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*Original paper*

# Two pathways of iron uptake in bovine spleen apoferritin dependent on iron concentration

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**Abstract**

Iron incorporation by bovine spleen apoferritin either with ferrous ammonium sulfate in different buffers or with ferrous ammonium sulfate and phosphate was studied. Iron uptake and iron autoxidation were recorded spectrophotomerically. The buffers [4-(2-hydroxyethyl)-1-piperazinyl]ethanesulphonic acid (Hepes) and tris(hydroxymethyl)aminoethane (Tris) exhibited pH-dependent iron autoxidation, with Tris showing less iron autoxidation than Hepes. An Eadie-Scatchard plot (v/[s] versus v) of the iron uptake rate in Hepes was a curved rather than a straight line, suggesting that there are two iron uptake pathways. On the other hand, the Eadie-Scatchard plots of Tris and of Hepes after the addition of phosphate showed a straight line. Phosphate accelerated the iron uptake rate. The iron loading kinetics of apoferritin in Hepes was dependent on apoferritin concentration. The $K_m$ value obtained from iron uptake kinetics was 4.5 $\mu$M, corresponding to the physiological iron concentration. These results demonstrate that iron loading of apoferritin was accomplished at physiological iron concentrations, which is essential for iron uptake, via two uptake pathways of dependent on iron concentration.

## Introduction

Ferritin is a ubiquitous iron-containing protein having a molecular weight of around 500,000 and it plays a major role in accumulating and detoxifying intracellular iron (Theil 1987; Harrison & Arosio 1996). Mammalian tissue ferritin is composed of 24 subunits, which are made up of two types of subunits termed H (heart), predominant in heart ferritins, and L (liver), predominant in liver and spleen ferritins (Arosio *et al.* 1978; Theil 1987; Worwood 1990; Harrison & Arosio 1996). Naturally-occurring ferritin does not contain more than 3000 Fe atoms/molecule (Harrison & Arosio 1996), although it can accommodate up to 4,500 atoms (Theil 1987).

Iron uptake experiments using recombinant ferritin H and L chains as well as natural isoferritins showed a functional difference exists between the H and L chains (Macara *et al.* 1973; Levi *et al.* 1988, 1989, 1992, 1994; Lawson *et al.* 1991; Sun & Chasteen 1992); the H chain showing an advantage in iron oxidation (Levi *et al.* 1988), and the L chain being involved in ferritin stability (Lawson *et al.* 1991) and iron-core nucleation (Levi *et al.* 1989, 1992, 1994). There are two ways for ferritin to incorporate iron (Sun & Chasteen 1992): the protein-catalyzed (ferroxidation) pathway and the mineral-surface (autoxidation) pathway, with the latter being important for core growth.

Ferritin is found as polynuclear ferric oxyhydroxide bound to some phosphates (Treffry & Harrison 1978; Cheng & Chasteen 1991; de Silva *et al.* 1993), although phosphate is not essential for ferritin core formation (Treffry & Harrison 1978; Cheng & Chasteen 1991). Phosphate accelerates iron deposition due

to the coordination of ferroxidase, which charges the redox potential of the iron(II) to a more negative value at the ferroxidase site (Cheng & Chasteen 1991). Alternatively, it may simply facilitate iron exchange (Cheng & Harrison 1991).

The iron concentrations of the labile iron pool of mammalian cells are in the range of 0.2–1.5 $\mu$M (Epsztejn *et al*. 1997). The $K_m$ values reported previously do not correspond to physiological iron concentrations (Macara *et al*. 1973; Sun & Chasteen 1992). We describe here iron uptake kinetics of bovine spleen apoferritin by iron and phosphate.

## Materials and methods

### *Bovine spleen and preparation of apoferritin*

The bovine spleen ferritin monomer was purified as described (Kakuta *et al*. 1997). Apoferritin was prepared by three dialyses of 500 ml of 100 mM thioglycollic acid in 100 mM sodium acetate (pH 5.5), followed by dialysis in phosphate-buffered saline (20 mM sodium phosphate, 150 mM NaCl, pH 7.0), in 100 mM Hepes (pH 7.0) or in 50 mM Tris (pH 7.0).

The protein concentration was determined by the method of Lowry *et al*. (1951) using bovine serum albumin (BSA) (Boeringer Mannheim, Germany) as the standard. The iron concentration of ferritin samples and the buffer were determined coulometrically as previously described (Kakuta *et al*. 1997).

### *SDS-PAGE*

SDS-PAGE was carried out according to the method of Laemmli (1970), using a 4.5% polyacrylamide stacking gel and a 12% polyacrylamide running gel. The densitometry of subunit bands stained with Coomassie Brilliant Blue R-250 was analyzed at 565 nm using a Flying Spot Scanner (Shimadzu CS9000) (Shimadzu, Kyoto, Japan).

### *Iron uptake*

Iron uptake was assayed by incubating 50 $\mu$g ml$^{-1}$ apoferritin in 100 mM Hepes (pH 7.0) and 50 mM Tris (pH 7.0) buffers after the addition of various concentrations of ferrous ammonium sulfate (Wako Pure Chem., Tokyo). The formation of amber products was determined by measuring absorbance at 310 nm for 3 min at 30 °C. No precipitation of protein took place under these conditions. The stock solution used was 100 mM phosphate buffer ($Na_2HPO_4/KH_2PO_4$, pH 7.0) for phosphate addition to the buffer. The initial rate of iron uptake was determined by the increase in absorbance within the first 30 sec of the reaction. The amounts of iron(III) oxidized were calculated using a molar extinction coefficient of 2517 (Macara *et al*. 1973; Levi *et al*. 1988). The kinetic parameters of iron uptake were determined by a pharmacokinetic analysis program (MULTI) using a non-linear square method (Yamaoka *et al*. 1981).

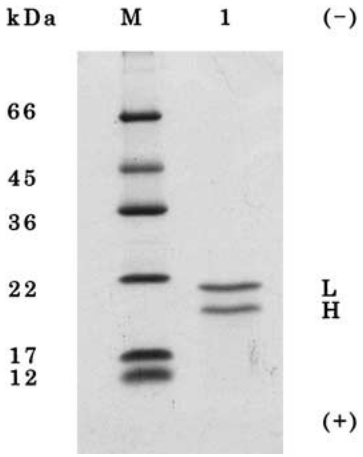


*Fig. 1.* SDS-PAGE of purified bovine spleen ferritin. The sample is bovine spleen ferritin (2 $\mu$g, lane 1). M represents marker proteins (2 $\mu$g each). L and H are liver- and heart-type ferritin subunits, respectively. Anode at bottom.

## Results

### *Preparations of bovine spleen ferritin and bovine spleen apoferritin*

Bovine spleen ferritin was purified and used for determining the kinetics of iron uptake. As shown in Figure 1, bovine spleen ferritin was separated into H and L subunits (H: 18.4 kDa; L: 20.5 kDa). The relative mobilities of the H and L subunits contrasted with those of other mammalian species due to a possible lower affinity of the L chains for SDS compared with those of previously described other animals (Orino *et al.* 1997). The L: H subunit ratio was 1.1: 1 as determined by densitometry. The iron concentration of bovine spleen apoferritin was very low (less than 0.1 ng/$\mu$g of protein), and the iron concentrations of the buffers used were less than 0.05 $\mu$g ml$^{-1}$.

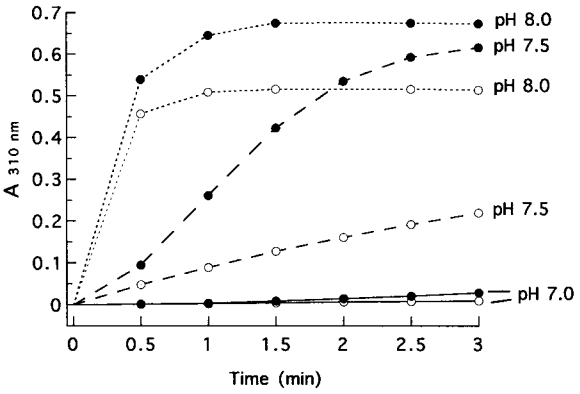


*Fig. 2.* pH-dependence of Fe (II) autoxidation in Hepes and Tris buffers. Conditions were 200 $\mu$M ferrous ammonium sulfate in 100 mM Hepes (●) and 50 mM Tris (○) buffers at pH 7.0, 7.5, and 8.0. Fe (II) autoxidation was monitored spectrophotometrically at 310 nm.

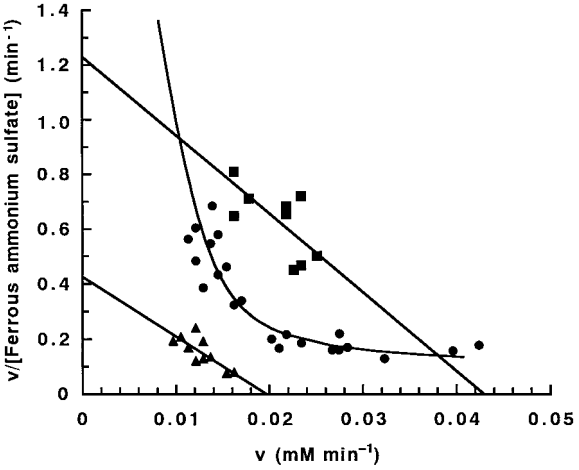


*Fig. 3.* Eadie-Scatchard plots for kinetics of iron uptake by bovine spleen apoferritin in Tris and Hepes buffers and after addition of phosphate to Hepes buffer. Ferrous ammonium sulfate was added to 0.1 $\mu$M apoferritin in Tris (▲) and Hepes (●) buffers and in Hepes added phosphate (0.8 mM) (■), at pH 7.0 and 30 °C. Iron incorporation was monitored spectrophotometrically at 310 nm. The range of ferrous iron concentration tested was 50–200 $\mu$M, 20–250 $\mu$M, and 20-50 $\mu$M in Tris, Hepes, and Hepes+phosphate, respectively.

### *Effect of buffers, pH, and phosphate addition on ferritin iron uptake or iron autoxidation*

Good's buffer is widely used in biological studies, with the initial velocity of iron deposition being higher in Hepes than in Mops and Mes at pH 7.0 (Pâques *et al*. 1980). Tris is known to retard iron autoxidation (Yang & Chasteen 1999). The iron uptake of bovine ferritin was low at a pH less than 7 (data not shown), in accordance with previous data (Pâques *et al*. 1980). In the present study, the iron autoxidation ability of the Hepes and Tris buffers exhibited a pH-dependence at a pH higher than 7 (Figure 2). Tris inhibited iron autoxidation more than Hepes. Although the physiological pH is about 7.4, it was impossible to measure the iron uptake of ferritin at pH 7.5, because not only iron uptake but also iron oxidation in both buffers was markedly increased at a pH higher than 7 (data not shown).

### *Kinetics analyses of ferritin iron uptake*

An Eadie-Scatchard (v/[s] versus v) plot in Hepes showed a curved line, suggesting the presence of two ferroxidase sites on the ferritin molecules (Figure 3). On the other hand, the Eadie-Scatchard plots of Tris and of Hepes after the addition of phosphate showed straight lines. The $K_m$ and $V_{max}$ obtained in Tris were 31 $\mu$M and 0.18 mmol/min/$\mu$mol of protein, respectively, and those obtained in the presence of phosphate were 35 $\mu$M and 0.43 mmol/min/$\mu$mol of protein, respectively. Phosphate thus accelerated ferritin iron uptake. Although bovine ferritin showed two pathways of iron uptake, we hypothesized that ferritin might incorporate automatically-oxidized iron in an iron concentration-dependent manner. Thus, ferritin-dependent iron uptake was examined. The iron uptake rate of ferritin was found to depend on ferritin concentration (Figure 4A). MULTI analysis of the Eadie-Scatchard plots showed two pathways of iron uptake of ferritin (Figure 4B, Table 1), indicating that the kinetic parameters depend on ferritin concentration. In additon, a lower Km was shown to correspond to physiological iron concentration. These results suggest that ferritin is unlikely to incorporate automatically-oxidized iron via two pathways of iron uptake, and that ferritin iron uptake is accomplished at physiological iron concentration.

## Discussion

Ferritin contains phosphate in the iron core, although phosphate exists mainly on the surface of ferritin (Treffry & Harrison 1978). Fe/Pi of normal mammalian ferritins varies from 1 to 8.4, and the absolute amount of phosphate decreased as the amount of iron in the core increased (de Silva *et al*. 1993). The iron uptake rate appears to coordinate Pi at the ferroxidase sites, shifting the redox potential of $Fe^{2+}$ to a more negative value (Cheng & Chasteen 1991). On the other hand, we found that phosphate increased iron autoxidation

*Table 1.* Kinetic parameters of ferritin iron uptake.

| Apoferritin concentration | $K_{m1}$ ($\mu$M) | $V_{max1}$ (mM $min^{-1}$) | $V_{max2}/K_{m2}$ ($min^{-1}$) |
|---|---|---|---|
| 50 $\mu$g $ml^{-1}$ | $4.5 \pm 2.5$ (56%) | $0.0126 \pm 0.0012$ (10%) | $0.093 \pm 0.0073$ (8%) |
| 75 $\mu$g $ml^{-1}$ | $4.5 \pm 2.5$ (56%) | $0.0203 \pm 0.0020$ (10%) | $0.015 \pm 0.0165$ (11%) |

Data are expressed as means $\pm$ SD ($n = 3$) and % shows CV.

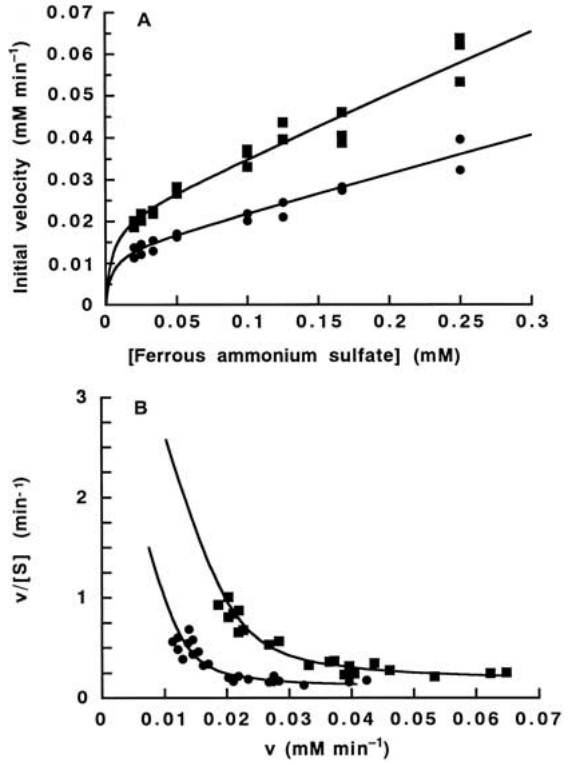


*Fig. 4.* Initial velocity of iron uptake (A) and Eadie-Scatchard (B) plots as a function of apoferritin concentration. Ferrous ammonium sulfate (20–250 $\mu$M) was added to 0.1 $\mu$M (●) or 0.15 $\mu$M (■) apoferritin in Hepes buffer, at pH 7.0 and 30 °C.

(data not shown). Therefore, the prompt iron uptake of ferritin by phosphate might be due to progressive core growth by phosphate rather than to the apparent increase in ferroxidase activity. The significant increase in iron autoxidation is problematic for the measurement of iron uptake using a spectrophotometer. Initial iron uptake by ferritin at pH 7.5 was increased by as much as 3-fold compared to uptake at pH 7.0 (data not shown). At physiological pH 7.4, iron uptake was expected to be initiated to a greater degree than at pH 7.0.

The iron uptake rate was found to depend on ferritin concentration (Figure 4A). In addition, MULTI analysis showed two pathways of iron uptake of ferritin in Hepes, and the kinetic parameters were dependent on apoferritin concentration (Table 1). These results suggest that ferritin is unlikely to incorporate automatically-oxidized iron that is dependent solely on iron concentration. Ferritin seemingly has two ferroxidase sites. Seven amino acid residues associated with a ferroxidase activity are found in human H chain (Andrews *et al.* 1992). Bovine H chain also showed residues identical to the ferroxidase active site (Orino *et al.* 1997). Whether alternate ferroxidase sites in the H or L chains exists remains to be elucidated. There is growing evidence that ferritin has two pathways for iron incorporation, the protein-catalyzed (ferroxidase) and the mineral surface (autoxidation) pathways (Sun & Chasteen 1992, Yang *et al.* 1998; Yang & Chasteen 1999). The L chain coordinates iron uptake with iron core formation induction in the presence of the H chain (Levi *et al.* 1989, 1992, 1994). Whether the growth of the mineral core accelerates iron autoxidation because of catalytic activity on the internal surface of the L remains to be confirmed by further studies. Although the intracellular labile iron pool ($Fe^{2+}$) of mammalian cells is very low (0.2–1.5 $\mu$M) (Epstzejn *et al.* 1997), the $K_m$ value reported here did not correspond with those reported previously (Macara *et al.* 1973, $K_m$ = 0.38 mM; Sun & Chasteen 1992, $K_m$ = 0.35 mM). MULTI analysis identified a lower $K_m$ at 4.5 $\mu$M, which corresponded to physiological iron concentration. Once the iron core was formed, the incorporation of iron into ferritin may have been promoted by activity of the catalytic surface on the growing mineral core rather than by ferroxidase activity. The $K_m$ in Tris was about 10 times higher than that in Hepes, suggesting that Tris has higher affinity for $Fe^{2+}$ than Hepes, because Tris inhibited iron autoxidation (Figure 1). This result indicates that Tris can compete with ferritin as an inhibitor of iron uptake. Iron uptake by ferritin ferroxidase activity was observed from the relatively low concentration of $Fe^{2+}$ (100 $\mu$M) without overriding the iron autoxidation reaction (Yang & Chasteen 1999). However, we believe that the physiological $K_m$ is possibly be masked by the catalytic activity of the mineral core at a higher $Fe^{2+}$ concentration.

Ferritin induced by oxidative stress showed a cytoprotective effect against such stress, suggesting that ferritin functions as one of the cytoprotectants against oxidative stress (Balla *et al*. 1992; Cairo *et al*. 1995; Orino *et al*. 1999). The ability of ferritin to incorporate iron at physiological iron concentration is important to the rapid sequestration of $Fe^{2+}$ in the cells that serves to protect against oxidative damage (Balla *et al*. 1992; Orino *et al*. 1999). Iron oxidization by intrinsic ferroxidase of ferritin may strongly influence the early uptake of iron.

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