Ferritin binds to light chain of human H-kininogen and inhibits kallikrein-mediated bradykinin release

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INTRODUCTION

Ferritin is an iron-storage protein that exists in both intracellular and extracellular compartments. We have previously identified H-kininogen (high-molecular-weight kininogen) as a ferritin-binding protein [Torti and Torti (1998) J. Biol. Chem. 273, 13630–13635]. H-Kininogen is a precursor of the potent pro-inflammatory peptide bradykinin, which is released from H-kininogen following cleavage of H-kininogen by the serine protease kallikrein. In this report, we demonstrate that binding of ferritin to H-kininogen occurs via the modified light chain of H-kininogen, and that ferritin binds preferentially to activated H-kininogen. We further demonstrate that binding of ferritin to H-kininogen retards the proteolytic cleavage of H-kininogen by kallikrein and its subsequent release of bradykinin from H-kininogen. Ferritin does not interfere with the ability of kallikrein to digest a synthetic substrate, suggesting that ferritin specifically impedes the ability of kallikrein to digest H-kininogen, perhaps by steric hindrance. Based on these results, we propose a model of sequential H-kininogen cleavage and ferritin binding. These results are consistent with the hypothesis that the binding of ferritin to H-kininogen may serve to modulate bradykinin release.

Key words: ferritin-binding protein, inflammation, protein interaction.
during inflammation [38–41]. Thus the binding of ferritin to kininogen and/or the effects of ferritin on bradykinin release may link ferritin and H-kininogen in the inflammatory response. The experiments presented here were designed to explore characteristics of the interaction between H-kininogen and ferritin. We report that the ferritin-binding site of H-kininogen is localized to the modified light chain of H-kininogen, and that binding of ferritin to H-kininogen both retards kallikrein cleavage of H-kininogen and inhibits bradykinin release.

MATERIALS AND METHODS

Ferritins, H-kininogen, kallikrein and antibodies

Human spleen ferritin (Scripps Laboratories), purified H-kininogen (Enzyme Research), kallikrein (Calbiochem) and horseradish peroxidase-conjugated anti-ferritin (Pierce) were purchased. Apoferritin was prepared from human spleen ferritin by treatment with thioglycolic acid [26].

Cleavage of H-kininogen by plasma kallikrein in the presence of ferritin

H-kininogen in 4 mM sodium acetate/0.15 M NaCl, pH 5.3, was added to an equal volume of 10 mM sodium phosphate buffer/0.3 M NaCl, pH 7.8, and treated with plasma kallikrein at a molar ratio of 600:1 [27] in the presence of 10 µg/ml dextran sulphate (≈ 500000 Da; Fisher Biotech) at room temperature for 5 min to produce nicked kininogen with bradykinin still attached to H-kininogen (see Figure 6, below). Dextran sulphate was used to provide an experimental platform for contact activation, as described previously [42]. Spleen ferritin or apoferritin was added in a 1:1 molar ratio with respect to H-kininogen. At intervals, aliquots were removed and heated at 95 °C to inactivate the enzyme and analysed for polypeptide cleavage using SDS/PAGE [10–20 % gradient gels; with or without 0.1 M dithiothreitol (DTT)] as described below, followed by staining with Coomassie Brilliant Blue (GELCODE Blue Stain; Pierce Chemicals). Band intensities were quantified by densitometry using a PD135 scanning densitometer (Amersham Bioscience).

Ligand blotting

Aliquots of cleavage products were subjected to SDS/PAGE, transferred to nitrocellulose, and incubated sequentially with spleen ferritin, horseradish peroxidase-conjugated anti-ferritin antibody and a chemiluminescent substrate (ECL*, Amersham Bioscience) as described previously [17].

Plate assay to assess the relative binding of intact versus kallikrein-cleaved H-kininogen towards ferritin

High-binding (4HBX) Immulon (Dynex) plates were coated with H-kininogen or fully cleaved (7 h) kallikrein-treated kininogen (5 µg/100 µl in each well) in PBS, overnight at 4 °C. The plates were washed with PBS and blocked with 2 % BSA in PBS. Biotinylated spleen ferritin (at different concentrations) was allowed to bind to kininogen-coated wells for 2 h at room temperature with shaking. The wells were washed extensively with PBS, treated with streptavidin-peroxidase for 1 h at room temperature and assayed with TMB substrate (3.3'5'-tetramethylbenzidine; Pierce) by measuring the absorbance at 450 nm in a microtitre plate reader (Dynateck). Control wells were coated with BSA. Biotinylation of ferritin was carried out using sulpho-N-hydroxysuccinimido-long-chain-biotin (sulpho-NHS-LC-biotin) and the EZ-Link Sulpho-NHS-LC-Biotinylation kit (Pierce).

Measurement of bradykinin release by HPLC

H-kininogen (120 µg, 1 nmol) was treated with kallikrein for 5 min at room temperature to produce nicked kininogen, as described above. The nicked kininogen was allowed to interact with spleen ferritin (500 µg, 1 nmol) or apoferritin (460 µg, 1 nmol) at room temperature. Aliquots were removed at different time intervals, adjusted to 0.1 % trifluoroacetic acid by adding concentrated acid (25 %), and analysed for bradykinin by HPLC. Bradykinin was separated on a reverse-phase C18 column (4.61 mm × 250 mm) equilibrated with 0.1 % trifluoroacetic acid and eluted with a gradient of 0.1 % trifluoroacetic acid and 0.08 % trifluoroacetic acid in 70 % acetonitrile. A flow rate of 1 ml/min was maintained and separation was monitored by measuring the absorbance at 215 nm. Bradykinin standard was purchased from Peninsula Laboratories and used to identify the bradykinin peak on the chromatogram (24.7 min). Released bradykinin was well separated from the digestion mixture containing H-kininogen, kallikrein and ferritin, all of which had retention times different from bradykinin (results not shown).

N-terminal sequence analysis

N-terminal sequence analysis of the H-kininogen 58 kDa light chain (produced by 5 min of nicking with kallikrein) and modified 45 kDa light chain (obtained by 7 h of digestion with kallikrein) was performed after electrophoretic transfer on to a PVDF membrane. The membrane was stained with Coomassie Brilliant Blue, the appropriate bands excised and the N-terminal sequence obtained by Edman degradation (Protein Analysis Core Laboratory of the Comprehensive Cancer Center of Wake Forest University School of Medicine, Winston-Salem, NC, U.S.A.).

Chromogenic assay for measurement of kallikrein activity

To examine the effect of ferritin on kallikrein activity, the chromogenic peptide substrate H-d-prolyl-l-phenylalanyl-l-arginine-p-nitroanilide dihydrochloride (S-2302; where H is a non-substituted amino acid; 20 nmol; Chromogenix) was incubated with kallikrein (enzyme/substrate ratio, 1:600) in the presence of dextran sulphate in 1 ml of 10 mM Tris/HCl, 100 mM NaCl, pH 7.8, at room temperature. The rate of p-nitroaniline formation was measured by the increase in absorbance at 405 nm at 15 s intervals [43]. A buffer blank containing the substrate without the enzyme was included. Apoferritin prepared from spleen ferritin by thioglycollate treatment was mixed with the reaction buffer containing the enzyme and the rate of p-nitroaniline formation from the substrate was measured. An appropriate blank with apoferritin and substrate lacking the enzyme was also included.

Gel electrophoresis

SDS/PAGE was performed as described by Laemmli [44] using 10–20 % gradient minigels (Bio-Rad). Protein samples were heated at 95 °C for 3 min in sample buffer containing 1 %, SDS, 30 mM Tris/HCl, pH 6.8, and 5 % glycerol, with or without 0.1 M DTT.
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Figure 1 Time course of digestion of H-kininogen with plasma kallikrein

(A) SDS/PAGE analysis (reducing conditions) of the time course of cleavage of human H-kininogen by plasma kallikrein. Single-chain human H-kininogen was incubated with plasma kallikrein at a molar ratio of 600:1 in the presence of dextran sulphate at 25°C for the indicated time intervals. The digest was reduced with 0.1 M DTT, heated at 95°C for 3 min to inactivate the enzyme and subjected to SDS/PAGE (10–20% gradient gels). The proteins were revealed by staining with Coomassie Brilliant Blue. (B) SDS/PAGE analysis (reducing conditions) of the time course of binding of human spleen ferritin to plasma kallikrein-cleaved polypeptide chains of H-kininogen. After digestion of human H-kininogen with plasma kallikrein and SDS/PAGE as described for (A), ferritin-binding proteins were detected by ligand blotting as described in the Materials and methods section. (C) SDS/PAGE analysis (non-reducing conditions) of the time course of cleavage of human H-kininogen by plasma kallikrein. After digestion of human H-kininogen with plasma kallikrein as described for (A), the proteins in the digest were analysed by SDS/PAGE. The proteins were visualized by staining with Coomassie Brilliant Blue. Note that, under non-reducing conditions the migration of intact kininogen was slightly retarded relative to reducing conditions (apparent molecular masses of 120 and 114 kDa respectively). (D) SDS/PAGE analysis (non-reducing conditions) of the time course of binding of human spleen ferritin to plasma kallikrein-cleaved polypeptide chains of H-kininogen. After digestion of human H-kininogen with plasma kallikrein and SDS/PAGE as described for (A), ferritin-binding proteins were detected by ligand blotting as described in the Materials and methods section.

RESULTS

Localization of ferritin binding in H-Kininogen

H-kininogen was digested with plasma kallikrein, subjected to SDS/PAGE under reducing conditions, and cleavage products revealed by Coomassie Brilliant Blue staining. Figure 1(A) shows the time course of this digestion. By 10 min, the H-kininogen (114 kDa) was digested and converted to its constituent chains; heavy chain (63 kDa), light chain (58 kDa) and modified light chain (45 kDa). As the digestion proceeded, the 58 kDa light chain was converted to 45 kDa modified light chain, and, upon complete digestion (7 h), the H-kininogen gave rise to only two chains, 63 kDa heavy chain and modified 45 kDa light chain. The cleavage pattern is consistent with previously published reports [22,42,45]. In order to determine which of these chains of H-kininogen possessed ferritin-binding activity, the proteins were transferred on to a PVDF membrane and analysed by ligand blotting with spleen ferritin. As shown in Figure 1 (B), ferritin-binding activity was associated with the intact 114 kDa H-kininogen and its light chains (58 and 45 kDa). The 63 kDa heavy-chain band did not bind to ferritin. The time course of digestion followed by ligand blotting indicated that the ferritin-binding site is localized on the modified 45 kDa light chain of H-kininogen (7 h digest). The identity of this modified light chain was confirmed further by determination of the N-terminal amino acid sequence, which was found to be GHGLGH(H)HEQ (where the H in parentheses was a tentative designation in the original analysis [17]), corresponding to amino acids 440–449 of H-kininogen [46]. Interestingly, there was apparent preferential binding of ferritin to reduced H-kininogen (Figure 1B) compared with intact H-kininogen (Figure 1D). Although we have no certain explanation for this finding, it is possible that disulphide-bond reduction modifies structural features of H-kininogen, facilitating access of ferritin.

Differential binding of ferritin to intact H-kininogen versus kallikrein-digested H-kininogen

Following kallikrein digestion during contact activation, H-kininogen undergoes a conformational change and exposes its light chain, facilitating binding to proteins involved in the coagulation cascade [47]. As the ferritin-binding site is localized to the light chain of H-kininogen, it was of interest to examine whether there were differences in the binding of ferritin to intact H-kininogen versus kallikrein-digested kininogen in the presence
of dextran sulphate, a surface used to initiate the intrinsic coagulation cascade. Figure 1(C) shows the time course of digestion of H-kininogen by plasma kallikrein as revealed by SDS/PAGE under non-reducing conditions followed by Coomassie Brilliant Blue staining. Consistent with reports published previously [25,45], with increasing digestion time, the single-chain 120 kDa H-kininogen was converted to 103 and 95 kDa cleavage products, with the 95 kDa band predominating on prolonged digestion. Ferritin ligand blotting (Figure 1D) of this gel showed a consistent stronger intensity of kallikrein-derived chains (103 and 95 kDa) compared with the intact 120 kDa chain. These results suggested that kallikrein digestion enhances the ability of H-kininogen to bind ferritin.

To quantitatively assess the differential binding of ferritin to intact versus kallikrein-digested kininogen, as well as to test whether the preferential binding of ferritin to nicked kininogen would also be observed under non-denaturing conditions, a plate assay was developed. The binding of biotinylated spleen ferritin to microtitre plates coated with intact kininogen was compared with that of kallikrein-digested kininogen. As shown in Figure 2, biotinylated spleen ferritin binds to kallikrein-digested kininogen in a dose-dependent manner. Furthermore, the binding of ferritin to kallikrein-digested kininogen is greater than binding to intact kininogen. For example, at 4 μg of kininogen, binding was 70-fold greater to nicked than intact kininogen (Figure 2).

Effect of ferritin on the cleavage of H-kininogen by kallikrein

To determine whether ferritin binding to H-kininogen might affect the cleavage of the latter by plasma kallikrein, single-chain H-kininogen was incubated with plasma kallikrein for 0–360 min in either the absence or presence of spleen ferritin. The pattern of cleavage was assessed by SDS/PAGE under non-reducing conditions using Coomassie Brilliant Blue staining. Figure 3(A) shows the time course of cleavage of single-chain H-kininogen by kallikrein in either the absence or presence of spleen ferritin. In the absence of ferritin, the intact 120 kDa H-kininogen band slowly disappeared with the concomitant appearance of 103 and 95 kDa bands during digestion with kallikrein. These bands both represent cleaved kininogen polypeptide chains [42]. In the presence of ferritin, the 120 kDa H-kininogen band persisted, even after 180 and 360 min of digestion, whereas it was absent in the control digests without ferritin. Densitometric analysis

Figure 3 (A) Effect of the addition of spleen ferritin and apoferritin on cleavage of human plasma H-kininogen and (B) densitometric quantification of the H-kininogen bands on SDS/PAGE gels in (A)

(A) Human plasma H-kininogen was digested with plasma kallikrein in either the absence or presence of spleen ferritin or apoferritin added in a 1:1 molar ratio 5 min following the addition of kallikrein. Digestion was continued for the additional indicated periods of time and stopped by heating. The digests were analysed by SDS/PAGE under non-reducing conditions and proteins were revealed by Coomassie Brilliant Blue staining. (B) Cleavage of H-kininogen by kallikrein was calculated (average of triplicate analyses) from the band intensities of intact kininogen (120 kDa band) and cleavage products (103 and 95 kDa bands).
confirmed that the presence of ferritin retarded kallikrein-mediated cleavage of intact H-kininogen (120 kDa) to its 103 and 95 kDa cleavage products (Figure 3B). Slowing of H-kininogen cleavage was related to the primary structure of the ferritin protein rather than its iron content, since apoferritin also retarded kallikrein cleavage of H-kininogen (Figure 3).

Effect of ferritin on the release of bradykinin from H-kininogen during digestion with plasma kallikrein

Bradykinin is a potent vasoactive nonapeptide that is released from H-kininogen upon proteolytic digestion with plasma kallikrein. Because the results above suggested that ferritin delays kallikrein cleavage of H-kininogen, we next wished to determine whether ferritin binding to H-kininogen would affect the release of bradykinin during plasma kallikrein digestion of H-kininogen. Figure 4 shows the time course of release of bradykinin from H-kininogen during digestion with plasma kallikrein in the absence or presence of spleen ferritin, as assessed by HPLC. There was a gradual time-dependent release of bradykinin. The release of bradykinin was complete (95\%) at the end of the 4 h digestion. In order to examine whether ferritin might affect the release of bradykinin from H-kininogen, H-kininogen was initially nicked by kallikrein treatment for 5 min followed by the immediate addition of spleen ferritin. The digestion was continued for various periods of time in the presence of ferritin, and bradykinin release was measured. As shown in Figure 4, there was no release of bradykinin during 5 min nicking of H-kininogen. Furthermore, N-terminal sequence analysis of the 58 kDa light chain generated after 5 min digestion gave rise to the sequence SSRIGEIK (corresponding to amino acids 372–379 of H-kininogen), consistent with the first scission of H-kininogen [21] on the C-terminal side of bradykinin (which is intact and still linked to the heavy chain through its N-terminus). HPLC analysis of bradykinin released in the presence of ferritin indicated that virtually no bradykinin was released in the presence of ferritin (Figure 4, lower panel). Furthermore, bradykinin was quantitatively recovered (100\%) if this peptide was included in a reaction.

Figure 4  Effect of ferritin on the kallikrein-mediated release of bradykinin from H-kininogen

Human plasma H-kininogen (1 nmol) was treated with plasma kallikrein for 5 min to produce nicked kininogen. Human spleen ferritin (1 nmol) was added to the digest and digestion continued for various periods of time, as indicated. An aliquot of these digests was analysed for bradykinin by reverse-phase HPLC as described in the Materials and methods section. Bradykinin synthetic peptide standard (Peninsula Laboratories) had the same retention time (24.7 min) as the bradykinin peak (BK) released from H-kininogen shown in the chromatogram. Bradykinin release was also measured in the presence of apoferritin prepared from spleen ferritin by thioglycollate treatment (results not shown).
mixture containing H-kininogen and ferritin (results not shown). This observation rules out the possibility of a physical association of bradykinin with ferritin hindering the release of this peptide in the presence of added ferritin.

Spleen ferritin contains higher proportions of iron than serum ferritin [48]. It was therefore of interest to examine whether apoferritin might also block the release of bradykinin. Bradykinin release was measured in the absence and presence of spleen apoferritin prepared by thioglycollate treatment. This experiment demonstrated that apo- and holo-spleen ferritin were indistinguishable in their ability to block the release of bradykinin from H-kininogen (results not shown).

Effect of ferritin on kallikrein activity

To test the possibility that ferritin might inhibit the release of bradykinin from H-kininogen by interfering with the enzymic activity of plasma kallikrein, we measured the effect of ferritin on kallikrein activity using a chromogenic kallikrein substrate, H-o-prolyl-L-phenylalanyl-L-arginine-p-nitroanilide dihydrochloride (S-2302). For these experiments, kallikrein digestion of substrate was measured in the presence or absence of apoferritin, added in a 1:1 molar ratio. These experiments utilized apoferritin because the absorbance of holoferitin interferes with spectrophotometric measurements of substrate cleavage. Figure 5 shows the time course of release of chromogen measured spectrophotometrically at 410 nm following kallikrein digestion of the substrate in either the presence or absence of spleen apoferritin. The results demonstrate that apoferritin does not affect the activity of kallikrein. Thus the inhibition of bradykinin release by ferritin is not attributable to a generalized inhibition of kallikrein activity by ferritin.

**DISCUSSION**

In our initial observation identifying H-kininogen as a ferritin-binding protein in human serum [17], we did not localize the ferritin-binding region in H-kininogen or determine whether ferritin-kininogen binding might affect the proteolytic cleavage of kininogen by kallikrein and hence bradykinin release. H-kininogen, on treatment with kallikrein, initially produces a nicked kininogen [49]: cleavage occurs at the C-terminal end of bradykinin, leaving the bradykinin peptide attached through its N-terminus to H-kininogen. Further digestion results in the release of bradykinin and, on reduction with DTT, H-kininogen is converted to a 63 kDa heavy chain and 58 kDa light chain. Prolonged cleavage of H-kininogen results in the conversion of the 58 kDa light chain to its modified light chain of 45 kDa [45,46]. Our previous results demonstrated that ferritin did not bind to L-kininogen (low-molecular-weight kininogen) [17]. Since L-kininogen and H-kininogen share identical heavy chains but differ in their light-chain moieties, we postulated that ferritin might bind to the light chain of H-kininogen. The results presented here provide direct evidence that ferritin indeed associates with the light chain of H-kininogen, and further delimit the binding domain to the C-terminal amino acids of H-kininogen that comprise the 45 kDa modified light chain. This region of H-kininogen contains domains 5 and 6, domains involved in cellular binding [50], prekallikrein binding [18] and inhibition of endothelial-cell proliferation and angiogenesis [50]. Kallikrein is a proteolytic enzyme that cleaves H-kininogen. Whereas ferritin does not affect the enzymic activity of kallikrein (Figure 5), we found that ferritin binding to H-kininogen retards kallikrein-mediated cleavage of H-kininogen (Figure 3). Further, ferritin binding to kininogen inhibits the release of bradykinin by kallikrein (Figure 4). Although our experiments utilized spleen ferritin and not serum ferritin (which is not readily available in quantities required for these biochemical experiments), spleen and serum ferritin share some similarities, including immunological cross-reactivity [51], as well as differences, notably in glycosylation [51] and the extent of iron binding [38]. Our experiments did not detect an effect of iron content on ferritin binding to kininogen; however, additional experiments will be required to test whether other differences affect the interaction between H-kininogen and ferritin.

Binding assays employing ferritin ligand blotting (Figure 1D) and plate assays (Figure 2) demonstrated preferential binding of ferritin to kallikrein-cleaved kininogen versus intact kininogen. The plate assay was performed under non-denaturing physiological conditions, whereas the ligand blot assay measures the binding of ferritin to kininogen under denaturing conditions. Therefore, it is reasonable to conclude that ferritin binds to native as well as denatured H-kininogen and their nicked counterparts.

Based on these composite results, we propose the following model for the effect of ferritin on kallikrein-mediated cleavage of H-kininogen and the subsequent release of bradykinin (Scheme 1). Following an initial activation step in which H-kininogen is nicked by kallikrein (at the C-terminus of the bradykinin peptide), the light chain of H-kininogen becomes available to bind ferritin. The large molecular mass of the bound ferritin (460–500 kDa) sterically hinders kallikrein from further cleavage at the N-terminus of bradykinin, thereby blocking the release of bradykinin.

The physiological concentration of ferritin in normal human serum or plasma is 20–100 ng/ml or ≈50–200 fmol/ml [4]. Furthermore, human serum has been shown to contain ferritin-binding proteins [8–11] other than H-kininogen. Therefore it is expected that the concentration of ferritin-bound kininogen in normal human serum or plasma is <50–200 fmol/ml. However, despite these low concentrations, ferritin-bound kininogen has the potential to exert a biologically important effect. Recent evidence suggests that H-kininogen localizes and perhaps is activated on the surface of endothelial cells; H-kininogen docked on the endothelial-cell surface might serve as a recruitment site for ferritin [52,53]. In addition, bradykinin, the potent vasoactive peptide produced through cleavage of H-kininogen by kallikrein, is present in normal plasma at concentrations of 2–8 fmol/ml [54–56]. Partially cleaved, kinin-containing H-kininogen has also
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Scheme 1 Proposed mechanism of blockage of kallikrein-mediated bradykinin release from H-kininogen by ferritin

K₁ indicates the initial site of cleavage of H-kininogen by plasma kallikrein, resulting in a nicked kininogen with bradykinin still attached to it. K₂ is the second site of cleavage releasing bradykinin. Ferritin binds to the light chain of nicked kininogen, poses steric hindrance for further cleavage and therefore bradykinin release is blocked. Ferritin also retards cleavage at K₁ (not shown).

BK, bradykinin.

been reported in plasma [49]. This suggests that a small proportion of H-kininogen is subject to cleavage and release of bradykinin under normal physiological conditions [18]. We hypothesize that it is this pool of activated kininogen, present at concentrations less than or equal to concentrations of serum ferritin, that may be regulated through interaction with ferritin. Recent reports that levels of bradykinin may be transiently increased 10–20-fold during cardiopulmonary bypass suggests that, under selected pathophysiological conditions, the potential of ferritin to buffer such a response may acquire increased importance [57]. Further experiments will be required to directly address the binding of ferritin to kininogen and its effects on bradykinin release in vivo.

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