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New insights into iron release from ferritin: direct observation of the neurotoxin 6-hydroxydopamine entering ferritin and reaching redox equilibrium with the iron core

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Iron release from the iron storage protein ferritin has been studied extensively because of its important role in oxidative stress and its possible role in the progression of Parkinson's disease. For many years external indicators, notably strong iron(II) chelators, have been used to investigate this reaction. Such chelators can, however, drastically affect the electrochemical and thermodynamic properties of iron. The present study is unique in that it has been possible to follow a reaction taking place *within* the ferritin shell. This was made possible by our serendipitous discovery that, at physiological pHs, the oxidation product of 6-hydroxydopamine (a deprotonated quinone) acts as its own indicator (G. N. L. Jameson and W. Linert, *J. Chem. Soc., Perkin Trans. 2*, 2001, 563–568). The redox equilibrium data and the kinetics of the formation of this red-coloured species can only be explained on the basis that reduction of the iron(III) takes place within the ferritin shell. This is, in fact, the first time that a reaction actually taking place inside the ferritin shell has been followed. It has also been established that, at least *in vitro*, all eight hydrophilic channels are capable of being simultaneously involved in the reaction. It has also been possible to calculate the rate of oxidation of the 6-hydroxydopamine within the ferritin and it is demonstrated that a redox equilibrium is established within the protein. Finally, evidence is provided confirming that chelators are in fact intrinsically linked to iron removal from ferritin.

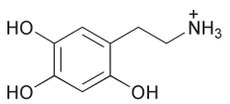
Introduction

Iron plays an important and complicated role in biological systems. Not only is it a redox catalyst but it is also involved in control systems and Lewis acid–base reactions, making it the one transition-metal common to all forms of life. The simple aquo-cations of iron(III) are, however, highly insoluble at physiological pHs and therefore any iron present in biological systems must be complexed before it can be transported and utilised (although soluble at physiological pHs, any free iron(II) aquo-ions present would in any case be rapidly oxidised to iron(III) unless complexed). In order to maintain solubilisation there is a pool of low molecular weight complexes lying between where the iron is stored and where it is to be employed. Little is known about the chelators involved but in mammals citrate seems a likely candidate, with nucleotides and amino acids also being suggested.[1]

The common iron storage protein in plants, bacteria and mammals is ferritin.[2] This is composed of 24 sub-units arranged in 432 symmetry to form a hollow protein shell enclosing a cavity 80 Å in diameter. It is within this cavity that up to 4500 iron atoms can be stored as an iron(III) oxy-hydroxide which, in the case of mammals, is similar in structure to ferrihydrate. However, it is as iron(II) that the iron enters and leaves the cavity – this is not surprising as iron(II) is by far the more labile of the two species. Reduction of iron within the cavity is therefore a pre-requisite to its release. But what the nature of the *in vivo* process actually is remains unknown; neither the reductant nor the mode of transport has been identified.

The way in which iron enters and leaves the ferritin is in part dictated by the structure of ferritin itself.[2] The sub-units are packed tightly together, leaving eight narrow channels around the 3-fold axes and six around the 4-fold axes. In mammalian ferritin the 3-fold channels are predominantly hydrophilic, whereas the 4-fold channels are highly hydrophobic with very little space around them. It is usually assumed that the eight hydrophilic 3-fold channels function as both entry and exit routes for iron. In this respect it is pertinent that Theil and co-workers[3,4] have shown that increased rates of iron release can be achieved by mutating conserved amino acids at the inter-helical junction of the 3-fold channels.

It has also been established that organic molecules can migrate into ferritin *via* the hydrophilic channels, and *in vitro* experiments[5,6] have been performed using organic spin probes to measure rates of entry into the cavity. These experiments have therefore confirmed that it is possible for a reductant to make use of the 3-fold channels. We have confined our studies to the potential reductant 6-hydroxydopamine, **1**, because it is a neurotoxin that is used in animal studies to produce neuronal damage similar to that seen during the development of Parkinson's disease.[7] Since it can be produced *via* a Fenton reaction from dopamine,[8,9] it has also been suggested that it may be more directly involved in the progression of Parkinson's disease.[10]



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We have already established the manner in which 6-hydroxydopamine reacts with inorganic iron(III)[11–13] and the present study of its interaction with ferritin relies on this knowledge. Importantly, 6-hydroxydopamine reacts with iron(III) almost exclusively *via* an outer-sphere mechanism at all pHs, *i.e.* electron exchange takes place *without the prior formation of a complex*. This is in stark contrast to the other catecholamines,[14–16] which form relatively stable bis- and tris-complexes at physiological pHs.[15] (Noradrenaline is an exception[17] in that at low pHs electron transfer takes place *via* the simultaneous use of both electron transfer mechanisms.)

The only product of 6-hydroxydopamine oxidation at pHs > 5 is the deprotonated quinone which itself arises from all three quinones that are produced by two successive one-electron transfers.[13] This deprotonated quinone, **2**, absorbs at 480 nm and the extinction coefficient is known.[12] It is therefore possible to measure directly the reduction of iron without the use of complex formation as an intermediary.

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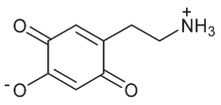

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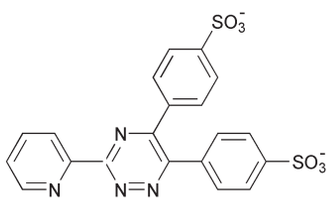
Here we report the result of using this method which has enabled us to establish that (i) it is certain that the reduction product, iron(II), is not automatically released but requires an external chelator for its removal; (ii) the reductant 6-hydroxydopamine enters the protein; and (iii) a redox equilibrium is set up within the ferritin shell.

Most previous investigations[18–22] have made use of an iron(II) chelator such as ferrozine [3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine],[23] **3**, to act as a colorimetric probe of the iron released.



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The question arises, however, as to what extent ferrozine (or any other iron chelating indicator) is influencing or disturbing these investigations, and indeed this has been questioned in the past.[24] The reduction of iron(III) using 6-hydroxydopamine as reductant was therefore also followed using ferrozine as an indicator under the same conditions. The results of these investigations show clearly the extent to which the use of ferrozine can lead to misleading conclusions.

Materials and methods

Chemicals

6-Hydroxydopamine was obtained from two sources (Fluka and Sigma-Aldrich), ferrozine [3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine] was obtained from Fluka, PIPES buffer (1,4-piperazin-bis-ethansulfonic acid) came from Aldrich, and all were used without further purification.

Ferritin preparation

Ferritin samples with constant iron-loading have been used because the reaction may well involve the interaction between solid and liquid interfaces (see below) and these properties will depend on the size of the ferritin ion core. Horse-spleen ferritin was bought from Sigma (108 mg ml^{-1}) and then separated into different iron-loadings on a caesium chloride density gradient using the methodology of Fischbach and Anderegg.[25] Caesium chloride was removed by dialysis and the concentrations were then checked – iron concentrations were measured by atomic absorption spectroscopy using a Perkin Elmer Zeeman/3030, and protein concentrations were measured spectrophotometrically using the Bio-Rad assay.

Stopped-flow

Experiments were carried out on an SX-17MV stopped-flow spectrometer with photo-multiplier detector from Applied Photophysics. Samples of ferritin of known concentration (both protein and iron known – approximately 520 iron atoms per ferritin) were prepared. Solutions of 6-hydroxydopamine of varying concentrations were also prepared, some containing ferrozine as the indicator. All solutions were buffered at a pH of 7.10 using PIPES buffer. The samples were de-oxygenated with argon and then transferred to the stopped-flow apparatus by means of Hamilton gas-tight syringes.

Results and theoretical interpretation

The molar extinction coefficients of the deprotonated-quinone ($\varepsilon = 2200$ M^{-1} cm^{-1}, $\lambda = 480$ nm[12]) and the ferrozine complex ($\varepsilon = 27\,900$ M^{-1} cm^{-1}, $\lambda = 562$ nm[23]) are both known, allowing the absorbance–time curves to be converted into concentration–time curves. Furthermore, the initial rapid increase in the deprotonated quinone ceases after only a relatively small amount of the 6-hydroxydopamine added has been consumed. The reaction that follows the initial 'burst' has been measured exclusively over longer periods of time in previous studies,[18–22,26] using ferrozine as the indicator. The present study, however, is confined to an investigation of this initial 'burst'.

The effect of using ferrozine as external indicator

Solutions of ferritin were reacted with (a) solutions of 6-hydroxydopamine of known concentrations, (b) solutions of 6-hydroxydopamine containing ferrozine as indicator, and (c) solutions of ferrozine alone. One ferrozine complex corresponds to one iron atom and two iron atoms create one quinone. This allows the curves to be directly compared by converting them into concentrations of iron. This is illustrated in Fig. 1 in which three experiments with the same concentration of ferritin have been overlaid.

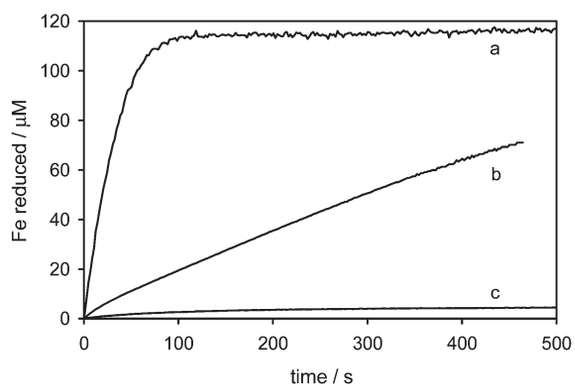


Fig. 1 The concentration of iron reduced within ferritin over time by 6-hydroxydopamine. a) Using 6-hydroxydopamine as its own indicator ($\lambda = 480$ nm); b) using ferrozine as indicator ($\lambda = 562$ nm); c) the addition of ferrozine alone as control. [ferritin] = 5.27 µM; [6-hydroxydopamine] = 0.265 mM; [ferrozine] = 1.46 mM; pH = 7.10.

Firstly, it can be seen that ferritin protects its iron from strong iron(II) chelators, for although ferrozine mixed with ferritin alone produces some iron-ferrozine complex, this is very slow and minimal. Secondly, and most importantly, the deprotonated quinone is formed very much faster than the ferrozine complex. (These two experiments had to be carried out independently because the band corresponding to the ferrozine complex at 562 nm quickly obscures the band at 480 nm corresponding to the deprotonated quinone.) Finally, the initial increase in the amount of iron(II) released (measured as the ferrozine complex) in the mixed ferrozine/6-hydroxydopamine solutions does not correspond to the amount of quinone produced by the 6-hydroxydopamine. However, over a much longer time scale (*i.e.* after the initial 'burst' the amount of quinone produced does start to rise again and is then tracked by the ferrozine indicator – this is the period followed in previous studies. The reason for the eventual tracking of the reduction of the iron is readily explained because, as is shown below, the ferrozine is disturbing the equilibria involving the ferritin by sequestering iron(II).

Interpretation of the end-point of the initial 'burst'

The rate and extent of formation of the quinone (curve a, Fig. 1) must represent the reduction of iron in the core and the kinetics involved are presented below. The fast initial part of this reaction has a distinct plateau that obviously represents an equilibrium and we demonstrate below that this arises from an equilibrium between 6-hydroxydopamine inside and outside the ferritin coupled with the equilibrium between 6-hydroxydopamine inside and the iron-containing core. Table 1 shows representative values of the concentration of quinone, $[Q]_{eq}$, present at this equilibrium with variation of the total 6-hydroxydopa-

Table 1 Representative values of kinetic and absorption data

$[fer]_T$/μM	$[L]_T$/μM	Abs. max. of Q	$[Q]_{eq}$/μM	$[L]_{free}$/μM	$[Q]^3/[fer]^4$/M^{-1}	k^{obs}/s^{-1}	k_1/s^{-1}	$V_{fer}[fer]k_1$/s^{-1}
1.32	473	0.0372	16.9	456	1.61×10^9	0.0760	0.0595	1.17×10^{-5}
1.32	709	—	—	—	—	0.120	0.103	2.04×10^{-5}
1.32	1000	0.0472	21.5	982	3.29×10^9	0.203	0.186	3.68×10^{-5}
1.32	1420	—	—	—	—	0.360	0.343	6.78×10^{-5}
1.32	1650	0.0500	22.7	1630	3.91×10^9	0.520	0.503	9.94×10^{-5}
1.32	2010	0.0577	26.2	1980	6.01×10^9	0.995	0.978	1.93×10^{-4}
1.32	2360	0.0542	24.6	2340	4.98×10^9	2.03	2.01	3.98×10^{-4}
1.32	4020	0.0670	30.5	3990	9.41×10^9	3.30	3.28	6.48×10^{-4}
1.32	5020	—	—	—	—	3.35	3.33	6.58×10^{-4}
2.63	52.0	0.0382	17.4	34.7	1.09×10^8	0.0190	0.00246	9.71×10^{-7}
2.63	260	0.0790	35.9	224	9.64×10^8	0.0290	0.0125	4.92×10^{-6}
2.63	416	—	—	—	—	0.0370	0.0205	8.08×10^{-6}
2.63	520	0.0700	31.8	489	6.71×10^8	0.0420	0.0255	1.01×10^{-5}
5.27	52.4	0.0682	31.0	22.0	3.87×10^7	0.0180	0.00146	1.15×10^{-6}
5.27	106	0.0985	44.8	61.3	1.17×10^8	0.0190	0.00261	2.06×10^{-6}
5.27	265	1.276	58.0	207	2.54×10^8	0.0230	0.00646	5.10×10^{-6}

mine concentration added, $[L]_T$, and the amount left free in solution at equilibrium, $[L]_{free} = [L]_T - [Q]_{eq}$. Results were obtained for three values of ferritin concentrations, [fer]. Examination of these shows (see Fig. 2) that they can be expressed in the form given in eqn. (1).

$$K_{exp} = \frac{[Q]_{eq}^3}{[L]_{free}[fer]^4} \quad (1)$$

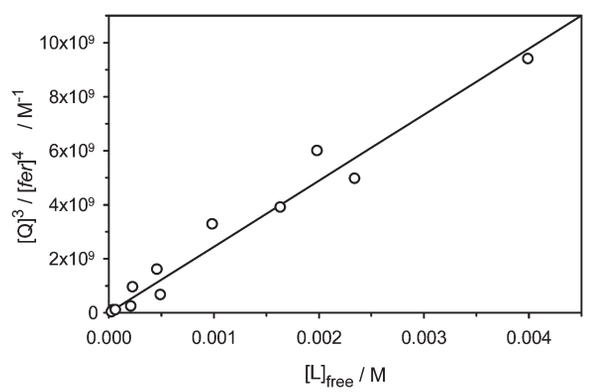


Fig. 2 Plot confirming that the quinone produced at equilibrium follows the relationship $K_{exp} = [Q]^3/[L]_{free}[fer]^4$. The regression line has a slope of $K_{exp} = 2.45 \times 10^{12}$ M^{-2}.

That this rather surprising equilibrium relationship *is only explicable if we assume that the quinone is produced in, and remains inside, the ferritin shells* is established as follows. If both the 6-hydroxydopamine entering and the quinone formed therein remain in the ferritin, then their concentrations must be corrected to allow for the fact that all measurements are made assuming the relevant species occupy the bulk solution. These corrections are given in eqns. (2) and (3).

$$[L]_{in} = [L]_{measured} \frac{\text{vol. of bulk soln.}}{\text{vol. enclosed by ferritin}} \quad (2)$$

$$[Q]_{in} = [Q]_{measured} \frac{\text{vol. of bulk soln.}}{\text{vol. enclosed by ferritin}} \quad (3)$$

But the number of moles of ferritin present = [fer] × vol. of bulk solution. Therefore, the total volume enclosed by the ferritin = number of moles of ferritin present × volume of one mole of ferritin cavities = [fer] × vol. of bulk solution × V_{fer}, where V_{fer} is the total volume of the cavities per mole of ferritin and equals 150 M^{-1} using a diameter of 80 Å and correcting for the space occupied by the iron core. The core was assumed to have the approximate formula $5Fe_2O_3 \cdot 9H_2O$ and have a density[27] of 3.5 g cm^{-3} and the samples of ferritin used had an iron content of 520 atoms. Hence for the experimental conditions we get the following expressions [eqns. (4) and (5)].

$$[L]_{in} = \frac{[L]_{in,\,measured}}{[fer]V_{fer}} \quad (4)$$

$$[Q]_{in} = \frac{[Q]_{in,\,measured}}{[fer]V_{fer}} \quad (5)$$

The equilibria involved (at constant pH) are given by eqns. (6) and (7). Note that in eqn. (7) K_Q is an undefined function of $[H^+]$, which is allowable because all measurements have been made at constant pH. Furthermore, because the iron(III) is a constituent of a solid, its activity can be assumed equal to 1 and because the stoichiometry requires 2[Fe(II)] = [Q] the expression for K_Q becomes as in eqn. (8). Making use of eqns. (4) and (6) then yields eqn. (9).

$$[L]_{out} \overset{K_L}{\rightleftharpoons} [L]_{in} \qquad K_L = \frac{[L]_{in}}{[L]_{out}} = \frac{[L]_{in}}{[L]_{free}} \quad (6)$$

$$[L]_{in} + 2Fe(III)_{in\,(s)} \overset{K_Q}{\rightleftharpoons} 2Fe(II)_{in} + [Q]_{in}$$

$$K_Q = \frac{[Fe(II)]_{in}^2[Q]_{in}}{[L]_{in}[Fe(III)]_{in\,(s)}^2} \quad (7)$$

$$K_Q = \frac{[Q]_{in}^3}{4[L]_{in}} \quad (8)$$

$$K_Q = \frac{[Q]_{in}^3}{4[L]_{free}V_{fer}[fer]} \quad (9)$$

Finally, by use of eqn. (5) and taking $4V_{fer}^4$ into the new constant we get eqn. (1) which is the required result.

Initial rates

The initial 'burst' was found to obey similar kinetics, albeit with a different initial rate, to that found with aqueous iron(III).[11] The observed first-order initial rate constants were therefore measured for three concentrations of ferritin and several concentrations of 6-hydroxydopamine (Table 1). In Fig. 3, k^{obs} is plotted against $[L]_T$ for low concentrations of 6-hydroxydopamine and the two higher concentrations of ferritin showing that k^{obs} depends inversely upon the concentration of ferritin and appears virtually linear with respect to $[L]_T$ with an intercept $k_2 = 0.0165$ s^{-1}. (At higher concentrations of 6-hydroxydopamine the plot becomes sigmoidal – see Fig. 4.) The constant intercept implies that the rate equation can be expressed by eqn. (10) and when the 6-hydroxydopamine and ferritin dependencies are taken into account k_1 is given by eqn. (11) in which A is a constant.

$$\frac{d[Q]}{dt} = k^{obs}[Q] = k_1[Q] + k_2 \quad (10)$$

$$k_1 = \frac{A[L]_T}{[fer]} \quad (11)$$

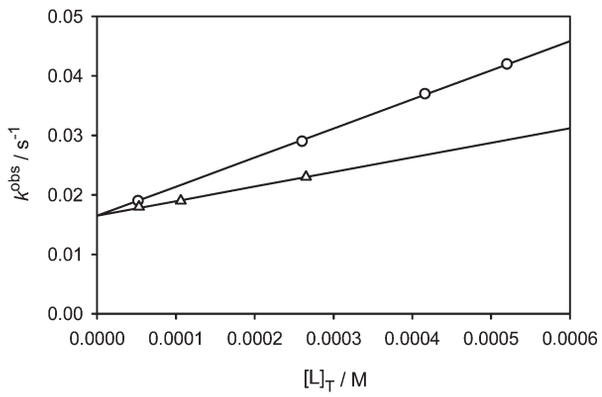
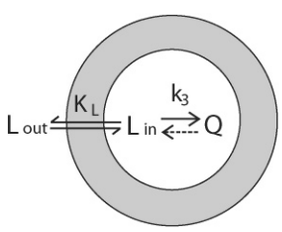


Fig. 3 Relationship between k^{obs} and $[L]_T$ at low $[L]_T$. ○ [fer] = 2.63 μM, slope = 48.9 M^{-1} s^{-1}; △ [fer] = 5.27 μM, slope = 24.5 M^{-1} s^{-1}. Both intercepts = k_2 = 0.0165 s^{-1}.

Because of its invariance we interpret k_2 (= 0.0165 s^{-1}) as representing the rate at which iron(III) is migrating to the surface of the ferritin where it is reduced by the 6-hydroxydopamine.

Consider one channel in a single ferritin. We assume that, for the initial stage of the reaction, the establishment of equilibrium (6) is rapid and is followed by reduction of the iron by the 6-hydroxydopamine within the protein shell (Scheme 1). We also assume that the initial rates are measuring the rate of the forward reaction. Since k_2 in eqn. (10) can be disregarded with respect to the redox reaction within the ferritin, the relevant relationship is given by eqn. (12).



Scheme 1 6-Hydroxydopamine diffuses reversibly into the ferritin and then reacts with the iron(III) core.

$$\frac{d[Q]}{dt} = k_1[Q] \quad (12)$$

Correcting for the fact that bulk measurements have been made leads to eqn. (13).

$$\frac{d[Q]}{dt} = \frac{k_3[Q]}{V_{fer}[fer]} \quad (13)$$

When a 6-hydroxydopamine molecule enters a channel we can refer to any ferritin involved as ferL and thus express equilibrium (6) in terms of 'complex formation' (14):

$$fer + L \underset{}{\overset{K_1}{\rightleftharpoons}} ferL \qquad K_1 = \frac{[ferL]}{[fer][L]} \quad (14)$$

The average number, $\bar{n}$, of L bound to fer is given by eqn. (15), assuming that L is in large excess.

$$\bar{n} = \frac{K_1[L]_T}{1 + K_1[L]_T} \quad (15)$$

Thus, because only the 'complex' ferL is involved in the reaction the rate equation is given by (16) and k_1 by eqn. (17). But since $K_1[L]_T$ is very small compared with 1, eqn. (17) is in agreement with eqn. (11) where $A = k_3K_1/V_{fer}$.

$$\frac{d[Q]}{dt} = \frac{\bar{n}k_3[Q]}{V_{fer}[fer]} \quad (16)$$

$$k_1 = \frac{k_3K_1[L]_T}{V_{fer}[fer](1 + K_1[L]_T)} \quad (17)$$

The sigmoidal dependence of $V_{fer}[fer]k_1$ on $[L]_T$ illustrated in Fig. 4 can be explained by extending the theory above to include ferritins employing more than one channel, *i.e.* we have a series of 'complexes' $ferL_n$, the distribution of which we can describe by means of equilibrium constants β_n given in eqn. (18).

$$fer + L \underset{}{\overset{\beta_1}{\rightleftharpoons}} ferL \qquad \beta_1 = K_1 = \frac{[ferL]}{[fer][L]}$$

$$fer + 2L \underset{}{\overset{\beta_2}{\rightleftharpoons}} ferL_2 \qquad \beta_2 = K_1K_2 = \frac{[ferL_2]}{[fer][L]^2}$$

.....

$$fer + 8L \underset{}{\overset{\beta_8}{\rightleftharpoons}} ferL_8 \qquad \beta_8 = K_1 \ldots K_8 = \frac{[ferL_8]}{[fer][L]^8} \quad (18)$$

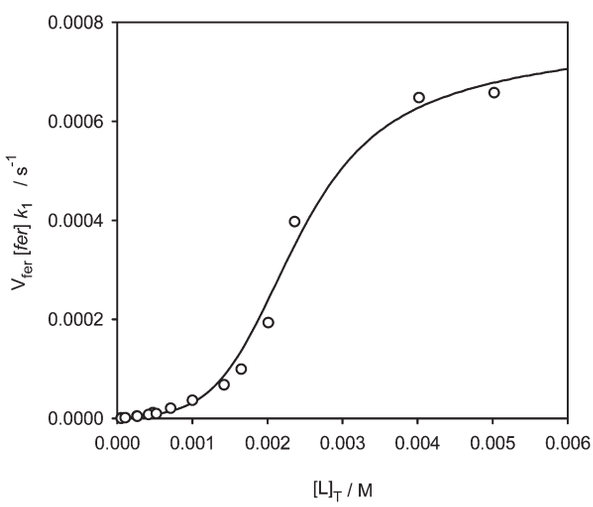


Fig. 4 Sigmoidal dependence of $V_{fer}[fer]k_1$ on $[L]_T$. The theoretical curve was calculated using eqn. (19), $k_3 = 1.22 \times 10^{-4}$ s^{-1}, $K = 11$ (see text), and $V_{fer} = 150$ M^{-1} for the ferritin used in this study.

Hence, $\bar{n}$ in eqn. (15) must be expanded to take into account more than one channel being used per ferritin. Furthermore, the steepness of the curve mapped by the experimental points in Fig. 4 demands that all eight C_3 channels into the ferritin are involved in this 'complex' formation and therefore eqn. (17) can be expanded to give eqn. (19).

$$V_{fer}[fer]k_1 = \frac{k_3\left(\beta_1[L] + 2\beta_2[L]^2 + \cdots + 8\beta_8[L]^8\right)}{\left(1 + \beta_1[L] + \beta_2[L]^2 + \cdots + \beta_8[L]^8\right)} \quad (19)$$

L is in very large excess enabling [L] to be replaced by $[L]_T$ in eqn. (19). Therefore, if K is defined as applying to the existence of a ferritin employing one channel, then the statistical distribution of identical species must be allowed for. Thus $K_1 = 8K$; $K_2 = 28K$; $K_3 = 56K$; $K_4 = 70K$; $K_5 = 56K$; $K_6 = 28K$; $K_7 = 8K$; $K_8 = K$, enabling the curve to be fitted using only two independent variables, namely k_3 and K. The best fit to the data is $k_3 = 1.22 \times 10^{-4}$ s^{-1} and $K = 11$. (The theoretical curve calculated on the basis of these values is included in Fig. 4.)

Discussion

From the above results it is seen that 6-hydroxydopamine enters the ferritin. This is accepted because the equilibrium data are only explicable if the concentration of the quinone is corrected to allow for it being exclusively within the ferritin shell and, furthermore, the interpretation of the kinetic data makes the same demand.

Chasteen and co-workers[5,6] have measured, in a rather elegant manner, the rate of entry and exit of several spin probes in and out of ferritin. Positively charged and neutral species are favoured, and a rate of entry into the ferritin can be obtained as approximately 3×10^{-4} s^{-1} (dependent on the spin probe). It was further shown that K_L varied from 0.73 to 1.25 according to the spin probe employed.

It is certain, on the other hand, that ferrozine does *not* enter because (i) it is very large (although it must be recalled that porphyrin is known to be able to diffuse into ferritin[28,29]), and (ii) it is carrying two ionized sulfonic acid groups. At physiological pHs it carries an overall charge of −1 due to protonation at one of the nitrogens[30] and hence the tris-iron(II) complex is negatively charged. In contrast, 6-hydroxydopamine is a mixture of positively charged and neutral species at physiological pHs.

It could, of course, be that over time the ferrozine does diffuse in, but this would be at a significantly slower rate. It seems certain, therefore, that over the experimental time scale described here, the 6-hydroxydopamine is entering the protein and reducing the iron whilst the ferrozine picks up the iron(II) as it slowly diffuses out.

The present work using 6-hydroxydopamine (a well-known neurotoxin, thought to have its toxicity through its well-documented ability to reduce the iron in ferritin) has shown that without the presence of a strong chelator, iron is not released. Thus, the idea that 6-hydroxydopamine could be a prime suspect in the production of iron overload[31] does not seem to be realistic, unless the removal of the iron(II) by another species can be substantiated under these conditions.

Simple electrochemical thermodynamics show that the presence of a chelator changes the redox potential of redox couples (for example, the redox potential of the iron(III)/iron(II) couple[32] can be changed from −0.30 V to +1.12 V by varying the complexing agent). The presence of strong chelators of iron(II) will, therefore, increase the potential-reducing abilities of any reductant. This can easily lead to the conclusion that (in the presence of ferrozine or analogues) substances lead to an iron release, which would under actual physiological conditions (*i.e.* in the absence of such extremely strong chelators) never be able to free iron. In fact, the presence of strong chelators such as transferrin may be used by nature to facilitate the release of iron from ferritin, but this has not been established. The claim that the reduction of the iron within ferritin can also be carried out by dopamine,[33] a neuro-transmitter, we find very suspect because, at physiological pHs, the bis- and tris-complexes predominate, and they are remarkably stable towards internal electron exchange.

From the present work, therefore, it appears that the 'release' of iron from ferritin relies on the interplay between the reducing and complexing abilities of two independent species. Thus, one can follow the rate of reduction by the reductant (in this work 6-hydroxydopamine) and the rate of 'release' of iron(III) by means of an independent complexing agent but these are not independent processes. In other words neither an efficient reducing agent nor a strong chelator alone are likely to throw much light on the biological processes involved.

Since the oxidation potential[34] of ferritin is −0.190 V and the two-electron oxidation potential of 6-hydroxydopamine is 0.371 V (measured[19] at pH = 7.2) the extent of reduction of iron(III) cannot be very large. In fact, the amount of iron reduced in this series of experiments never exceeded 8% of the core iron present. In other words, 6-hydroxydopamine is a poor reductant when within the ferritin shell but an external complexing agent such as ferrozine will increase the extent of oxidation by removing a product of the reaction, namely iron(II).

Because of the importance of the studies of iron-related oxidative stress in connection with aging processes, and with diseases such as Parkinsonionism, these findings are of great importance and should stimulate further work on the study of reactions taking place within the ferritin shell. However, it must be emphasized that these are *in vitro* studies and cannot in any way be interpreted as implying that *in vivo* reduction makes simultaneous use of more than one channel in the ferritin. Furthermore, the data obtained in the present study involved concentrations of reductant (6-hydroxydopamine) of up to 10^3 times the concentration of ferritin! It does, however, illustrate the remarkable stability of the ferritin moiety.

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