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The Release of Iron from Horse Spleen Ferritin by Reduced Flavins

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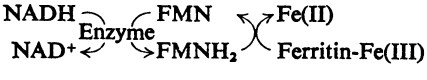
Ferritin-Fe(III) was rapidly and quantitatively reduced and liberated as Fe(II) by $FMNH_2$, $FADH_2$ and reduced riboflavin. Dithionite also released Fe(II) from ferritin but at less than 1% of the rate with $FMNH_2$. Cysteine, glutathione and ascorbate gave a similar slower rate and yielded less than 20% of the total iron from ferritin within a few hours. The reduction of ferritin-Fe(III) by the three riboflavin compounds gave complex second-order kinetics with overlapping fast and slow reactions. The fast reaction appeared to be non-specific and may be due to a reduction of Fe(III) of a lower degree of polymerization, equilibrated with ferritin iron. The amount of this Fe^{3+} ion initially reduced was small, less than 0.3% of the total iron. Addition of FMN to the ferritin–dithionite system enhanced the reduction; this is due to the reduction of FMN by dithionite to form $FMNH_2$ which then reduces ferritin-Fe(III). A comparison of the thermodynamic parameters of $FMNH_2$–ferritin and dithionite–ferritin complex formation showed that $FMNH_2$ required a lower activation energy and a negative entropy change, whereas dithionite required 50% more activation energy and showed a positive entropy change in ferritin reduction. The effectiveness of $FMNH_2$ in ferritin–Fe(III) reduction may be due to a specific binding of the riboflavin moiety to the protein portion of the ferritin molecule.

Reductive mechanisms have dominated thinking and experimentation on the release of iron from ferritin (Frieden & Osaki, 1974). Michaelis *et al.* (1943) reported that iron was liberated from ferritin *in vitro* by the reducing action of sodium dithionite. Later, Tanaka (1950) reported that milk xanthine oxidase and a diaphorase preparation from pig heart muscle were active in the release of iron from ferritin. He suggested that the key enzyme was either a pyridino- or a flavo-protein. A more detailed mechanism for the release of iron from ferritin was proposed by Mazur and co-workers (Green & Mazur, 1957; Mazur *et al.*, 1958). Correlations of xanthine oxidase activity and iron release in normal and developmental systems were presented (Mazur & Carleton, 1965). It was suggested that ferritin could accept electrons from reduced xanthine oxidase and thereby release its iron as Fe^{2+} ion.

However, Kozma *et al.* (1967) reported that liver xanthine oxidase activity could be substantially decreased by allopurinol (a powerful inhibitor of xanthine oxidase) without noticeably affecting iron metabolism. Grace *et al.* (1970) also showed that the suppression of xanthine oxidase activity by allopurinol did not influence the mobilization of hepatic iron. Osaki & Sirivech (1971) also found that in liver homogenates, xanthine oxidase substrates did not produce significant release of iron from ferritin; neither did milk xanthine oxidase, nor chicken liver xanthine dehydrogenase. However, we remained convinced from observations on the mobilization of iron from perfused livers (Osaki *et al.*, 1971), that a reductive mechanism must be available for the release of ferritin iron.

Osaki & Sirivech (1971) demonstrated an enzyme system which can reduce ferritin-Fe(III) in homogenates from the livers of various species of vertebrates. This system catalyses the complete reduction of ferritin-bound Fe(III) to free Fe(II) in the presence of NADH and FMN, FAD or riboflavin, at O_2 concentrations of less than 3 μM. It was inferred that reduced flavin reacted directly with ferritin:



This direct reduction of ferritin can be studied independently as a separate reaction. A comparison of the reaction of horse spleen ferritin with riboflavin derivatives and typical tissue reducing agents has revealed that only the riboflavins can reduce ferritin-Fe(III) at a rate and to an extent that is likely to be significant physiologically.

Materials and Methods

Materials

Ferritin (equine spleen; twice crystallized) was obtained from Calbiochem, San Diego, Calif., U.S.A. Trizma base [tris(hydroxymethyl)aminomethane], Trizma–HCl, flavin mononucleotide, flavin adenine dinucleotide, cysteine hydrochloride and glucose

oxidase were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. D-Glucose, $\alpha\alpha$-bipyridyl and platinum asbestos were from Fisher Scientific Co., Fair Lawn, N.J., U.S.A., sodium dithionite from Matheson, Coleman and Bell, Rutherford, N.J., U.S.A., GSH was from Mann Research Laboratories Inc., New York, N.Y., U.S.A.; ascorbic acid, U.S.P., was from Merck and Co., Rahway, N.J., U.S.A.

Preparation of reduced flavins

$FMNH_2$, $FADH_2$ and reduced riboflavin were prepared by adding a small amount of platinum asbestos to each reaction mixture (10.0ml) which contained 50μM of either FAD, FMN or riboflavin in 25mM Tris–HCl buffer, pH7.4. The reaction mixture was flushed with H_2 gas for 10–15min until the yellow colour of the flavin had disappeared. The platinum asbestos was removed anaerobically from reduced flavin solution by filtration. The reduced flavins were used immediately.

Assay

Slow reactions were measured in the usual way in a Cary 15 spectrophotometer. Rapid reactions of ferritin with reduced flavins were studied in a Durrum stopped-flow spectrophotometer (model D-109). The reaction mixture was first prepared in two separate modified Thunberg tubes; tube A contained 25.0mM-Tris–HCl buffer, pH7.4, 0.20% ferritin, 1.0mM-D-glucose, 100μl of glucose oxidase (1300 units/ml), 1.28mM-$\alpha\alpha$-bipyridyl in a total reaction mixture of volume 10.0ml. The addition of glucose and glucose oxidase were intended to remove contamination by O_2, and any H_2O_2 produced would be eliminated by catalase (the glucose oxidase obtained from Sigma Chemical Co. contains catalase impurity, approx. 0.5% by weight). The glucose–glucose oxidase system does not interfere in the reduction of iron from ferritin by any reducing agents. The tube was evacuated and flushed with N_2 gas several times. Tube B contained the anaerobic solution of reducing agents such as $FMNH_2$ (50μM), $FADH_2$ (50μM), reduced riboflavin (50μM) and sodium dithionite (2.0mM) in 25mM-Tris–HCl buffer, pH7.4. The reaction mixtures in tubes A and B were transferred to the reservoir syringes of the stopped-flow apparatus in an anaerobic chamber constantly flushed with N_2 gas. The syringes were anaerobically returned to the spectrophotometer. The reaction of ferritin with reducing agents was measured by following the colour formation of the complex, Fe(II)–$\alpha\alpha$-bipyridyl, with a maximum absorption at 530nm. All experiments were carried out at 15°C unless otherwise indicated. The rate constant (k) for the second-order reaction can be obtained from the slope of the plot log (AB_0/BA_0) versus time (Fig. 1). The reaction of Fe(II) with $\alpha\alpha$-bipyridyl was fast enough ($k = 1.56\times10^5 M^{-1}\cdot s^{-1}$ at 15°C) so that it was not the rate-determining step of any reaction studied.

Results and Discussion

Effectiveness of various reducing agents on the reduction of ferritin

Ferritin-Fe(III)$_n$ can be reduced by several reducing agents such as $FMNH_2$, reduced riboflavin, $FADH_2$, ascorbate, reduced glutathione, cysteine and sodium dithionite, etc. Table 1 shows the rate of ferritin reduction by $FMNH_2$, $FADH_2$, reduced riboflavin and sodium dithionite in a Durrum stopped-flow apparatus. The results from Table 1 show that $FMNH_2$ and reduced riboflavin had almost the same rate constants for ferritin reduction, but $FADH_2$ was somewhat slower. Sodium dithionite, glutathione, cysteine and ascorbic acid exhibited a low rate of ferritin reduction. The slow rate of dithionite reduction of ferritin was not caused by interference with the reaction of Fe(II) and $\alpha\alpha$-bipyridyl because of the presence of 100μM-Fe(II) in the reaction mixture (80mM-Tris–HCl buffer, pH7.4) instantaneously completes the reaction with $\alpha\alpha$-bipyridyl with or without 2.85mM-sodium dithionite.

The reduction of ferritin by sodium dithionite, $FADH_2$, $FMNH_2$, and reduced riboflavin exhibited a non-linearity in a typical second-order reaction plot (Fig. 1). The first portion of the graph in all cases was non-linear, probably because of a reduction of loosely bound Fe(III) equilibrated with the tightly bound iron of the ferritin molecule. The amount of this loosely bound Fe(III) was 0.05–0.3% of the total iron and was considered negligible compared with the total reduction of ferritin iron. The values in Table 1 are computed from the slower linear portions of the plots in Fig. 1.

Table 1. *Rates and rate constants for the reduction of ferritin by various reducing agents*

The values for reduced flavins and sodium dithionite were obtained at 15°C by using a stopped-flow apparatus. The results for ascorbate, cysteine and glutathione were obtained at 30°C in a Cary 15 spectrophotometer. The ferritin concentration was 4.74mM as Fe(III) and the pH was 7.4 in 25mM-Tris buffer.

Compounds	Concentration (μM)	$10^3\times$Rate ($\Delta E_{530}s^{-1}$)	k ($M^{-1}s^{-1}$)
$FMNH_2$	50	7.1	10.8
$FADH_2$	50	2.5	3.1
Reduced riboflavin	50	7.6	13.0
Dithionite	2000	2.4	0.052
Ascorbate	100	0.16	—
Cysteine	100	0.24	—
Glutathione	100	0.23	—

The fraction (41 nmol) of the total iron in ferritin reduced by four reductants in various times is shown in Table 2. In these experiments, the amount of Fe(II) released was followed by the $\alpha\alpha$-bipyridyl reaction until no further release could be detected for 60min. At one-half the concentration, $FMNH_2$ reduced all 43μmol of total iron in 10min. Sodium dithionite required 4h for complete reduction; ascorbate and glutathione reduced less than 20% of the total iron in 4 and 6h respectively.

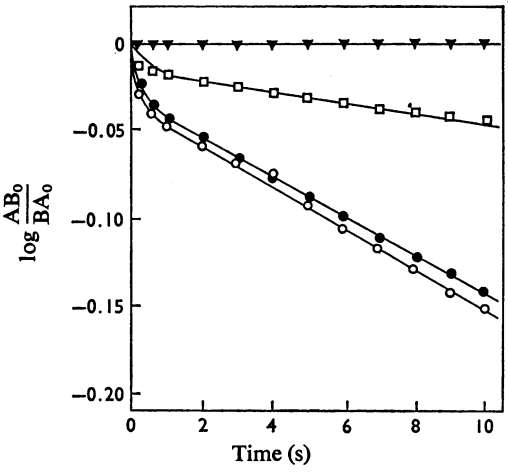


Fig. 1. *Reduction of ferritin-Fe(III)$_n$ by reduced riboflavin, $FMNH_2$, $FADH_2$ and sodium dithionite*

The concentration of ferritin-Fe(III)$_n$ in the reaction mixture was 4.74mM as Fe(III) and the concentrations of reduced riboflavin (○), $FMNH_2$ (●), $FADH_2$ (□) and sodium dithionite (▼) were 50, 50, 50 and 2000μM respectively. A, A_0, B, B_0, represent the concentration of ferritin as Fe(III) in mM at time t, the initial concentration of ferritin as Fe(III), the concentration of reducing agent at time t and the initial concentration of reducing agent respectively. The experiments were carried out at 15°C.

Effect of FMN on the sodium dithionite–ferritin system

FMN added to the sodium dithionite–ferritin system affected the rate of ferritin reduction. The addition of increasing amounts of FMN to a constant amount of sodium dithionite (2.0mM) mixed with ferritin [4.74mM as Fe(III)] in 25.0mM-Tris–HCl buffer, pH7.4, increased the rate of reduction (Table 3). The reactions were carried out in 100mM-Tris–HCl buffer, pH7.4, at 15°C, and the concentrations of FMN added to the sodium dithionite–ferritin system were 0, 10, 50 and 100μM respectively. It is well known that dithionite reduces FMN rapidly. The k values listed in the table were calculated with respect to dithionite concentration. Because it was not known how much FMN was reduced to $FMNH_2$ under steady-state conditions, it was impossible to obtain the k value with respect to $FMNH_2$. However, if the FMN is assumed to be fully reduced at its lowest concentration (10μM) the estimated k value with respect to FMN would be

$$\frac{0.064 \times 2000\ (\text{dithionite})}{10\ (\text{FMN})} = 12.8\,\text{M}^{-1}\cdot\text{s}^{-1}$$

which is in good agreement with the value shown in Table 1. This also indicates that 2mM-dithionite did not interfere with the reaction of Fe(II) with $\alpha\alpha$-

Table 3. *Effect of FMN addition on the rate constants of ferritin reduction by sodium dithionite*

The second-order rate constants (k) were obtained at 15°C, pH7.4, in a stopped-flow apparatus. The k values were calculated with respect to sodium dithionite (2mM). The concentration of ferritin was 4.74mM as Fe(III).

Concentration of FMN added (μM)	k ($M^{-1}\cdot s^{-1}$)
0	0.032
10	0.064
50	0.122
100	0.190

Table 2. *Extent of the anaerobic reduction of iron in ferritin by reducing agents at pH 7.4*

Measurements of change in absorbance owing to Fe(II)–$\alpha\alpha$-bipyridyl complex ($\varepsilon^{530nm}_{1mM} = 8.65$) in an anaerobic reaction mixture were done at 30°C by using a Cary 15 spectrophotometer. Ferritin (41 nmol) as Fe(III) was used. The second column lists the times necessary to reach the constant absorbance reported in the third column. The absorbance was then constant for at least 1h.

Reductant (μM)	Time required to reach constant absorbance (h)	ΔE_{530}	Reduced Fe equivalent (nmol)	% reduction
$FMNH_2$ (200)	0.17	0.38	43	104
Sodium dithionite (400)	4.0	0.35	40	98
Ascorbic acid (400)	4.0	0.064	7.4	18
Glutathione (400)	6.0	0.06	6.9	17

bipyridyl. The enhancement of ferritin reduction with dithionite by addition of FMN could be explained by assuming two consecutive reactions:

$$2H_2O + Na_2S_2O_4 + FMN \rightarrow FMNH_2 + 2NaHSO_3$$

$$FMNH_2 + \text{Ferritin-Fe(III)}_2 \rightarrow 2Fe(II) + FMN + 2H^+ + \text{Ferritin}$$

With no addition of FMN, the rate of reduction was small. The results together with the data in Tables 1 and 2 suggest that $FMNH_2$ is more specific and effective in ferritin reduction.

Thermodynamic parameters

The thermodynamic parameters for the reactions of ferritin and $FMNH_2$, and ferritin and sodium dithionite were obtained from the measurements of the second-order rate constants at various temperatures. An Arrhenius plot of the data is shown in Fig. 2. By assuming that ferritin reacts with reducing agents to form an activated complex which then gives the products according to the theory of absolute reaction rates, the values of ΔH^*, ΔF^* and ΔS^* for $FMNH_2$–ferritin and sodium dithionite–ferritin complex formation can be obtained from the slope in Fig. 2 and rate constants obtained previously. The calculated

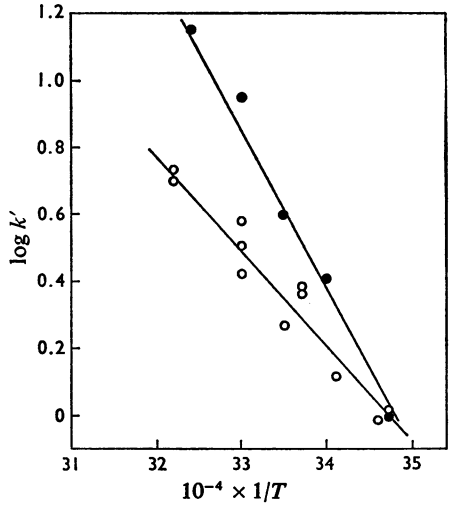


Fig. 2. *Arrhenius plot of the reaction of ferritin and $FMNH_2$, and ferritin and sodium dithionite at various temperatures between 15° and 35°C*

○, 50μM-$FMNH_2$ in 25.0mM-Tris–HCl buffer, pH7.4; ●, 2.0mM-sodium dithionite in the same buffer. The concentration of ferritin was kept constant at 6.65mM as Fe(III) in both reductions by $FMNH_2$ and sodium dithionite. The k value for the reaction at 15°C was assumed to be 1.0 to compute relative k values (k') for other temperatures.

Table 4. *A comparison of the thermodynamic parameters for reactions of ferritin with $FMNH_2$ and sodium dithionite*

The assay was performed as described in the Materials and Methods section. The values for ΔH^*, ΔF^* and ΔS^* were calculated at 30°C.

	ΔH^* (kcal/mol)	ΔF^* (kcal/mol)	ΔS^* (cal/mol per °C)
$FMNH_2$	13.0	16.0	−9.9
Sodium dithionite	23.0	18.7	14.1

results are shown in Table 4. The ΔH^* value for the $FMNH_2$–ferritin complex formation was smaller than the ΔH^* for the sodium dithionite–ferritin complex formation. This suggests that ferritin reacts with $FMNH_2$ more readily than with sodium dithionite. The entropy (ΔS^*) change was negative for the formation of an activated $FMNH_2$–ferritin complex and positive for sodium dithionite–ferritin complex formation, suggesting that the reaction of ferritin and $FMNH_2$ probably formed a rather specific complex, whereas the reaction between ferritin and sodium dithionite formed a more random complex. The free energy change (ΔF^*) for the two reaction systems was found to be approximately the same. The formation of a possible specific complex between $FMNH_2$ and ferritin will be discussed below.

Relative rates of the reduction of ferritin-Fe(III)

Michaelis *et al.* (1943) reported a reduction of iron from ferritin *in vitro* by the action of sodium dithionite. Mazur *et al.* (1955) showed that naturally occurring reducing agents such as glutathione, ascorbic acid, or cysteine liberate Fe^{2+} ion when incubated with ferritin at pH7.4 and at 37.5°C for 30min. Cysteine was most effective in releasing Fe(II) from ferritin. Our data also show that cysteine, glutathione and ascorbate released Fe(II) from ferritin at a slow rate, but we found that $FMNH_2$ and reduced riboflavin were much more effective in both the rate of ferritin reduction and the extent of Fe(II) released. $FADH_2$ and a 40 times higher concentration of dithionite reduced ferritin iron at a moderate rate, i.e. about 10 times as fast as cysteine.

Mazur and his co-workers also studied the release of iron from tissue ferritin in rat liver slices *in vitro* (Green & Mazur, 1957) and in rabbits, guinea pigs and dogs *in vivo* (Mazur *et al.*, 1958). They postulated that xanthine oxidase was the enzyme system responsible for the release of hepatic iron. From their data, we estimated that the amount of ferritin iron reduced by xanthine oxidase was less than 1% of the total ferritin iron. This reduction of ferritin was probably the reduction of free Fe(III) equilibrated with bound iron or loosely bound Fe(III), which we found to be

0.05–0.3% of the total iron. The reduction and release of Fe(III) from ferritin by a 1,4-naphthaquinone–milk xanthine oxidase system was reported by Duggan & Streeter (1973) and is also at a rate only comparable with cysteine, etc. Moreover, the extent of the reaction was not reported for this non-physiological reductant. We have already cited additional evidence against the involvement of xanthine oxidase in iron release from ferritin. Jones & Johnston (1967) and Hoy *et al.* (1974) attempted to release iron from ferritin by ascorbate or photo-reduction. The rate of the reduction observed was considerably less than the present findings for flavins.

Mechanism of ferritin iron reduction

Niederer (1970) suggested the 'penetration hypothesis' for ferritin in iron incorporation and iron release. He pointed out that the hollow apoferritin sphere is riddled with holes; the nascent Fe^{3+} ions readily form an intramolecular complex and soon the growing FeOOH micelle becomes too large to escape. He demonstrated that catalytic iron oxidation occurred at a specific site(s) located on the inside of the apoferritin molecule. Niederer (1970) also proposed that the reduction of ferritin iron involved the same active site as iron uptake. We can explain the mechanism and the specificity of ferritin reduction by the 'penetration hypothesis' in that a molecule of reducing agent such as $FMNH_2$, $FADH_2$, reduced riboflavin etc. can penetrate into the inside of a ferritin molecule; the reduction would occur at an active site on the inner surface of the protein shell where flavins are bound in a specific way to facilitate the electron transfer. A negative entropy change for activated complex formation supports a specific binding. The ability of various reducing agents to reduce ferritin may depend on both their specificity and the ability of each one to penetrate the ferritin molecule. Dithionite, ascorbate and glutathione can be assumed to be small enough to penetrate ferritin molecules, but because of the specificity of the sites they may be unable to transfer their electrons to the reducing site as efficiently as $FMNH_2$.

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