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Kinetic analysis of bovine spleen apoferritin and recombinant H and L chain homopolymers: Iron uptake suggests early stage H chain ferroxidase activity and second stage L chain cooperation

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Abstract

Ferritin utilizes ferroxidase activity to incorporate iron. Iron uptake kinetics of bovine spleen apoferritin (H : L=1 : 1.1) were compared with those of recombinant H chain ferritin and L chain ferritin homopolymers. H chain ferritin homopolymer showed an iron uptake rate identical to bovine spleen apoferritin (0.19 and 0.21 mmol/min/μmol of protein, respectively), and both showed iron concentration-dependent uptake. In contrast, the L chain homopolymer, which lacks ferroxidase, did not incorporate iron and showed the same level of iron autoxidation in the absence of ferritin. Bovine spleen apoferritin was shown to have two iron concentration-dependent uptake pathways over a range of 0.02–0.25 mM ferrous ammonium sulfate (FAS) by an Eadie-Scatchard plot (v/[FAS] versus v), whereas the H chain ferritin homopolymer was found to have only one pathway. Of the two Km values found in bovine spleen apoferritin, the lower mean Km value was 9.0 μM, while that of the H chain homopolymer was 11.0 μM. H chain ferritin homopolymer reached a saturating iron uptake rate at 0.1 mM FAS, while bovine spleen apoferritin incorporated more iron even at 0.25 mM FAS. These results suggest that the intrinsic ferroxidase of ferritin plays a significant role in iron uptake, and the L chain cooperates with the H chain to increase iron uptake.

Introduction

Ferritin is a ubiquitous iron-containing protein made up of 24 subunits with a major role in accumulating and detoxifying intracellular iron (Theil 1987; Andrews *et al*. 1992; Cairo *et al*. 1995; Harrison & Arosio 1996). Mammalian ferritin is composed of a variable proportion of two subunits termed H (heart, 21 kDa) and L (liver, 19 kDa) (Arosio *et al*. 1978; Theil 1987; Andrews *et al*. 1992; Harrison & Arosio 1996). Intracellular ferritin contains 2000 to 3000 Fe(III) atoms/molecule under normal conditions (Harrison & Arosio 1996) although levels as high as 4,500 Fe(III) atoms/molecule have been observed (Theil 1987). The labile iron concentration (Fe(II)) within mammalian cells is very low, in the range of 0.2–1.5 μM (Epsztejn *et al*. 1997).

H and L chains are functionally different; the H chain shows ferroxidase activity (Levi *et al*. 1988; Andrews *et al*. 1992; Harrison & Arosio 1996) and the L chain provides nucleation sites for the formation of the iron core (Andrews *et al*. 1992; Levi *et al*. 1989, 1992, 1994). There are two pathways for ferritin to incorporate iron: protein-catalyzed ferroxidation and mineral core surface-oxidation (Sun & Chasteen 1992; Yang *et al*. 1998). Recently, we also found that the two pathways of iron uptake in bovine spleen apoferritin depend on iron concentration in the range of 0.02–0.25 mM ferrous ammonium sulfate (FAS), and the lower Michaelis constant (Km, 4.5 μM) of the two Kms calculated from kinetics programs is suggested

to correspond to physiological iron concentrations (Orino *et al*. 2002a). However, whether a higher Km of ferroxidase activity in the other pathway is due to mineral core iron oxidation or a different ferroxidase has not been clarified.

To elucidate the two pathways of ferritin iron uptake, we compared the kinetic parameters of bovine spleen apoferritin iron uptake with recombinant bovine H and L chain ferritin homopolymer iron uptakes.

Materials and methods

Ferritin samples

Bovine spleen ferritin was purified from bovine spleen as described previously (Kakuta *et al*. 1997) and its apoferritin was prepared from the spleen ferritin followed by dialysis with 100 mM thioglycolic acid in sodium acetate buffer (pH 5.5) followed by dialysis with phosphate-buffered saline (150 mM NaCl, 20 mM sodium phosphate, pH 7.0) for protein measurement or 100 mM [4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) (pH 7.0) for ferritin iron uptake experiments. Bovine ferritin H and L cDNAs were expressed in *Spodoptera frugiperda* using a baculovirus expression system as previously described (Orino *et al*. 1997).

Protein determination

Protein concentrations of ferritin samples were determined using the method of Lowry *et al*. (1951) with bovine serum albumin as the standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli (1970) using a 12% polyacrylamide running gel with a 4.5% polyacrylamide stacking gel. Purified bovine spleen ferritin (2 μg) and recombinant bovine H and L chain homopolymers (1 μg each) were loaded on the gel. Ferritin subunits bands stained with Coomassie Brilliant Blue R-250 were analyzed by densitometry at 565 nm using a Flying Spot Scanner (Shimadzu CS-9000)(Shimadzu, Kyoto, Japan).

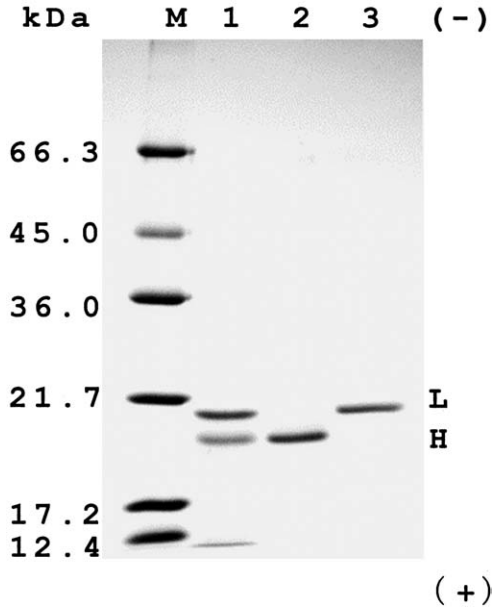

Fig. 1. SDS-PAGE of purified bovine spleen ferritin and recombinant bovine H and L chain ferritin homopolymers. Bovine spleen ferritin (2 μg, lane 1) and recombinant bovine H (1 μg, lane 2) and L (1 μg, lane 3) chain ferritin homopolymers were run against marker proteins (M, 2 μg each). H and L are heart- and liver- type ferritin subunits, respectively. Anode at bottom.

Iron uptake

Iron uptake was analyzed by incubating 0.1 μM ferritin samples in 100 mM HEPES (pH 7.0) with various concentrations (0.02–0.25 mM) of FAS (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Absorbance was measured at 310 nm on a HITACHI U-2010 spectrophotometer (HITACHI, Tokyo, Japan) at 30 °C. Initial iron uptake was determined by the increase in absorbance units (A) at 310 nm within the first 30 sec of the reaction, and the concentration of oxidized iron was calculated using a molar extinction coefficient of 2,475 M^{-1} cm^{-1} (Macara *et al*. 1973; Levi *et al*. 1988). The kinetic parameters of ferritin iron uptake were determined by pharmacokinetic analysis (MULTI) using the nonlinear square method (Yamaoka *et al*. 1981).

Results

Characterization of bovine spleen and recombinant bovine H and L chain ferritin homopolymers

The H (19 kDa) and L (21 kDa) chains of purified bovine ferritin were separated by SDS-PAGE (Fig-

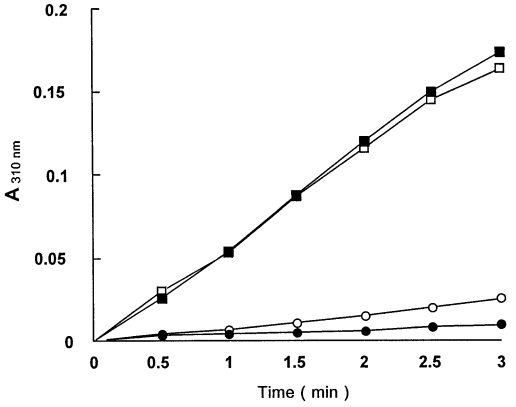

Fig. 2. Iron uptake of bovine spleen apoferritin (□) and recombinant bovine H (■) and L (●) chain ferritin homopolymers. Ferritin samples (0.1 μM) in 100 mM HEPES at pH 7.0 were incubated with 0.1 mM FAS at 30 °C, and absorbance at 310 nm (A_{310} nm) was measured at 30 sec intervals for 3 minutes. Buffer alone (○) was the control.

ure 1), and the H: L subunit ratio was determined to be 1: 1.1 by densitometry. The relative mobilities of the H and L subunits differed from those of other mammalian species; bovine L chain possibly has a lower affinity for SDS compared with those previously described (Orino *et al.* 1997). Expressed H and L chain homopolymers showed almost identical mobilities to the corresponding subunits of bovine spleen ferritin and spontaneously formed 24-mer proteins containing little iron (<0.4 ng/μg of protein), as previously described (Orino *et al.* 2002b).

Iron uptake analyses of bovine spleen apoferritin and recombinant bovine H and L chain ferritin homopolymers

Recombinant bovine H chain ferritin homopolymers showed the same iron uptake rate as bovine spleen apoferritin (0.19 and 0.21 mmol/min/μmol of protein, respectively), but the L chain ferritin homopolymer did not show iron uptake and exhibited the same curve as buffer alone (Fe(II) autoxidation) (Figure 2). A Michaelis-Menten plot shows that bovine spleen apoferritin did not reach iron-saturation at 0.25 mM FAS (Figure 3A), and an Eadie-Scatchard plot of apoferritin uptake shows the iron concentration dependence of the curve (Figure 3B). The H chain ferritin homopolymer also showed iron concentration-dependent uptake, but the H chain ferritin homopolymer reached its saturation point within the range tested (Figure 4A), and the Eadie–Scatchard plot was analyzed as a straight line (Figure 4B). Bovine spleen apoferritin

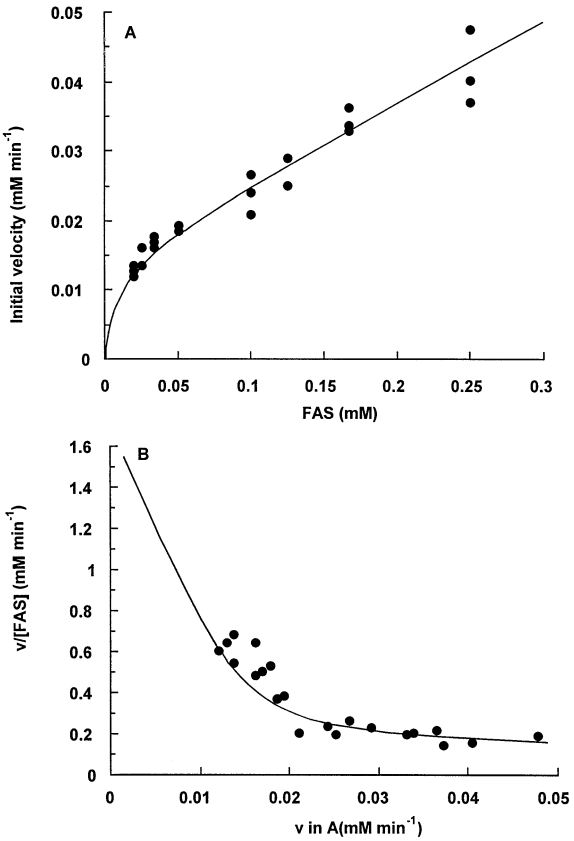

Fig. 3. Michaelis-Menten plot of initial velocity of iron uptake (A) and Eadie-Scatchard plot (B) of bovine spleen apoferritin. Bovine spleen apoferritin (0.1 μM) in HEPES buffer (pH 7.0) was incubated with FAS (0.02–0.25 mM) at 30 °C. Iron incorporation was monitored spectrophotometrically at 310 nm.

is expected to have two ferroxidase activities based on pharmakokinetic analysis in accordance with previous data (Orino *et al.* 2002a), while the H chain ferritin homopolymer has only one ferroxidase. The lower Km of the two Kms of the spleen apoferritin (9.0 μM) was identical to the Km of the H chain ferritin homopolymer (11.0 μM) (Table 1).

Discussion

The H chain ferritin homopolymer showed the same initial uptake rate as bovine spleen apoferritin (L/H = 1.1). This seems to be independent on H chain content of bovine ferritin iron uptake. In contrast, ferritin iron uptake has been shown to be H-content dependent in human and mouse ferritin heteropolymers genetically engineered variable subunit composition; H chain ferritin homopolymers show 20% faster iron uptake than

Table 1. Kinetic parameters of iron uptake in bovine spleen apoferritin and H chain ferritin homopolymer.

	Km1 (μM)	Vmax1 (mM min^{-1})	Vmax2/km2 (min^{-1})
Spleen apoferritin	9.0 ± 1.0 (11%)	0.0143 ± 0.0008 (6%)	0.116 ± 0.0076 (7%)
H chain homopolymer	11.0 ± 1.9 (17%)	0.0186 ± 0.0007 (4%)	–

Data are shown as the mean $\pm$ SD ($n = 3$), and % CV is shown in parentheses.

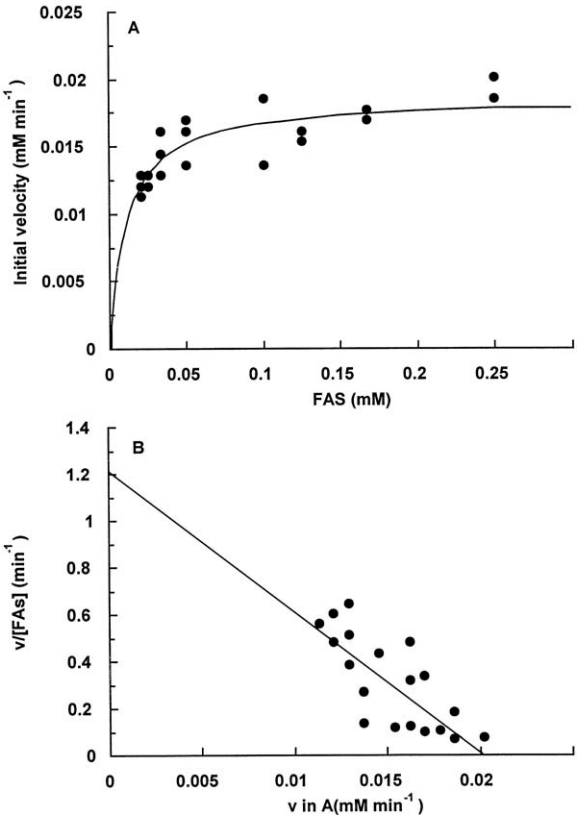

Fig. 4. Michaelis-Menten plot of initial velocity of iron uptake (A) and Eadie-Scatchard plot (B) of recombinant H chain ferritin homopolymer. H chain ferritin homoplolymer (0.1 μM) in HEPES buffer (pH = 7.0) was incubated with FAS (0.02–0.25 mM) at 30 °C. Iron incorporation was monitored spectrophotometrically at 310 nm.

ferritins with 50% H content (Rucker *et al.* 1996). Amino acid residues among mammalian ferritin subunits are very highly conserved (H chain, >91%; L chain, >83%) (Andrews *et al.* 1992; Orino *et al.* 1997), and seven amino acids involved in ferroxidase activity are perfectly conserved (Andrews *et al.* 1992; Harrison & Arosio 1996; Orino *et al.* 1997). The fact that bovine spleen apoferritin (48% H content) has the same initial iron uptake rate as recombinant H chain ferritin homopolymer contradicts previous data. Uptake rates for human H chain homopolymers are 20% faster than for mouse H chain homopolymers. Although the specific amino acids which contribute to the 20% faster iron uptake in human and mouse H chain ferritin homopolymers were not identified, the carboxyl-terminal domains of the H chain are suggested to cause a functional difference (Rucker *et al.* 1996). It has not been determined whether the iron uptake rates of bovine H chain ferritin homopolymers are different from human and mouse ferritin H chain homopolymers or whether bovine ferritin shows H content dependent-iron uptake.

An Eadie-Scatchard plot was used to determine the number of enzymes catalyzing the same reaction. Bovine spleen apoferritin shows two different pathways of iron uptake, depending on the iron concentration. The lower Km value (9.0 μM) of the two ferroxidase activities presented in this study was found twice in previous data (Orino *et al.* 2002a). However, because the spectrometric assay is not highly sensitive, ferritin iron uptake could not be measured for FAS concentrations less than 0.02 mM. If the ferritin iron uptake were eventually stopped by an iron chelating reagent, and the amount of iron incorporated into ferritin were measured using radioactive ferrous iron, the exact Km could be obtained through iron uptake kinetic analysis although ferritin iron uptake has not been measured using the radioactive iron simultaneously. Ferritin iron uptake experiments are usually performed in higher iron concentrations (Macara *et al.* 1973; Levi *et al.* 1988, 1989, 1992, 1994). Although intracellular labile ferrous iron concentrations are very low (0.2–1.5 μM) (Epsztejn *et al.* 1997), the lower Km value of ferroxidase seen in bovine spleen ferritin and the Km value seen in the H chain homopolymer are considered to correspond to physiological conditions.

There are two pathways of ferritin iron uptake: ferroxidase and mineral core-surface catalyzed iron oxidation (Sun & Chasteen 1992; Yang *et al.* 1998). The ferritin concentration-dependence of iron uptake shown by previous data (Orino *et al.* 2002a)

demonstrates a specific uptake reaction other than Fe(II) autoxidation even in high iron concentrations. Whether iron uptake seen under high iron concentrations is due to mineral core-catalyzed iron oxidation or another ferroxidase is not still clear. If the H chain ferritin homopolymer has two pathways as in bovine spleen apoferritin, the Vmax2/Km2 value of the H chain homopolymer as analyzed by MULTI should be one-tenth (0.011 ± 0.008 min^{-1}) that of bovine spleen ferritin, indicating that the H chain employs a mechanism of iron uptake that differs from the two pathways of iron uptake found in bovine spleen apoferritin. The H chain ferritin homopolymer shows a constant uptake rate at concentrations higher than 0.1 mM FAS, while bovine spleen apoferritin shows a tendency to increase iron uptake even at 0.25 mM FAS. H chain ferritin homopolymer, with only one mechanism of iron uptake, can not switch to another pathway of iron uptake under higher iron concentrations. The L chain, with functional residues involved in iron nucleation, provides nucleation sites within the ferritin molecule (Levi *et al*. 1989, 1992, 1994; Andrews *et al*. 1992), allowing the L chain to play a cooperative role with the H chain in iron uptake. Therefore, the L chain seems to be associated with alternative ferritin iron uptake by core-surface iron oxidation, suggesting that the L chain adjusts the microenvironment of iron core growth within the ferritin shell. The human L chain homopolymers show iron uptake at iron concentrations (0.1 mM) higher than physiological iron concentration under longer incubation times (3 h) (Levi *et al*. 1989). Once Fe(II) is oxidized within the ferritin molecule (even L chain homopolymer) in higher iron concentrations, ferritin may promote core growth to store up iron within the molecule without H chain.

An H-chain gene deletion is lethal to the early mouse embryo (Ferreira *et al*. 2000). Ferritin detoxifies Fe(II), a cause of the Harber-Weisse reaction (McCord 1996) by segregating the iron (Cairo *et al*. 1995; Orino *et al*. 1999). This study shows that the H chain plays a significant role in incorporating iron using intrinsic ferroxidase. In this study, cooperative iron uptake by H and L chains is not observed at physiological iron concentration. Further study is needed to elucidate the cooperative roles played by H and L chains at physiological iron concentration.

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