Binding Analysis of Horse and Bovine Ferritins using Beads Immobilized with Alpha-Casein: Detection of Ferritin Binding or Nonbinding with Heme using Alpha-Casein-Immobilized Beads

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Abstract

Ferritin, iron storage protein, binds heme on its surface although it's physiological role has not been elucidated yet. Firstly, this study analyzed the bindings of bovine and horse spleen ferritins to beads immobilized with α-casein (CasB) with the heme-mediated ferritin-binding capacity of α-casein (Cas). These spleen ferritins were added to solutions containing CasB, and the supernatant and beads obtained by centrifugation was subjected to polyacrylamide gel electrophoresis (SDS-PAGE or Native-PAGE) to detect ferritin. In case of horse spleen ferritin, CasB partly bound ferritin, and CasB-bound ferritin was detected only in the pelleted beads before the second addition of CasB to the first supernatant obtained before washing the beads, while the amount of ferritin detected in the first and second supernatants was similar. After addition of bovine spleen ferritin to CasB, some ferritin was also detected in the pelleted beads as CasB-binding protein, and the other ferritin was also detected in the supernatant as ferritin with no heme. On the other hand, the serum ferritin in fetal bovine serum showed little binding in the binding with CasB. These results suggest the following: CasB selectively detects heme-binding ferritin in tissue and serum ferritin, circulating ferritin contains little heme, and ferritin is intracellularly compartmented in heme catabolism as well as protection against its oxidative stress.

Background

Transient iron is an essential trace element for all living things and is required in various metabolic processes such as electron transport, the citric acid cycle, and DNA synthesis.[1,2] Heme (ferriprotoporphyrin) serves as a cofactor in the active sites of several enzymes classified as heme-binding proteins such as hemoglobin, myoglobin, microsomal and mitochondrial cytochromes, catalase-peroxidase, and transcriptional factors.[1,2] Ferritin is a 24-mer globular protein composed of two subunits, H (heavy or heart) and L (light or liver), and this hollow nanocage can accommodate maximum of 4,500 ferric irons for the double function of storing and oxidizes ferrous iron to ferric iron, and thus H subunit is involved in the incorporation of ferric iron into ferritin. L subunit lacks conservative amino acids associated with ferroxidase activity but contributes to ferritin stability and is more amenable to long term iron storage.[1] Mammalian and avian ferritin possesses a heme-binding pocket on the surface as well as bacteria ferritin,[2-5] suggesting that ferritin is involved in heme metabolism. Serum ferritin circulates relatively at low level (<1 μg/ml), but, in the circulation of bovine fetus, serum ferritin levels is remarkably high[1,6] although further study needs to clarify whether serum ferritin binds heme as in tissue ferritin. α-Casein (Cas) has the predicted global secondary structure and forms the anionic clusters to bind calcium ions.[7] It also binds ferritin through

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heme-mediated binding that depends on the iron in the heme ring, and may be capable of detecting ferritin bound or not bound to heme.[2,8] However, it remains to clarify to what extent tissue and serum ferritins possess hemin on their molecular surfaces. Poly-L-lysine (PL) also binds hemin and iron,[9] but it remains unclear whether PL binds ferritin or not. In bead methods, metal-binding protein and protein-protein interactions can be studied using beads with immobilized metal ions, fibrinogen, α-casein (CasB) or PL (PLB).[9,10] This study, firstly, conducted the binding analysis with CasB using commercial available horse spleen ferritin and purified bovine spleen ferritin. Commercial bovine fetal serum (FBS) contains high levels of ferritin with its high iron content (approximately 2,000 iron atoms a ferritin molecule).[6] Next, we provide preliminary data showing that CasB can discriminate trance amounts of serum ferritin bound to heme from ferritin not bound to heme in FBS. Intra- and extracellular ferritins are likely to be involved in heme-metabolism or protection against heme-mediated oxidative stress by heme-binding as well as iron-binding.

MATERIALS AND METHODS

Chemicals

Bovine α-casein (≥70% purity) and horse spleen ferritin monomer was purchased from Sigma (St. Louis, MO, USA). CNBr-activated Sepharose 4B and Sepharose 4B were purchased from GE Healthcare (Columbus, OH, USA). Fetal bovine serum (FBS) was purchased from Sanko Junyaku Co., Ltd. (Tokyo, Japan), SAFC Biosciences (Lenexa, KS, USA), and PAN-Biotech GmbH (Gewerbepark, Aidenbach, Germany). Block Ace (BA) was purchased from DS Farma Biomedical Co., Ltd (Osaka, Japan). Coomassie Plus-The Better Bradford Assay kit, Immuno Plate Maxisorp F96 microplate, and alkaline phosphatase (ALP)-conjugated NeutrAvidin were purchased from Thermo Fisher Scientific Inc. (Rockford, IL, USA). Bovine serum albumin (BSA) was purchased from Roche Life Science Inc. (South San Francisco, CA, USA). ALP-conjugated goat affinity purified antibody to rabbit IgG Fc fragment was purchased from MP Biomedicals (Irvine, CA, USA). All other reagents were of the highest grade available. Pure water (Elix water) was produced from tap water using an Elix Advantage Water Purification System (Millipore, Billerica, MA, USA).

Preparation of CasB

CasB was prepared using CNBr-activated Sepharose 4B according to the method described previously.[10]

Ferritin and apoferritin preparation

Horse spleen ferritin was purified from commercial horse spleen ferritin as described previously.[11] Bovine spleen ferritin was purified from pieces of frozen bovine spleen as described previously.[12] Apoferritin was prepared by dialysis of holoferritin against 100 mM thioglycolic acid in 100 mM acetate buffer (pH 5.5), followed by phosphate-buffered saline (PBS, 150 mM NaCl, 20 mM sodium phosphate, pH 7.2), to release heme and iron, as described previously.[8] Protein concentrations were determined by a Coomassie Plus-The Better Bradford Assay kit using BSA as the standard according to the manufacturer's micro microplate protocol.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), native-PAGE, and immunoblotting

SDS-PAGE was carried out according to the method of [13] using a 4.5% stacking gel and 12.5% running gel. Native-PAGE was performed using a 5% slab gel and the buffer system described by[14] with buffer system of 375 mM Tris/HCl buffer (pH 8.9). Sample buffer (5X) consists of 25% glycerol, 0.01% bromophenol blue, 10% SDS, 200 mM dithiothreitol, and 250 mM Tris/HCl, pH 6.8, for SDS-PAGE, and the same sample buffer (5X) without SDS and dithiothreitol was used for native-PAGE. Protein bands separated by SDS-PAGE were stained with Coomassie Brilliant Blue R250. Protein samples separated on a native-PAGE gel were transferred to a polyvinylidene fluoride (PVDF) membrane at 12 V for 12 min using an Invitrogen iBlot 2 Gel Transfer Device (Carlsbad, CA, USA). Briefly, the transferred proteins were

exposed to rabbit anti-bovine spleen ferritin antiserum[6] followed by ALP-labeled polyclonal antibodies specific for the rabbit IgG Fc fragment. ALP-labeled antibodies bound on the PVDF membrane were detected with 100 mM Tris/HCl (pH 7.6) containing 5 mM $MgCl_2$, 0.39 mM nitro blue tetrazolium, and 0.38 mM5-bromo-4-chloro-3-indolyl phosphate.

Ferritin measurement

Ferritin in FBS was measured by sandwich ELISA according to a previously described method using BA in place of gelatin.[8] Briefly, 100 µl of anti-bovine spleen ferritin antibody (200 ng/m1) in PBS was added to each well of a microtiter plate. After washing the wells with PBS containing 0.5% (v/v) Tween 20 (PBST), a 300-µl aliquot of 1% BA (w/v) was added to each well, and the plate was held at room temperature for 1 hr to block non-specific adsorption of ferritin protein with BA. Following another wash with PBST, 100-µl aliquots of bovine spleen ferritin standards (0.156-10 ng/ml) in PBS containing 0.5 M $(NH_4)_2SO_4$ were added, and the plate was incubated at 37°C for 2 hr. Serum samples were appropriately diluted with the same buffer used to prepare the standard solution. Aliquots (100 µl) of serum samples were pipetted into the plate wells along with the ferritin standard series. After washing with PBST, 100 µl of biotinylated rabbit anti-bovine spleen ferritin antibody (125 ng/ml) diluted in 0.1% (w/v) BA was added to each well, and the plate was incubated at 37°C for 1.5 hr. After washing, 100 µl of ALP-conjugated NeutrAvidin (1 µg/ml) diluted in 0.1% (w/v) BA was added to each well, and the plate was incubated at 37°C for 1.5 hr. After washing, the ALP enzyme reaction was carried out using disodium p-nitrophenyl phosphate as previously described.[11] The concentration of p-nitrophenol produced by the ALP reaction was determined by measuring the absorbance at 405 nm.

Binding of tissue and serum ferritin to CasB

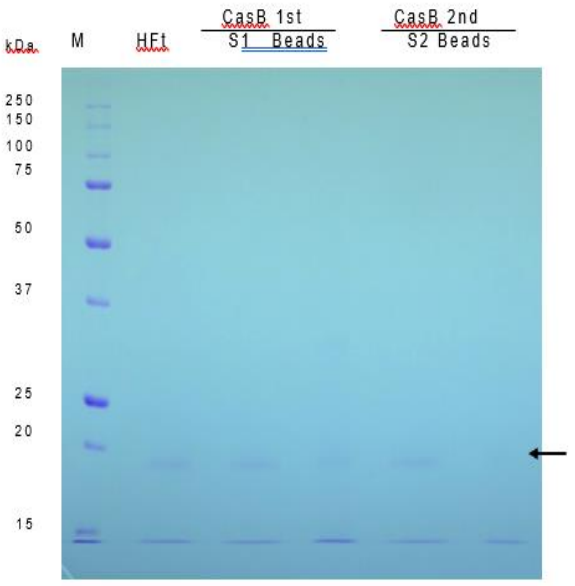
Purified commercial horse ferritin (20 µg) were dissolved in 1 ml of PBS (net volume of beads per sample: 20 µl) containing CasB or Sepharose 4B as control beads (CB), and the mixture was rotated at 4°C for 30 min. The mixture was centrifuged at 16,000 x g for 5 min at 4°C. After recovering the first supernatant, the pelleted beads were re-suspended with 1 ml of PBS followed by centrifugation at 16,000 x g for 5 min at 4°C. The pelleted beads were washed three times with 1 ml of PBS under the same conditions as described above. Forty-eight microliters of aliquots was removed from the supernatant collected after the first centrifugation before washing the beads, and CasB was again added to the rest of the supernatant (net volume of beads per sample: 20 µl). The mixture was rotated at 4°C for 30 min again, centrifuged as described above, and the supernatant was again collected after the first centrifugation before washing the beads. The pelleted beads were also washed using the same centrifugation conditions as described above. The supernatant and beads obtained in the two steps were subjected to SDS-PAGE, followed by staining with Coomassie Brilliant Blue R250. Bovine spleen ferritin and the measured ferritin in FBS were prepared by dissolving spleen ferritin and FBS ferritin (2.5 µg and 2 µg spleen ferritin and ferritin in FBS, respectively) in 1 ml of PBS (net volume of beads per sample: 20 µl) containing CasB or CB, and the mixture was rotated at 4°C for 30 min. The mixture was centrifuged at 16,000 x g for 5 min at 4°C. After recovering the first supernatant, the pelleted beads were re-suspended with 1 ml of PBS followed by centrifugation at 16,000 x g for 5 min at 4°C. The pelleted beads were washed three times with 1 ml of PBS under the same conditions as described above. The first supernatant and the beads were subjected to native-PAGE followed by ferritin detection by immunoblotting with rabbit anti-bovine spleen antiserum as previously described.[8,15] FBS was added to solution containing beads to be same amount of ferritin based on ferritin levels determined by sandwich ELISA as described above. Supernatant obtained was prepared with the corresponding sample buffer (4:1) as described above, and the mixture (20 µl) was applied to SDS-PAGE or native-PAGE. The precipitated beads were resuspended to be 50 µl with the corresponding native-PAGE. The precipitated beads were resuspended to be 50 µl with the corresponding SDS-PAGE or native-PAGE.

RESULTS

Binding of horse ferritin and its apoferritin form, and bovine spleen ferritin to CasB

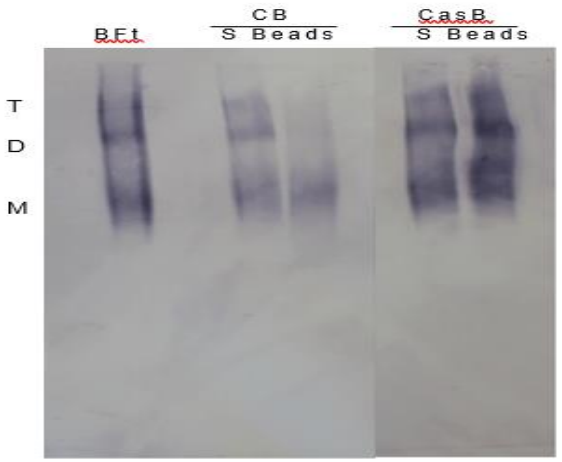
Firstly, in this study, the binding analysis of horse spleen ferritin and its apoferritin, which lacks iron and heme, with PLB was also performed by its heme-binding capacity[9] as in Cas[8,9] (Supplementary Data 1). Purified commercial horse spleen ferritin is composed almost exclusively of L subunit, with an apparent molecular mass of 19 kDa, as described previously.[12] Horse spleen ferritin in the holo form did not bind to CB and was detected exclusively in the supernatant (S) obtained following the first centrifugation after incubation, but holoferritin showed the binding with PLB (A). Horse spleen apoferritin also bound with PLB, similar to holoferritin, but was absent from the supernatant (B). Apoferritin did not bind with CasB and was detected only in the supernatant, as with CB (B), according with previous data that Cas did not bind bovine spleen apoferritin.[8] Next, holo-horse ferritin and CasB was used to develop a method for the detection of heme-binding ferritin (Figure 1) Holo-horse spleen ferritin partly bound to CasB, and was detected in both the supernatant and the pelleted beads (CasB 1st in Figure 1). After the first addition of horse ferritin to CasB, the same amount of CasB was added to the supernatant obtained the first time to examine the extra of CasB-binding ferritin after removing a 48 μl sample (CasB 1st) for SDS-PAGE. The pelleted beads were washed as described above and subjected to SDS-PAGE together with the first pelleted beads. Subsequently, the supernatant and the pelleted beads obtained under the same conditions were applied to an SDS-PAGE gel together with samples obtained the first time. After the second round of pelleting and washing, CasB-bound ferritin was not detected, in contrast to the first addition of ferritin to CasB, and the amount of ferritin in the first and second supernatants appeared similar (CasB 2nd in Figure 1).

Figure 1: Detection method of heme-binding ferritin by the binding of commercial horse ferritin binding with CasB. Forty micrograms of commercial horse spleen ferritin was added to 1 ml PBS containing CasB (net volume of beads per sample: 20 μl), and the mixture was rotated at 4°C overnight. The mixture was centrifuged at 16,000 x g for 5 min at 4°C, and then the pelleted beads were suspended with 1 ml of PBS. The mixture was again centrifuged under the same centrifugation conditions and the pelleted beads were washed three times with 1 ml of PBS under the same centrifugation conditions. The supernatant (S) was collected after the first centrifugation before washing the beads. The pelleted beads obtained were washed using the same centrifugation conditions. The supernatant (S1 in CasB 1st) was collected after the first centrifugation before washing the beads, an aliquot (48 μl) of S1 was removed, and CasB was again added to the rest of S1 (net volume of beads per sample: 20 μl). The mixture was rotated at 4°C for 30 min again, centrifuged as described above, and the supernatant (S2 in CasB 2nd) was again collected after the first centrifugation before washing the beads. The pelleted beads were also washed using the same centrifugation conditions as described above. The supernatant and beads obtained in the two steps (CasB 1st and 2nd) were subjected to SDS-PAGE. Horse spleen ferritin (HFt) was separately applied to the gel (2 μg/lane). Arrow indicates horse spleen ferritin. M represents marker proteins.



Bovine spleen ferritin was added to the solution containing CasB, and detected by immunoblotting (Figure 2). Oligomerization of the bovine spleen ferritin 24-mer was detected as dimers (D) and trimers (T) in addition to ladder bands of the ferritin monomer (M) due to complex combination of H and L subunit heteropolymers, as previously described.[14-16] Bovine spleen ferritin bound non-specifically with CB because ferritin (M, D, and T) was detected in the supernatant. Although bovine spleen ferritin showed non-specific binding to CB, more CasB-binding ferritin oligomers (M, D, and T) were detected in the pelleted beads as compared with CB, suggesting that the ferritin bands detected in the supernatant is ferritin oligomers nonspecific binding to beads and with no heme.

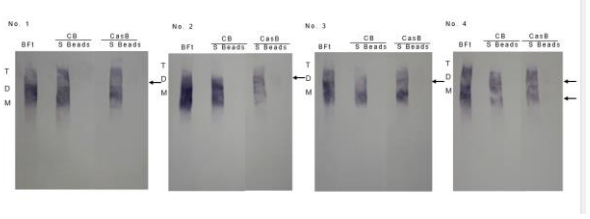
Figure 2: Partial binding of bovine spleen ferritin with CasB. Two micrograms of bovine spleen ferritin was added to 1 ml PBS containing CasB (net volume of beads per sample: 20 μl), and the mixture was rotated at 4°C for 30 min. The mixture was centrifuged as described in Figure 1, and the pelleted beads were washed three times with PBS using the same centrifugation conditions as described in Figure 1. The supernatant (S) was collected after the first centrifugation before washing the beads. The pelleted beads were subjected to native-PAGE together with S, followed by the detection of ferritin by immunoblotting. Bovine spleen ferritin (BFt) was separately applied to the gel (40 ng/lane). M, D, and T represent monomer, dimer, and trimer, respectively.



Binding of ferritin in FBS with CasB

This study also showed the presence of heme-binding ferritin extracellular ferritin. Four lots of commercial FBS were used for binding analysis of ferritin in FBS with CasB (Figure 3), and 2.5 μg FBS ferritin was added per sample. FBS ferritin in the four lots was detected in the supernatants from CB and CasB. No FBS ferritin was detected in the pelleted CB, but a trace amount of CasB-binding ferritin was detected in ferritin dimers except for No. 4 detected both ferritin monomer and dimer.

Figure 3: Binding of a trace amount of ferritin in FBS with CasB. A measured amount of ferritin from 4 lots (Nos. 1-4) of FBS was added to 1 ml PBS containing CasB (net volume of beads per sample: 20 μl) to a final concentration of 2 μg/ml. The mixture was rotated at 4°C for 30 min. The mixture was centrifuged, and the supernatant (S) was collected after the first centrifugation before washing the beads by suspension in PBS. The pelleted beads were washed three times with 1 ml of PBS as described in Figure 1. The pelleted beads were subjected to native-PAGE together with S. Bovine spleen ferritin (BFt) was separately applied to the gel (40 ng/lane). M, D, and T represent monomer, dimer, and trimer, respectively. Arrows indicate ferritin monomer or dimer in the precipitated beads.



DISCUSSION

The reduction of holoferritin releases iron from ferritin and also releases heme (Sugawara et al., 2009), indicating that apoferritin is available as ferritin nonbinding with heme. CasB has a possibility to detect heme-binding ferritin because Cas binds bovine ferritin but not its apoferritin, which lacks iron and heme, by heme-mediated binding[8,17] Although PL binds heme,[15] horse spleen ferritin and its apoferritin form bind to PLB

different from Cas, suggesting that PLB binds ferritin directly, likely through hydrophobic interactions, similar to the binding of PLB and bovine lactoferrin[10] On the other hand, CasB partly bound horse ferritin but did not bind the apoferritin form. As shown in Figure 1, no CasB-bound ferritin was detected in pelleted beads after the addition of CasB to the first supernatant obtained by centrifugation. We found the same amount of horse ferritin in both the first and second supernatants obtained after the addition of CasB to the first supernatant. This result indicates that heme-binding ferritin was removed by the first incubation with CasB. Although we did not measure the heme content of heme-binding ferritin or the binding affinity between ferritin and CasB, CasB may be able to separate ferritin not bound to heme from heme-binding ferritin, also indicating that not all ferritin in tissues necessarily binds heme. A combination of native-PAGE and immunoblotting detected bovine ferritin more sensitively than did SDS-PAGE (data not shown), indicating that this method is applicable for the detection of serum ferritin. In the CasB experiment, bovine spleen ferritin comprises heme-binding ferritin and heme-free ferritin. In native-PAGE, a previous report demonstrated that feline serum ferritin-binding protein strongly bound feline ferritin, but ferritin-binding proteins were released as determined by native-PAGE, allowing their purification after binding with ferritin-immobilized beads.[15] This study also indicated that CasB-binding ferritin is released from the beads by native-PAGE. Mammalian ferritins likely bind heme through heme-binding pockets on the ferritin surface, although demetallation causes the release of iron from heme, followed by iron uptake by ferritin.[3,18] It remains unclear why purified ferritin retains heme, and how a part of intracellular ferritin is involved in heme metabolism. Macrophages especially play an important role in heme clearance to degrade heme after uptake of senescent erythrocyte, and the heme-binding capacity of spleen ferritin may influence heme-oxygenase activity to modulate immunosuppression.[19] These results suggest that, although intracellular ferritin plays important role to sequester ion for iron storage or protection of irom-mediated oxidative stress, spleen ferritins associate with heme segration through its heme-binding as another way which binds iron. Iron chaperone poly(rC)-binding protein 2(PCBP2) is involved in iron transfer by binding ferrous iron after the reduction of iron in the course of heme degradation.[20] Further study needs to clarify the carry heme. CasB may serve as a tool for measuring heme-binding ferritin and reveal its physiological function. FBS ferritin contains a high amount of iron, as in tissue ferritin, and FBS ferritin is proposed to circulate and carry iron to meet the needs of fetal growth, as indicated by the high levels of ferritin in the circulation.[6] A small amount of CasB-binding ferritin was preferentially detected in ferritin dimers, suggesting that FBS ferritin binds trace amounts of heme on its surface. Further study needs to clarify a tendency to more heme in ferritin dimers. This finding suggests that circulating serum ferritin probably binds heme after secretion, and that serum ferritin segregates heme for the production of heme-mediated reactive-oxygen species, and is involved in inhibiting bacterial proliferation to require heme for an iron source[2]. The presence of ferritin that does not bind heme suggests that intracellular ferritin may bind heme in the compartment likely formed by droplets although it remains to be clarified whether iron and/or heme metabolism occur during liquid-liquid phase separation[21]. Further studies are required to clarify the effect of iron and/or heme bound to circulating ferritin on ferroptosis, and on cancer development and progression.[22-24]

CONCLUSIONS

In the present study, CasB can differentiate heme-binding ferritin from ferritin not bound to heme. Serum ferritin slightly binds heme as compared with spleen ferritin. These results suggest that intracellular ferritin is compartmented in various forms such as sequestering form of released iron from heme degradation, hem-binding form to protect heme degradation, and secretion form. This study also showed that PLB binds directly to ferritin regardless of iron- and/or heme-binding.

Competing Interests State

Authors declare no conflict of interests for this article.

Author Contribution

Conceptualization, K. O., R. H., and Y. Y.; methodology, K. O. and R. H.; validation, K. O., R. H., and Y. Y.; formal analysis, K. O. and R. H.; investigation, A. T. and R. H.; data curation, K. O.

Ethics statement

All experiments were conducted following the established guidelines for animal welfare and approved by the Committee on the Ethics of Animal Experiments of Kitasato University (permit no.: 20-048)

Supplementary information

The online version supplementary data available at…

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