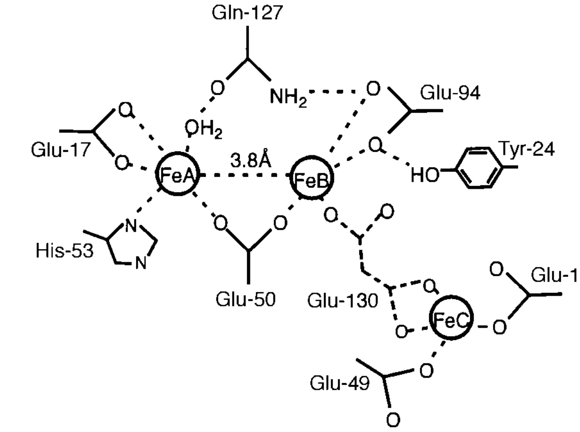

Fig. 1 Schematic diagram of the iron binding sites in EcFTN

* Based on the presentation given at Dalton Discussion No. 2, 2nd–5th September 1997, University of East Anglia, UK.

† Rapid kinetic experiments were carried out in an Applied Photophysics (Leatherhead, UK) SX.17 MV stopped-flow instrument.[2,3] All assays involved adding an equal volume of ammonium iron(II) sulfate in 50 μM H_2SO_4 to an aerobic solution of the iron-free protein (final concentration 1 μM) in 0.2 M 2-morpholinoethanesulfonic acid buffer, pH 6.5, to give 48 Fe^{II} per molecule.

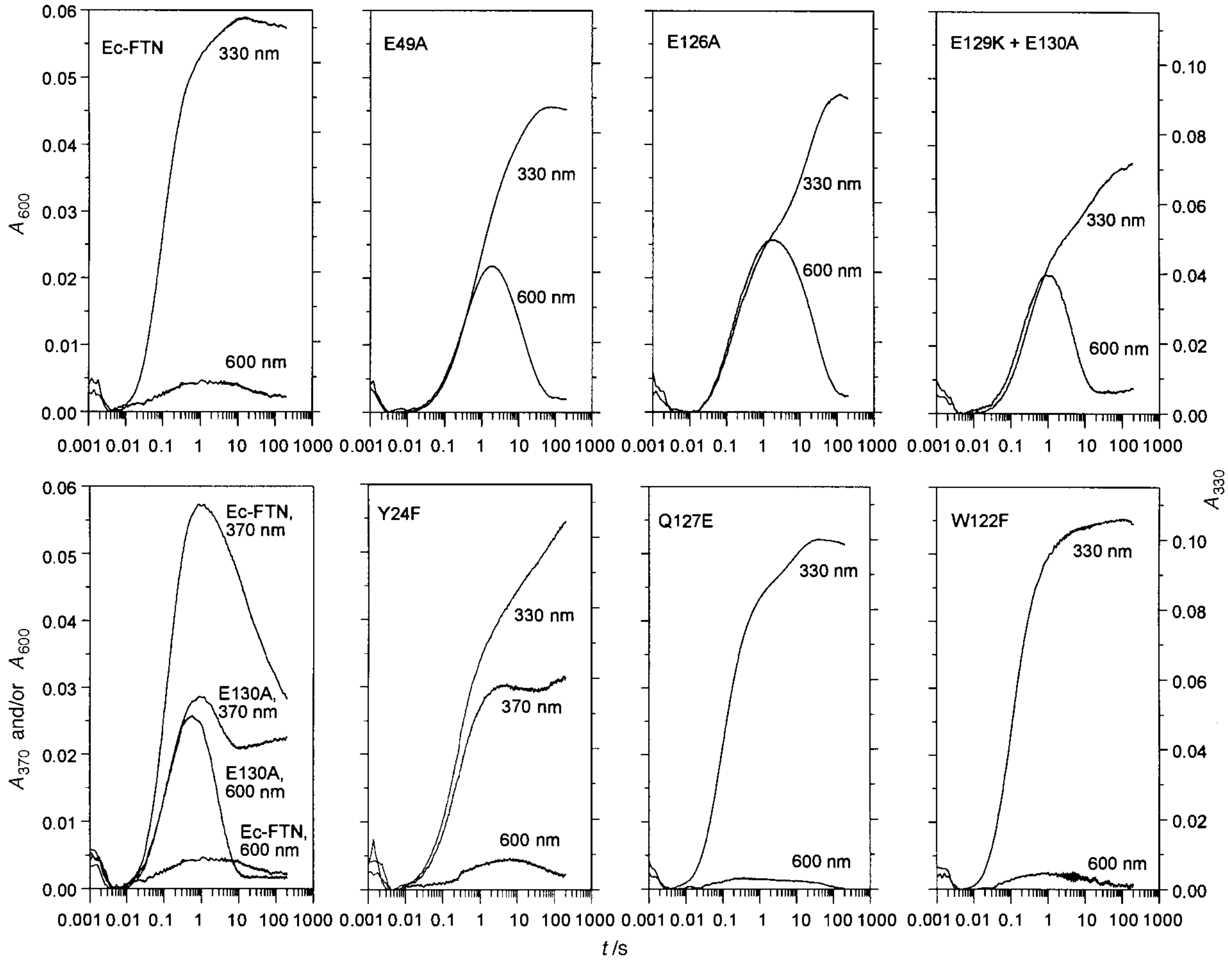

Fig. 2 Stopped-flow kinetics of iron(II) oxidation by EcFTN and its site-directed variants. Variants E130A and E129K + E130A give virtually identical absorptions at all three wavelengths. Key for the amino acid residues: A = alanine, E = glutamate, F = phenylalanine, K = lysine, Q = glutamine, W = tryptophan, Y = tyrosine; 'E49A' means that the glutamic acid residue at position 49 has been replaced with alanine

absorption is due neither to a tyrosine→Fe^{III} charge-transfer complex nor to a tryptophan radical. It is likely that it is due to a ligand-to-metal charge-transfer (LMCT) transition of a diiron(III) peroxo complex such as has been identified in a number of diiron proteins and model compounds.[10–12] If so, how can this conclusion be reconciled with the Raman-based identification of the coloured BfLF complex as an iron(III) tyrosinate?[9] The answer may be that the transient species observed by stopped-flow spectroscopy with HuHF,[2] BfLF[8] and now EcFTN is not the same as that identified by Raman spectroscopy. It should be noted that the Raman spectra were observed under totally different conditions [iron(II) concentration 80 times that of Fig. 2, limiting dioxygen concentration and spectral measurements made at 5 min after oxygen exposure] from those pertaining in the stopped-flow experiments. Although the species giving the Raman modes also gave a UV/VIS spectrum with A_{max} at 550 nm, the absorption at 550 nm was maximal at 6–7 min and it decayed slowly over 25 min or more.

If correct, the assignment of the blue intermediate as a diiron(III) peroxo complex is consistent with the conclusion that the mechanism of oxidation in EcFTN and HuHF involves the transfer of two electrons from a dioxygen molecule bound to a pair of iron(II) atoms at the dinuclear centre.[2] The geometry of the complex needs further investigation. The ferritin EcBFR also contains a dimetal site[13,14] and it seems likely that the initial oxidation step proceeds by a similar mechanism to that occurring in other ferritins.

Acknowledgements

We thank the Wellcome Trust for financial support.

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Received 28*th May* 1997; *Communication* 7/03675B