Binding Analysis of Ferritin with Heme Using α-Casein and Biotinylated-Hemin: Detection of Heme-Binding Capacity of Dpr Derived from Heme Synthesis-Deficient Streptococcus mutans

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(Received 21 February 2013/Accepted 19 March 2013/Published online in J-STAGE 2 April 2013)

ABSTRACT. Bacterial and mammalian ferritins are known to bind heme. The use of α-casein and biotinylated hemin could be applicable to detection of protein-bound heme and of proteins with heme-binding capacity, respectively. Although commercial horse spleen ferritin and purified horse spleen ferritin (L:H subunit ratio=4) bound to an α-casein-coated plate, and this binding could be inhibited by hemin, recombinant iron-binding protein (rDpr), derived from heme-deficient Streptococcus mutans and expressed in Escherichia coli, did not bind to an α-casein-coated plate. Both horse spleen ferritins bound to α-casein-immobilized beads. Commercial horse spleen ferritin and rDpr showed direct binding to hemin-agarose beads. After preincubation of commercial horse spleen ferritin or rDpr with biotinylated hemin, they showed indirect binding to avidin-immobilized beads through biotinylated hemin. These results demonstrate that α-casein is useful for detection of heme-binding ferritin and that both hemin-agarose and the combination of biotinylated hemin and avidin-beads are useful for detection of the heme-binding capacity of ferritin. In addition, this study also revealed that Dpr, a decameric iron-binding protein, from heme-deficient cells binds heme.

KEYWORDS: α-casein, biotinylated hemin, Dpr, ferritin, heme.


Iron is an essential trace element required as a cofactor of active sites of several enzymes involved in electron transport, the citric cycle, DNA synthesis and oxygen transport [1, 5, 14, 20]. Heme, ferriprotoporphyrin IX, enhances iron catalytic activity [2, 18]. However, the high redox potential of iron and heme causes oxidative damage at the higher concentrations than required within cells [5, 16, 18]. The intracellular pool of iron and heme is tightly regulated [2, 5, 18]. Ferritin, a ubiquitous iron-binding protein, is found in animals, plants and bacteria [1, 5, 20]. The function of ferritins is to store a bioavailable and nontoxic form of iron to prevent its use in the production of highly toxic hydroxyl radicals through the iron-mediated Fenton reaction [5, 14, 16]. Mammalian ferritins are 24-mer proteins, composed of two types of subunits named the H (heart or heavy) and L (liver or light) subunits [1, 5, 15, 20]. Chicken and bacterial ferritin subunits possess ferroxidase activity (iron-binding site) as in mammalian H subunits [1, 3, 7, 13, 23].

Mammalian, avian and bacterial ferritins bind heme [8, 9, 13, 23]. Mammalian ferritin H subunits are likely to preferentially bind heme as compared with L subunits [13, 23]. Avian species are known to express only the H subunit, and chicken H subunit homopolymers bind more heme than the mammalian ferritins [13, 23]. Bacterial DNA-binding protein from starved cells (Dps) is a decameric iron-binding protein that belongs to the ferritin superfamily, and its iron-binding capacity protects DNA from oxidative stress [11, 12]. Recently, the heme binding of Dps from Porphyromonas gingivalis was demonstrated to protect DNA from heme-mediated oxidative stress, although the iron-binding protein has no DNA-binding capacity [4]. Dps possesses separate iron- and heme-binding sites, and sequestration of either iron or heme leads to protection from oxidative damage [4]. Additionally, the dpr (Dps-like peroxide resistance) gene product, Dpr, from heme-deficient Streptococcus mutans is unable to bind DNA, but its iron-binding limits the iron-catalyzed Fenton reaction [24, 25]; however, its heme-binding has not been revealed yet.

Bovine milk α-casein could be applicable to detection and isolation of iron-binding proteins and hemoproteins owing to its iron- and heme-binding capacity in the plate method [22]. Hemin-agarose is used for affinity chromatography of hemoproteins [21], and biotinylated hemin is also applicable to the detection and isolation of hemoproteins with heme-binding capacity in combination with streptavidin magnetic beads or streptavidin agarose beads [6]. Specific binding of biotinylated hemin with apohemoprotein can be spectroscopically monitored [6]. Although the use of beads was found to produce a nonspecific interaction between the protein and agarose resin [6], use of the beads can be applicable to the immobilization of various ligands and to isolation of more desired substances that interact with the ligands as compared with the plate method. The aim of this
study was to examine heme binding of horse spleen ferritin using α-casein-immobilized beads, hemin-immobilized beads and the combination of biotinylated hemin and avidin-immobilized beads. In addition, these assays allowed the detection of heme-binding capacity of Dpr derived from heme-deficient Strepococcus mutans [24, 25].

Ferritin monomers (24-mer proteins) were further purified from commercial horse spleen ferritin (Sigma-Aldrich Corp., St. Louis, MO, U.S.A.) according to a previously described method [15]. Horse spleen ferritin monomers were also purified from frozen bovine spleen as described previously [15]. The Dpr (decameric iron-binding protein) gene was transformed into Escherichia coli BL21 (DE3) cells (Life Technologies Japan Ltd., Osaka, Japan) using the pET28b expression vector (Life Technologies Japan Ltd.), and the protein was expressed and purified as described previously [24]. Protein concentrations were determined according to the method of Lowry et al. (1951) [10]. Rabbit anti-serum to commercial horse spleen ferritin and to Dpr was prepared according to the method previously described [15, 25]. Antibodies to commercial horse spleen ferritin were purified by affinity chromatography, and a part of the purified antibodies was biotinylated as described previously [22]. Bovine α-casein-Sepharose 4B was prepared by coupling 50 mg of α-casein (≥70% αs-casein, 90% purity by electrophoresis, Sigma-Aldrich Corp.) to 10 ml of CNBr-activated Sepharose 4B (GE Healthcare, U.S.A.) according to the manufacturer's instructions as previously described [19]. Hemin (ferritroporphyrin IX chloride) and biotinylated hemin were prepared as previously described [13].

Aliquots (100 µl) of α-casein protein solution in 20 mM Tris-HCl buffer (pH 7.0) (Buffer A) were added to the wells (21 pmol/well) of an immuno plate (MaxiSorp microtiter plate, Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.) and kept overnight at 4°C. After washing with 10 mM Tris-HCl buffer containing 0.05% Tween 20 (TT buffer), 300 µl of 20 mM Buffer A containing 0.1% Tween 20 and 0.025% gelatin (ELISA buffer), was added to each well and rinsed with gelatin for 1 h at room temperature to prevent nonspecific binding. One hundred microliters of horse spleen ferritin (10 nM) or rDpr (21 nM) in Buffer A were added to the separate wells for each sample, and the plate was incubated at 37°C for 2 h. After washing with TT buffer, 100-µl aliquots of biotinylated rabbit anti-commercial horse spleen ferritin polyclonal antibody (125 ng/ml) and rabbit anti-Dpr anti-serum (0.1%) diluted with ELISA buffer were added to the wells of the plate for the detection of ferritin and rDpr, respectively, and the plate was incubated at 37°C for 1.5 h. After washing, 100 µl of a solution containing 2 µg/ml of ALP-labeled avidin (Thermo Fisher Scientific Inc.) and 1 µg/ml of ALP-labeled goat anti-rabbit IgG antibody (Bethyl Laboratories, Montgomery, TX, U.S.A.) in ELISA buffer was added to the wells of the plate for the detection of biotinylated antibody and IgG antibody bound to the wells, respectively, and the plate was incubated at 37°C for 1 h. After washing, the enzyme reaction was carried out with disodium p-nitrophenyl phosphate as previously described [17]. The concentration of p-nitrophenol produced by the ALP reaction was determined by measuring the absorbance at 405 nm as the α-casein-binding activity. Hemin was used as an inhibitor in the binding of horse ferritins; hemin was added to horse spleen ferritin solution (10 nM each) to produce a final concentration of 10 µM and was added such that the NaOH concentration of the resulting solution of horse spleen ferritin was 0.1 mM.

One milliliter of horse spleen ferritin solution (10 nM each) in Buffer A containing 40 µl of 50% (v/v) α-casein-Sepharose 4B beads or Sepharose 4B beads (GE Healthcare) was added to a glass centrifuge tube, and the tube was rotated at 4°C. After mixing on a rotator overnight at 4°C, the tube was centrifuged at 2,000 × g for 5 min. The supernatant was discarded, and the precipitated beads were suspended with 1 ml of Buffer A followed by centrifugation at the same speed for 5 min. The supernatant was discarded, and the precipitated beads were suspended in the same volume of the same buffer and centrifuged under the same conditions. This washing was repeated three times, and the resultant precipitated beads were suspended with 1 ml of biotinylated rabbit anti-commercial horse spleen ferritin polyclonal antibody (125 ng/ml) diluted with ELISA buffer, and the mixture was incubated at 37°C for 1.5 h. After incubation, the mixture was centrifuged at 2,000 × g for 5 min, and the resultant precipitated beads were washed with 1 ml of the above buffer three times by centrifugation as described above. The precipitated beads were suspended with 1 ml of ALP-labeled avidin (2 µg/ml) diluted with ELISA buffer, and the mixture was incubated at 37°C for 1.5 h. After incubation, the mixture was centrifuged, and the resultant precipitated beads were washed three times by centrifugation as described above. The precipitated beads were suspended with substrate solution, and the mixture was centrifuged at 2,000 × g for 5 min. The released p-nitrophenol of the resultant supernatant was measured as described above.

In the detection of direct hemin-binding, 1 milliliter of horse spleen ferritin solution (10 nM) or rDpr (21 nM) in Buffer A containing 40 µl of 50% (v/v) hemin-agarose beads (Sigma-Aldrich Corp.) or agarose beads (Sigma-Aldrich Corp.) was added to a glass centrifuge tube, and the tube was rotated at 4°C. The mixture was treated with the method described above. Detection of the horse ferritin was performed as described above. The detection of Dpr was performed with rabbit anti-Dpr anti-serum (0.1%) diluted with ELISA buffer followed by subsequent reaction with 1 µg/ml of ALP-goat anti-rabbit IgG antibody in ELISA buffer. The released p-nitrophenol was measured as the direct hemin-binding activity as described above.

Biotinylated hemin was added to produce a final concentration of 0.2 µM in 1 ml of Buffer A containing horse spleen ferritin solution (10 nM) or rDpr (21 nM), the mixture was added to a glass centrifuge tube, and the tube was rotated at 4°C. After mixing on a rotator overnight at 4°C, 40 µl of 50% (v/v) Streptavidin Sepharose High Performance beads (GE Healthcare) or Sepharose 4B beads in Buffer A was added to the tube (net 20 µl each), and the mixture was incubated at 37°C for 1.5 h. After incubation, the mixture was centrifuged at the same speed for 5 min, and the super-
Commercial horse spleen ferritin was mostly comprised of L subunits, and purified horse spleen ferritin contained both H and L subunits with an L/H subunit ratio of four [13, 15]. Commercial holoferritin showed significantly lower binding activity to an α-casein-coated plate than the purified holoferritin, and this binding could be inhibited by hemin (Fig. 1A). This heme-mediated binding was in accordance with a previous report [13]. However, rDpr expressed in E. coli did not bind to an α-casein-coated plate (Fig. 1B), suggesting that rDpr did not bind heme when expressed in E. coli. Additionally, both the commercial holoferritin and the purified holoferritin showed significantly higher binding to an α-casein-immobilized beads than to the control beads (Fig. 1C), although rDpr did not bind to the immobilized α-casein as in the plate method (data not shown).

Hemin has previously been used as a ligand for affinity chromatography, and hemin-agarose can be used for the detection and isolation of hemoprotein [21]. Biotinylated hemin has also been used for the detection and isolation of hemoproteins in combination with avidin beads [6]. Commercial horse spleen ferritin and rDpr were shown to bind directly with hemin-agarose in Fig. 2A and 2B. Following the incubation of biotinylated hemin with the commercial horse spleen ferritin and rDpr, both of these iron-binding proteins were detected through heme-mediated binding by examination with streptavidin Sepharose, which trapped their biotinylated complexes (Fig. 3A and 3B).

The purified horse spleen ferritin with a subunit L/H ratio of four showed significantly higher binding to an α-casein-coated plate than the commercial horse spleen ferritin mostly composed of L subunits due to selective loss of H subunits by cadmium sulfate precipitation during purification [15]. The binding of both of these proteins to coated α-casein was inhibited by hemin. These findings agree with the previous results showing that the H subunit has higher affinity to hemin than the L subunit [13, 23], and that the binding of ferritin with α-casein is heme-mediated [22]. In addition, with the use of α-casein-immobilized beads, it is possible to obtain more iron- or heme-binding protein than
on an α-casein-coated plate. This study demonstrated that both the commercial horse spleen ferritin and the purified horse spleen ferritin bound more strongly to α-casein beads as compared with the control beads without the α-casein coating, although the commercial horse spleen ferritin and the purified horse spleen ferritin bound to α-casein beads to the same extent. In this study, use of beads seemed to cause nonspecific binding, probably by electrostatic interaction, as compared with the microtiter plate method. The lack of a significant difference between the commercial horse spleen ferritin and the purified horse spleen ferritin in the binding to α-casein beads may be due to some interactions with the beads themselves. In addition, α-casein has phosphoryl serine groups interacting with metal ions [22], and the pfds of ferritin H and L subunits differ from each other [1, 5]. This result may also be due to the differences in the conformational change between coated and immobilized α-caseins and/or ferritin subunit charges. Ferritin is present in the sera of mammals, although at a relatively low concentration (<1 µg/ml) [14], and it remains to be clarified whether serum ferritin binds heme. The present study, the first to report on the binding of ferritin with α-casein beads, suggests that α-casein beads could be applicable to detection or isolation of the heme-binding ferritin, even when it is present in trace amounts. Further studies are needed for measurement of heme-binding ferritin in serum and tissue samples using the method developed in this study.

rDpr is a decameric iron-binding protein that prevents oxidative stress by sequestering iron (Fe²⁺), and it is derived from the heme synthesis-deficient Streptococcus mutans [24, 25]. Use of α-casein-plate demonstrated that rDpr expressed in E. coli did not bind heme. On the other hand, rDpr bound to hemin-agarose beads in the same way as the commercial horse spleen ferritin. Additionally, following the binding of biotinylated heme to these 24-meric and decameric iron-binding proteins, both of these proteins bound to streptavidin beads through avidin-biotin complex formation. Although the mechanism of how intracellular iron-binding proteins bind heme remains to be clarified, this study demonstrated that these iron-binding proteins possess heme-binding capacity. Horse ferritin possesses heme-binding pocket coordinating heme [13, 23], and streptavidin beads seemed to bind biotinylated heme embedded into the heme-binding pocket. However, α-casein can recognize iron within the heme ring [22]. If heme iron is buried in the heme-binding pocket on the surface of the ferritin molecule, it is unlikely that α-casein is accessible to iron directly within the heme ring. Reconstitution of biotinylated heme with apomyoglobin revealed the existence of biotinylated heme incorporated in heme pocket based on spectroscopic data and functional analysis [6]. However, heme-binding analyses of ferritin using α-casein beads and biotinylated heme leads to the unexpected heme-binding mechanism. A further study is needed to clarify the binding mechanism of these iron-binding proteins with heme.
The α-casein beads can be used for detection of proteins binding iron or heme [19], and hemin beads and biotinylated hemin can be used for detection of proteins with the heme-binding ability. Although the amino acid homology between S. mutans Dpr and P. gingivalis Dps or horse L subunit is low (<20%) [1, 4, 24, 25], these iron-binding proteins may have developed as a divergent heme-binding family. Although heme binding is likely to be common in iron-binding proteins forming nanocages, this heme binding may affect cellular redox homeostasis and heme metabolism by a unique heme-binding mechanism.

REFERENCES


