Heavy Chain of Human High Molecular Weight and Low Molecular Weight Kininogens Binds Calcium Ion†

Shigeki Higashiyama, Iwao Ohkubo,* Hiroshi Ishiguro, and Makoto Sasaki

Department of Biochemistry, Nagoya City University Medical School, Mizuho-ku, Nagoya 467, Japan

Tsukasa Matsuda and Ryo Nakamura

Department of Food Science and Technology, Faculty of Agriculture, Nagoya University, Chikusa-ku, Nagoya 464, Japan

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ABSTRACT: An antibody subpopulation, anti high molecular weight (anti-HMW) kininogen–Ca^{2+} antibody able to bind specifically to the HMW kininogen–Ca^{2+} complex, was isolated from anti-HMW kininogen antiserum. Partially purified anti-HMW kininogen antibody was applied to a HMW kininogen–Sepharose column equilibrated with 40 mM tris(hydroxymethyl)aminomethane hydrochloride buffer, pH 7.5, containing 1.0 M NaCl and 1 mM $CaCl_2$, and anti-HMW kininogen–Ca^{2+} antibody was eluted with 5 mM ethylenediaminetetraacetic acid. As a result of characterization by enzyme-linked immunosorbent assay, this antibody specifically recognized the cyanogen bromide cleaved fragment 1 (CB-1) region (1–160 amino acid sequence) of the heavy chain of kininogen molecules in the presence of Ca^{2+} or Mg^{2+}. Furthermore, circular dichroism (CD) experiments showed that the conformational changes of HMW kininogen and heavy chain were induced by metal ions such as Ca^{2+} and Mg^{2+} and that these changes were due to the conformational change of the CB-1 region of the heavy chain. The dissociation constant (K_d) for the heavy chain–Ca^{2+} measured by CD analysis at 214 nm was found to be 0.33 ± 0.09 mM (mean ± SD). The number of Ca^{2+}-binding sites of heavy chain calculated from the Hill plot was 1.15 ± 0.04 (mean ± SD). Then, a possible Ca^{2+}-binding site was found in the amino-terminal portion of the heavy chain of kininogen molecules.

Kininogens, the precursor molecules of kinins, are glycoproteins present in plasma (Kato et al., 1981). Kininogens, i.e., high molecular weight (HMW)[1] kininogen and low molecular weight (LMW) kininogen, have, so far, been found in mammalian plasma. In addition to their function as kinin precursors, the two kininogens play an important role in the inhibition of thiol proteinases (Ohkubo et al., 1984; Sueyoshi et al., 1985; Müller-Esterl et al., 1985; Higashiyama et al., 1986). This latter function is mediated by their heavy-chain molecules which are identical for the HMW and LMW kininogens (Ohkubo et al., 1984; Takagaki et al., 1985). The heavy chain contains three cystatin-like repeat sequences termed domains 1, 2, and 3 resulting from gene triplication (Salvesen et al., 1986). Each of the reactive sites of the thiol proteinase inhibitor is located in domains 2 and 3 (Ohkubo et al., 1984; Salvesen et al., 1986). However, the physiological function of domain 1 still remains unknown.

On the other hand, HMW kininogen, which forms complexes with factor XI (Thompson et al., 1977) or prekallikrein (Mandle et al., 1976; Tait & Fujikawa, 1986) on the carboxyl-terminal region of the light chain in blood circulation, functions as a cofactor in the initial phase reactions of the intrinsic blood coagulation cascade by binding to negatively charged surfaces through a so-called histidine-rich fragment, fragment 1·2 moiety (Griffin & Cochrane, 1976; Davie et al., 1979; Heimark et al., 1980; Sugo et al., 1980). Furthermore, it has also been demonstrated that Zn^{2+}, Co^{2+}, Cu^{2+}, and Ni^{2+}, which accelerate the contact activation of intrinsic coagulation, bind to the fragment 1·2 moiety, but Ca^{2+} and Mg^{2+} do not (Shimada et al., 1984).

Recently, it has been reported that the conformation-specific antibodies directed against metal-binding protein–metal complexes, e.g., prothrombin–Ca^{2+} complex (Furie & Furie, 1979), factor IX–Ca^{2+} complex (Liebman et al., 1985), and thrombospondin–Ca^{2+} complex (Dixit et al., 1986), were produced during immunization. In order to obtain the specific antibodies against the fragment 1·2 region since this region could bind metal ions such as Zn^{2+}, Co^{2+}, Cu^{2+}, or Ni^{2+}, we tried to prepare anti-HMW kininogen–Zn^{2+} antibody with the same procedure used to obtain the antibodies against the protein–metal ion complexes described above. Actually, we obtained an anti-HMW kininogen–Zn^{2+} antibody, but the antibody did not recognize the fragment 1·2 region. Interestingly, we obtained the antibody (anti-HMW kininogen–Ca^{2+} antibody) recognizing the conformational change which was induced by Ca^{2+} and also found that the antibody did not recognize the fragment 1·2 region.

In the present studies, we show that the anti-HMW kininogen–Ca^{2+} antibody was specifically raised against the domain 1 region, which underwent a Ca^{2+}-induced conformational change, of heavy chain. Further, we speculate that there is a possible Ca^{2+}-binding site in the amino-terminal portion

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* Correspondence should be addressed to this author.

[1] Abbreviations: HMW kininogen, high molecular weight kininogen; LMW kininogen, low molecular weight kininogen; TPI, thiol proteinase inhibitor; SDS, sodium dodecyl sulfate; *p*-APMSF, (*p*-amidinophenyl)-methanesulfonyl fluoride; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; HRPO, horseradish peroxidase; Tris, tris(hydroxymethyl)aminomethane; CNBr, cyanogen bromide; CD, circular dichroism; CB-1, cyanogen bromide cleaved fragment 1; FPLC, fast protein liquid chromatography; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; PVP, poly(vinylpyrrolidone).

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of the heavy chain of kininogens.

Materials and Methods

Materials

Fresh human plasma was separated as immediately as possible from citrated whole blood obtained from volunteer students at this medical school. Human plasma was also separated from outdated citrated whole blood supplied by the Aichi Red Cross Blood Center, Nagoya, Japan. Trypsin, chymotrypsin, and cyanogen bromide (CNBr) were obtained from Sigma Chemical Co., St. Louis, MO. DEAE-Sephadex A-50, Red Sepharose, Sephacryl S-300, and Sephadex G-75 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. The membrane filter Molcot II GC was a product of Nihon Millipore Ltd., Tokyo, Japan. Zn chelate–Sepharose was prepared as described previously (Porath et al., 1975). Peroxidase-conjugated goat anti-rabbit γ-globulin was obtained from Cooper Biomedical, Inc., Malvern, PA. *o*-Phenylenediamine, complete Freund's adjuvant, (*p*-amidinophenyl)-methanesulfonyl fluoride (*p*-APMSF), and phenylmethanesulfonyl fluoride (PMSF) were products of Wako Pure Chemical Industries, Ltd., Osaka, Japan. Polystyrene Microtiter plates were purchased from A/S Nunc, Roskilde, Denmark.

All other chemicals were of analytical grade.

Methods

Proteins. HMW kininogen was isolated from fresh citrated human plasma within 12 h by two-step column chromatographies on DEAE-Sephadex A-50 and Zn chelate–Sepharose 4B as described previously (Higashiyama et al., 1986). Kinin-free HMW kininogen was also purified from outdated human plasma by a procedure similar to that used for native HMW kininogen (Higashiyama et al., 1986). Kinin-free LMW kininogen, which corresponds to α_2TPI, was also purified as described previously (Ohkubo et al., 1984). All samples except for HMW kininogen migrated as a homogeneous single band on SDS–polyacrylamide slab gel electrophoresis. The purified HMW kininogen gave two bands on nonreduced SDS–polyacrylamide slab gel electrophoresis, a major band of a native form and a minor band of a nicked or kinin-free form as described in a previous paper (Higashiyama et al., 1986).

Isolation of Heavy Chain and Fragment 1·2–Light Chain of HMW Kininogen. Heavy chain and fragment 1·2–light chain were isolated from kinin-free HMW kininogen on a Zn chelate–Sepharose 4B column and a Sephacryl S-300 column as follows. Ten milligrams of kinin-free HMW kininogen in 10 mL of 25 mM Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl was incubated overnight with 1.4 mM β-mercaptoethanol at room temperature. The sample was directly applied to a Zn chelate–Sepharose 4B column (1.2 × 5 cm) equilibrated with the above buffer. Only heavy chain passed through the column, and fragment 1·2–light chain and unreduced kinin-free HMW kininogen bound to it. The pass-through protein was dialyzed against a large volume of 20 mM Tris-HCl buffer, pH 7.5, without reducing reagent for 24 h at 4 °C to achieve mild reoxidation. The proteins bound to the column were eluted with the same buffer containing 50 mM EDTA. This procedure on the column was repeated twice. After being concentrated with Molcot II GC, the fraction eluted with EDTA was chromatographed on a Sephacryl S-300 column (2 × 140 cm) with 20 mM Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl. The isolated heavy and fragment 1·2–light chains gave a single band on SDS–polyacrylamide gel electrophoresis.

Isolation of Heavy-Chain Fragments. The fragment which was composed of the amino-terminal region (1–160 amino acid sequence) of the heavy chain and light chain (372–409 amino acid sequence) of LMW kininogen held together with a disulfide bond, tentatively named CB-1, was obtained from kinin-free LMW kininogen (α_2TPI) by CNBr treatment as follows. Ten milligrams of kinin-free LMW kininogen was dissolved in 5 mL of 70% formic acid, and 10 mg of CNBr was added to the solution. Digestion was performed overnight in a sealed tube at room temperature. The reaction mixture was chromatographed by gel filtration on a Sephadex G-75 superfine column (3 × 142 cm) equilibrated with 20 mM Tris-HCl buffer, pH 7.5. The fractions containing CB-1 fragment were collected and further applied on a TSK gel DEAE-5PW column (Toyo Soda, Tokyo, Japan) using the Pharmacia fast protein liquid chromatography system (FPLC) (Pharmacia, Uppsala, Sweden).

Domain 2 (123–244 amino acid sequence) and domain 3 (245–362 amino acid sequence) of the heavy chain were isolated from kinin-free LMW kininogen according to the procedure of Salvesen et al. (1986). Fifty milligrams of kinin-free LMW kininogen was incubated with 100 μg of trypsin in 15 mL of 50 mM Tris-HCl buffer, pH 7.5, for 90 min at 30 °C. The digestion was stopped by the addition of *p*-APMSF to give a final concentration of 2 mM. The digested sample was chromatographed successively on a Sephadex G-75 superfine column and a TSK gel DEAE-5PW column. The tryptic peptide with a molecular weight of 20 000 was isolated as a domain 3. The tryptic peptide with a molecular weight of 40 000 copurified with the above columns was further digested with chymotrypsin at a chymotrypsin to peptide weight ratio of 1:25 in 50 mM Tris-HCl buffer, pH 7.5, for 6 h at 30 °C, followed by the addition of PMSF to give a final concentration of 2 mM. Chymotrypsin-digested fragment, domain 2, was also isolated with a Superose column (Pharmacia, Uppsala, Sweden) using the FPLC system. Preparation methods of CB-1, domain 2, and domain 3 are summarized in Figure 1. The amino acid compositions and N-terminal amino acids of CB-1, domain 2, and domain 3 corresponded to those of sequences 1–160 and 372–409, 123–244, and 245–362 of LMW kininogen, respectively. The results of domains 2 and 3 also corresponded to those reported by Salvesen et al. (1986).

Preparation of HMW Kininogen Covalently Bound to Sepharose. HMW kininogen was coupled to cyanogen bromide activated Sepharose using standard methods (Cuatrecasas et al., 1968).

Preparation of Antibody Subpopulations. Antibody against HMW kininogen was prepared from New Zealand white rabbits according to the procedure described in Ohkubo et al. (1984). The antiserum (75 mL) obtained from the rabbits was partially purified by subsequent ammonium sulfate fractionation and DEAE-Sephacel column chromatography. The γ-globulin fraction was then dialyzed extensively against 40 mM Tris-HCl buffer, pH 7.4, containing 1 M NaCl and 1 mM $CaCl_2$. The sample was applied to a HMW kininogen–Sepharose column (2 × 10 cm) equilibrated with the same buffer. Antibody that bound to the column was eluted with 40 mM Tris-HCl buffer, pH 7.4, containing 1 M NaCl and 5 mM EDTA. The antibody subpopulation eluted with this buffer was designated as anti-HMW kininogen–Ca^{2+} antibody. The remaining antibody was eluted with 4 M guanidine hydrochloride and immediately dialyzed at 4 °C against 40 mM Tris-HCl-buffered saline, pH 7.4.

Enzyme-Linked Immunosorbent Assay (ELISA). Enzyme-linked immunosorbent assay (ELISA) was essentially

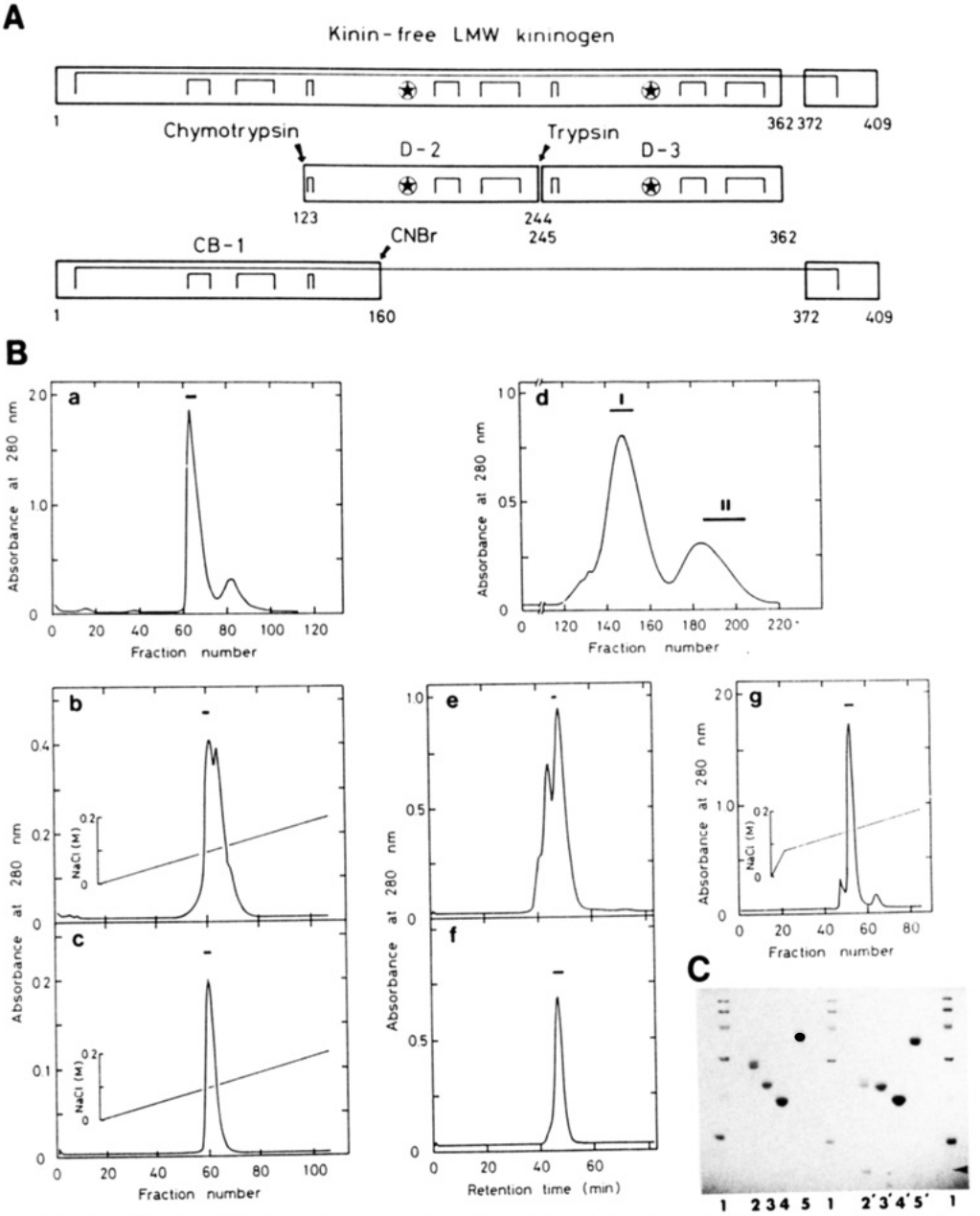

FIGURE 1: Linear model and purification of kininogen fragments, CB-1, domain 2, and domain 3. (A) Linear model of kinin-free LMW kininogen and its fragments produced by cleavages with trypsin, chymotrypsin, or CNBr. The two stars indicate predicted reactive sites for inhibition of thiol proteinases (Ohkubo et al., 1984). (B) Purification of CB-1, domain 2, and domain 3. (a) shows Sephadex G-75 column chromatography of the CNBr digest of kinin-free LMW kininogen. Fractions containing CB-1 were collected. The bar indicates the fractions pooled. (b) and (c) show TSK gel DEAE-5PW column chromatography and rechromatography of the CB-1 fraction described above using the FPLC system, respectively. Bars indicate the fractions pooled. (d) shows Sephadex G-75 column chromatography of the tryptic digest of kinin-free LMW kininogen. Bars indicate the fractions collected: I, domains 1·2–light chain (1–244 and 372–409 amino acid sequences held together with a disulfide bond); II, domain 3. (e) and (f) show Superose column chromatography and rechromatography of the chymotrypsin digest of domains 1·2–light chain (I), respectively. Bars indicate the fractions pooled. (g) shows TSK gel DEAE-5PW column chromatography of domain 3 fraction (II). The bar indicates the fractions collected. (C) SDS–polyacrylamide slab gel electrophoresis of CB-1, domain 2, domain 3, and domains 1·2. Electrophoresis was carried out on a 15% polyacrylamide slab gel in the presence of 0.1% SDS, and the gel was stained with Coomassie brilliant blue R-250. Lanes 2, 3, 4, and 5 were 3 μg of CB-1 (c), domain 2 (f), domain 3 (g), and domains 1·2–light chain (d, I), respectively, before reduction with β-mercaptoethanol; lanes 2′, 3′, 4′, and 5′ were 3 μg of fragments described above after reduction; lane 1 included the following standard proteins: phosphorylase *b* (M_r 94 000), albumin (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), soybean trypsin inhibitor (M_r 20 000), and α-lactalbumin (M_r 14 000). The arrowhead indicates the light chain of kinin-free LMW kininogen.

performed following the method of Engvall and Perlmann (1971). Polystyrene microtiter plates were coated overnight at 4 °C with 100 μL of protein solution (125 ng/mL) in 20 mM Tris-HCl buffer, pH 7.5, and washed 3 times with 20 mM Tris-HCl-buffered saline, pH 7.5, containing 0.05% Tween 20 and 3 mM EDTA (TBS–Tween/EDTA) or 20 mM Tris-HCl-buffered saline, pH 7.5, containing 0.05% Tween 20 and 3 mM $CaCl_2$ (TBS–Tween/$CaCl_2$). One hundred microliters of antibody solution appropriately diluted with TBS–Tween/EDTA or TBS–Tween/$CaCl_2$ containing 2% PVP and 0.2% BSA was added to each well, and the plates were incubated for 120 min at room temperature. The plates were then washed 4 times with TBS–Tween/EDTA or TBS–Tween/$CaCl_2$. One hundred microliters of peroxidase-coupled goat anti-rabbit immunoglobulins appropriately diluted with TBS–Tween/EDTA or TBS–Tween/$CaCl_2$ containing 2%

PVP and 0.2% BSA was added to each well. The reaction mixture was incubated for 120 min and washed thoroughly with TBS–Tween/EDTA or TBS–Tween/$CaCl_2$. After the reaction mixture was washed, 100 μL of substrate (0.04% *o*-phenylenediamine and 0.003% H_2O_2 in 0.1 M citrate–phosphate buffer, pH 5.0) was added, and the plates were incubated for 15 min at room temperature. The reaction was stopped by the addition of 50 μL of 2 N H_2SO_4. The amount of peroxidase products in each well was estimated spectrophotometrically at a wavelength of 492 nm.

Circular Dichroism Measurements. Circular dichroism (CD) experiments were carried out at an ambient temperature at 20 ± 2 °C with a JASCO J-40CS instrument equipped with a data processor (Japan Spectroscopic Co., Ltd., Tokyo, Japan) under the conditions of a 1.0-mm path length and a scan rate of 2 nm/min. All of the proteins were dissolved in 20 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA and centrifuged to remove dust particles. In the preparation of the samples for Ca^{2+} titration analysis, the proteins were dialyzed in plastic containers against 5 mM EDTA in 20 mM Tris-HCl-buffered saline, pH 7.5, to remove traces of Ca^{2+}. EDTA was then removed by extensive dialysis against 20 mM Tris-HCl-buffered saline, pH 7.5, which was freed from Ca^{2+} by Chelax (Bio-Rad Laboratories, Richmond, CA). The dialysis bags were pretreated with heavy-metal cleaning solution (Spectrum Medical Industries Inc., Los Angeles, CA). Base lines were subtracted from the experimental spectra by using the data processor. The instrument was calibrated according to manufacturer's specifications with the use of (+)-10-camphorsulfonic acid. The data were expressed as mean residue ellipticities in degrees centimeter squared per decimole, taking a mean residue weight of 115 in all cases.

Determination of Protein Concentration. The concentration of the proteins, including heavy chain, CB-1, domain 2, and domain 3, used in CD experiments was determined by the method of Lowry et al. (1951). Bovine serum albumin was used as a standard protein. In all experiments except for CD experiments, the concentrations of HMW kininogen, LMW kininogen, and immunoglobulin G were determined by employing $E^{1\%}_{280nm}$ values of 7.3 for HMW kininogen, 7.8 for LMW kininogen (Müller-Esterl & Fritz, 1984), and 13.5 for immunoglobulin G (Stevenson & Dorrington, 1970).

Prediction of Secondary Structure. Secondary structure was predicted according to the method of Chou and Fasman (1974).

Polyacrylamide Gel Electrophoresis. Slab gel electrophoresis was carried out on 15% polyacrylamide gels in 25 mM Tris, 192 mM glycine, pH 8.3, and 0.1% SDS by the method of Laemmli (1970).

RESULTS

Preparation of Anti-HMW Kininogen–Ca^{2+} Antibody. Rabbit immunoglobulin G fraction was applied to a HMW kininogen–Sepharose column in the presence of 1 mM $CaCl_2$. After removal of unbound protein, anti-HMW kininogen–Ca^{2+} antibody was eluted with 5 mM EDTA (Figure 2). The remaining anti-HMW kininogen antibody was eluted with 4 M guanidine hydrochloride. The anti-HMW kininogen–Ca^{2+} antibody was about 8% of the antibody raised against HMW kininogen.

Characterization of Anti-HMW Kininogen–Ca^{2+} Antibody. The effect of Ca^{2+} on the interaction of anti-HMW kininogen–Ca^{2+} antibody with HMW kininogen was studied. Anti-HMW kininogen–Ca^{2+} antibody reacted slightly with HMW kininogen in the presence of 3 mM EDTA. When Ca^{2+} was substituted for EDTA, an approximately 5-fold increase

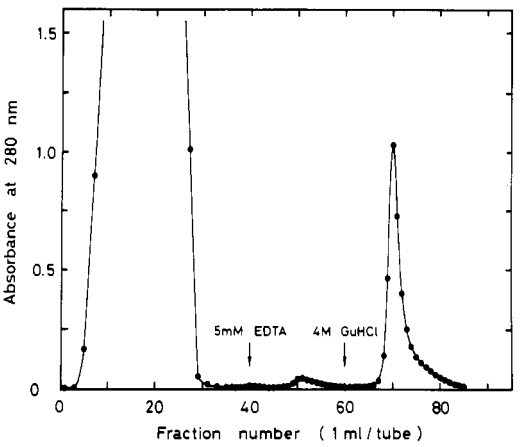

FIGURE 2: Purification of anti-HMW kininogen antibody specific to HMW kininogen–Ca^{2+} complex. Immunoglobulin G fractions were applied on a HMW kininogen–Sepharose column equilibrated with 40 mM Tris-HCl buffer, pH 7.4, containing 1.0 M NaCl and 1 mM $CaCl_2$. Anti-HMW kininogen–Ca^{2+} antibody, which recognized the conformational change of HMW kininogen induced by Ca^{2+}, was eluted with 5 mM EDTA. The remaining anti-HMW kininogen antibody was eluted with 4 M guanidine hydrochloride (GuHCl).

of the reactivity was observed (Figure 3A). On the other hand, Ca^{2+} gave no effects on the interaction of the anti-HMW kininogen antibody eluted with 4 M guanidine hydrochloride and HMW kininogen. These data indicate that the anti-HMW kininogen–Ca^{2+} antibody recognizes the conformational change of HMW kininogen induced by metal ion such as Ca^{2+} and Mg^{2+}, even though its change is not so drastic.

The characteristics of the anti-HMW kininogen–Ca^{2+} antibody were studied by ELISA with the heavy chain and the fragment 1·2–light chain of HMW kininogen as antigens. Under the conditions used, anti-HMW kininogen–Ca^{2+} antibody was capable of binding to the HMW kininogen and the heavy chain, but not in the least to the fragment 1·2–light chain (Figure 3B). Furthermore, in order to investigate the localization of antigenic site(s) on the heavy chain recognized by the anti-HMW kininogen–Ca^{2+} antibody, reactivities between the antibody and CB-1, domain 2, or domain 3 were studied by ELISA in the presence of 3 mM $CaCl_2$. As shown in Figure 3C, in the presence of 3 mM $CaCl_2$, the antibody was capable of interacting with CB-1 but not with domain 2 and domain 3. Magnesium also gave the same effect on the interaction between the antibody and CB-1, but Zn^{2+} did not (Figure 3D). On the basis of these data, it is strongly suggested that anti-HMW kininogen–Ca^{2+} antibody was raised against the conformational change of the domain 1 region (1–122 amino acid sequence) induced by metal ions such as Ca^{2+} and Mg^{2+}.

Ca^{2+}-Induced CD Changes of HMW Kininogen. The CD spectra of HMW kininogen in the presence and absence of 3 mM $CaCl_2$ are shown in Figure 4A. The spectrum of HMW kininogen has a minimum at 206 nm with a mean residue ellipticity of −4100 in the absence of $CaCl_2$. In the presence of 3 mM $CaCl_2$, the spectrum has a minimum at 206 nm with a mean residue ellipticity of −4700. The spectra of HMW kininogen in the presence and absence of 3 mM $CaCl_2$ have shoulders centered around 226 nm. The spectrum in the presence of 3 mM $CaCl_2$ has a slight increase in negative magnitude as compared with the spectrum in the absence of $CaCl_2$. When we look at the spectra in detail in the region of 216–240 nm, a small but real difference is apparent between both CD spectra in the presence and absence of 3 mM $CaCl_2$.

Metal Ion Induced CD Changes of the Heavy Chain. The effect of metal ions such as Ca^{2+}, Mg^{2+}, and Zn^{2+} on the CD

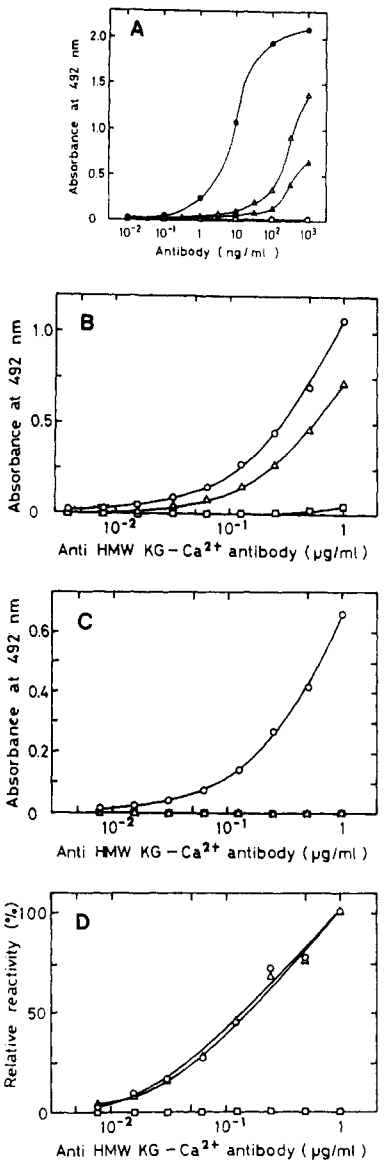

FIGURE 3: Interactions of anti-HMW kininogen–Ca^{2+} antibody with HMW kininogen and its fragments. (A) Interactions of anti-HMW kininogen antibody subpopulations with HMW kininogen in the presence and absence of 3 mM $CaCl_2$. The bindings of anti-HMW kininogen antibody (○, ●) and anti-HMW kininogen–Ca^{2+} antibody (△, ▲) to HMW kininogen (100 μL of 125 ng/mL) were estimated in the presence of 3 mM $CaCl_2$ (○, △) or 3 mM EDTA (●, ▲) by ELISA system. Nonspecific binding was symbolized with open squares (□). (B) Interactions of anti-HMW kininogen–Ca^{2+} antibody with HMW kininogen (○), heavy chain (△), and fragment 1-2–light chain (□) were investigated by ELISA in the presence of 3 mM $CaCl_2$. Experiments were performed as described under Methods except that the antigen solution used was 5×10^{-8} M. (C) Interactions of anti-HMW kininogen–Ca^{2+} antibody with CB-1 (○), domain 2 (△), and domain 3 (□) were investigated by ELISA in the presence of 3 mM $CaCl_2$. Experiments were performed as described under Methods except that the antigen solution used was 2.5×10^{-8} M. (D) Effects of metal ions on the interaction of anti-HMW kininogen–Ca^{2+} antibody with CB-1. Effects of Ca^{2+} (○), Mg^{2+} (△), and Zn^{2+} (□) on the interaction of anti-HMW kininogen–Ca^{2+} antibody with CB-1 were investigated by ELISA. The maximum difference between the reactivities of anti-HMW kininogen–Ca^{2+} antibody with CB-1 in the presence and absence of 3 mM $CaCl_2$ is defined as 100%, and these data are expressed as relative antigen–antibody reactivity. Experiments were performed as described under Methods except that the antigen solution used was 2.5×10^{-8} M and the washing buffer containing 3 mM $MgCl_2$ or $ZnCl_2$ instead of $CaCl_2$ was used.

spectrum of the heavy chain is shown in Figure 4B. The spectrum of the heavy chain in the presence of 3 mM $CaCl_2$, $MgCl_2$, or $ZnCl_2$ differs from the spectrum of the protein in

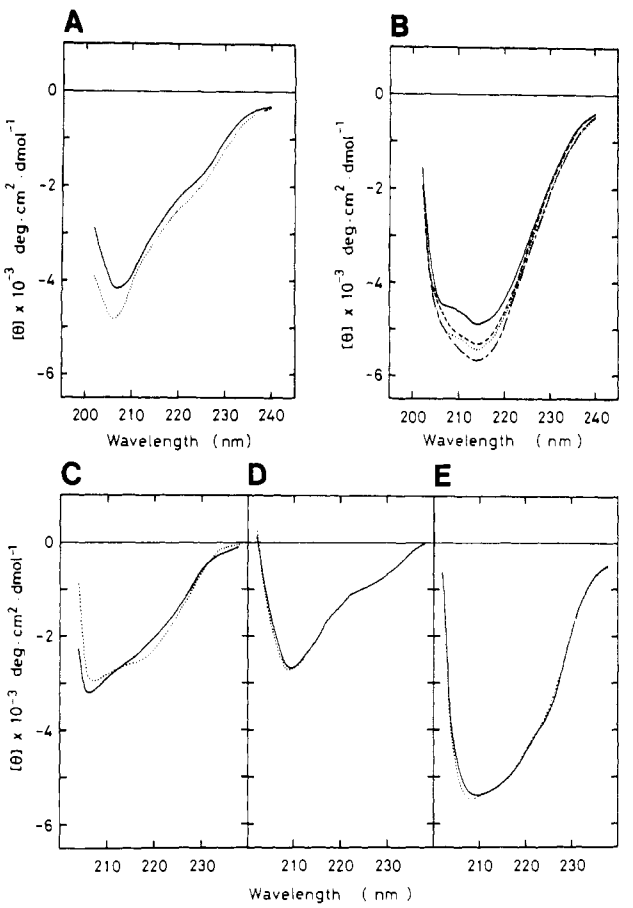

FIGURE 4: Circular dichroism spectrum of HMW kininogen and its fragments. (A) CD spectrum of HMW kininogen was measured in 20 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA with (···) and without (—) 3 mM $CaCl_2$. [θ] is the mean residue ellipticity. (B) Effects of metal ions on the circular dichroism spectrum of heavy chain. The measurement of the CD spectrum of heavy chain was carried out in 20 mM Tris-HCl buffer, pH 7.5, in the presence of 1 mM EDTA (—), 3 mM $CaCl_2$ (···), 3 mM $MgCl_2$ (- - -), or 3 mM $ZnCl_2$ (-·-·-). (C–E) CD spectra of purified peptide chains (C, CB-1; D, domain 2; E, domain 3) were measured in 20 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA with (···) and without (—) 3 mM $CaCl_2$.

the absence of them. In the absence of metal ions, the CD spectrum of the heavy chain was characterized by a shoulder centered at 206 nm and by a trough at 214 nm with a mean residue ellipticity of −4900. On the other hand, the heavy chain gave almost identical spectra characterized by a trough at 214 nm with a mean residue ellipticity of −5400 or −5300 and by a shoulder centered at 208 nm in the presence of 3 mM $CaCl_2$ and $MgCl_2$, respectively. However, the spectrum of the heavy chain in the presence of 3 mM $ZnCl_2$ has no shoulder centered at 206 to 208 nm which is characterized by the other three spectra. The magnitude at 214 nm in the presence of Zn^{2+} is the greatest with a mean residue ellipticity of −5700 among the four spectra. On the basis of these data, it is obvious that the conformational change of the heavy chain was induced by the binding of Ca^{2+}, Mg^{2+}, or Zn^{2+}. However, the conformational change of the heavy chain induced by Ca^{2+} or Mg^{2+} appears to be different from that induced by Zn^{2+}.

Ca^{2+}-Induced CD Changes of CB-1, Domain 2, and Domain 3. The CD spectral changes of HMW kininogen and the heavy chain reflect the conformational change induced by binding of metal ions, and the changes indicate that the metal-binding domain would exist in the heavy chain of HMW kininogen and LMW kininogen. Accordingly, we tried to locate the metal-binding domain by pursuing in detail the metal-induced

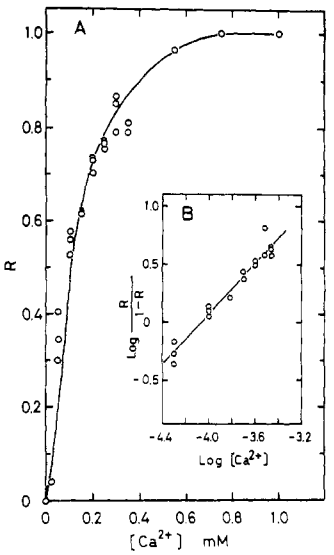

FIGURE 5: Ca^{2+} titration analysis of heavy chain. (A) Ca^{2+} titration of heavy chain was carried out by CD at 214 nm. R is defined as $([\theta] - [\theta]_0)/([\theta]_\infty - [\theta]_0)$, where $[\theta]$ is the observed mean residue ellipticity, $[\theta]_0$ is the value without Ca^{2+}, and $[\theta]_\infty$ is the value obtained when no further change in the spectrum occurs upon Ca^{2+} addition. (B) Hill plot of the data from (A). The intercept and slope are 0.11 mM and 1.08, respectively.

CD spectral changes of the heavy-chain fragments, namely, CB-1, domain 2, and domain 3 (Figure 4C–E).

Within experimental error, the CD spectra of domain 2 in the presence and absence of 3 mM $CaCl_2$ were identical and were characterized by a trough at 208 nm with a mean residue ellipticity of −2700 and two slight shoulders centered around 220 and 228 nm. Domain 3 also gave identical spectra characterized by a trough at 208 nm with a mean residue ellipticity of −5400 in the presence and absence of 3 mM $CaCl_2$. However, the CD spectra of CB-1 in the presence and absence of 3 mM $CaCl_2$ showed a small but real difference. In the CD spectrum of CB-1 in the presence of 3 mM $CaCl_2$, the ellipticity of the trough at 206 nm decreased in comparison with the result of the CD spectrum in the absence of $CaCl_2$. The amplitude around 220 nm increased, and a shoulder centered around 220 nm was formed. This is probably due to the increase of α-helical contents and the decrease of β-structure and/or random-coil contents induced by Ca^{2+} binding. On the basis of these data, it is speculated that the metal-binding domain would be located in the domain 1 region (1–122 amino acid sequence) of HMW and LMW kininogens.

Ca^{2+} Titration of the Heavy Chain by CD at 214 nm. Heavy chain was titrated with Ca^{2+} by following the change in the CD spectrum at 214 nm. This wavelength was chosen primarily because of the relatively large Ca^{2+}-induced change and because of the relatively high signal-to-noise ratio in this region of the CD spectrum. The spectra were recorded in the region of 205–225 nm, and the mean residue ellipticity at 214 nm was determined. The titration curve was plotted in terms of R vs Ca^{2+} concentration (Figure 5A). Here R is defined as $([\theta] - [\theta]_0)/([\theta]_\infty - [\theta]_0)$, where $[\theta]$ is the observed mean residue ellipticity, $[\theta]_0$ is the value without Ca^{2+}, and $[\theta]_\infty$ is the value obtained when no further change in the spectrum occurs upon Ca^{2+} addition. Since the titration curve was sigmoidal, it could be expressed as a Hill plot (Figure 5B). The Hill coefficient, which corresponds to the slope of the Hill plot, is related to the number of Ca^{2+}-binding sites.

As shown in Figure 5, the concentration of Ca^{2+} at which the midpoint of the transition is reached is 0.11 mM, and the Hill coefficient is 1.08. Furthermore, the dissociation constant (K_d) and the number of binding sites calculated by nonlinear least-squares analysis were 0.33 ± 0.09 mM (mean ± SD) and 1.15 ± 0.04 (mean ± SD) (approximately 1), respectively. These values are nearly identical with the values obtained from the solid lines in Figure 5.

Discussion

The use of conformationally specific antibodies as probes for the analysis of protein structure has yielded considerable information: the conformational motility of polypeptides (Sachs et al., 1972; Furie et al., 1975; Hurrell et al., 1977); the effects of ligands (Furie et al., 1975), temperature (Furie et al., 1975), light (Balkema & Drager, 1985), and prosthetic groups (Hurrell et al., 1977) on protein structure; the pathways of refolding of denatured protein (Chavez & Scheraga, 1977); and the implications of amino acid substitutions on the antigenic properties of the protein surface (Noble et al., 1972; Young et al., 1975). It has been also reported that Ca^{2+}-induced conformation-specific antibodies against the blood coagulation factors, factor IX (Liebman et al., 1985), prothrombin (Furie & Furie, 1979; Tai et al., 1980; Lewis et al., 1983), and thrombospondin (Dixit et al., 1986) were useful as probes for structure analyses of these proteins and capable of applying for large-scale purification (Liebman et al., 1985).

In the present study, we obtained anti-HMW kininogen–Ca^{2+} antibody and investigated the characteristics of this antibody and the Ca^{2+}-binding properties of kininogens.

On the basis of the obtained data, it was thought that the anti-HMW kininogen–Ca^{2+} antibody would recognize the conformational change of the domain 1 region of the heavy chain induced by binding of metal ions such as Ca^{2+} and Mg^{2+}. Although Zn^{2+} induced the conformational change of the heavy chain, this change was different from the conformational change recognized by anti-HMW kininogen–Ca^{2+} antibody. Accordingly, it is speculated that the binding site of Zn^{2+} might differ from that of Ca^{2+} or Mg^{2+}. From the results of CD studies and the characteristics of the anti-HMW kininogen–Ca^{2+} antibody, it was concluded that domain 1 contains a metal-binding site.

EF hand domains of parvalbumin, calmodulin, troponin C, etc. (Kretsinger & Nockolds, 1973; Barker et al., 1977; Goodman, 1980; Kretsinger, 1980), γ-carboxyglutamic acid domains of vitamin K dependent proteins such as factors VII, IX, and X and prothrombin (Stenflo, 1978; Davie et al., 1979), and phosphoresidues of phosphoproteins including casein, lipovitellin, and phosvitin (Taborsky, 1974) are well-known as typical structures with metal-binding capacity. Recently, the complete amino acid sequence deduced from the nucleotide sequence of LMW kininogen was determined by Ohkubo et al. (1984) and that of HMW kininogen by Takagaki et al. (1985). However, there were no indications about the metal-binding domain in the sequences of both proteins. We searched the sequences of the proteins whether they have metal-binding sequences or not and found the sequence to be similar to those of Ca^{2+}-binding sites, namely, EF hand sites, of sarcoplasmic Ca^{2+}-binding proteins (SCPs) from the protochrodate *Amphioxus* (Takagi et al., 1986) in the amino-terminal portion of the heavy chain of HMW and LMW kininogens (Figure 6).

According to Gariepy and Hodges (1983) and Haiech and Sallantin (1985), the 12-residue-long center loop in the EF hand site displays strong constraints in positions 1, 3, 4, 5, 6, 8, and 12, much lower constraints in positions 9, 10, and 11, and no constraints in positions 2 and 7. The segment of Asp-X-Asx-Gly-Asx-Gly from positions 1 to 6 represents the most conserved sequence of the EF hand site and is predicted

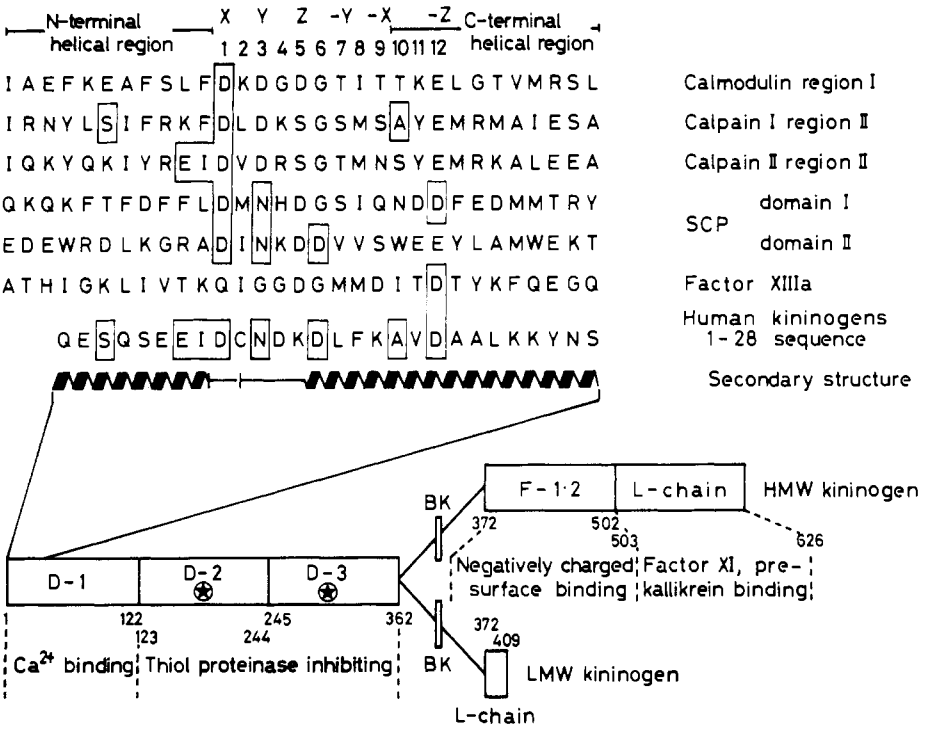

FIGURE 6: Comparison of the amino acid sequence of the amino-terminal 28 residues of kininogens with the EF hand site sequences of Ca^{2+}-binding protein II, and the linear model of domain structure and functions of kininogens. The two stars indicate predicted reactive sites for inhibition of thiol proteinases (Ohkubo et al., 1984). The sequence of calmodulin region I is described as a typical EF hand site. *X*, *Y*, *Z*, $-Y$, $-X$, and $-Z$ refer to vertices of the calcium coordination octahedron. Predicted secondary structure of the amino-terminal sequence of human kininogens was determined by the method of Chou and Fasman (1974). Designations are (〰) α-helix and (⊢) β-turn. The sequences of calmodulin, calpain I, calpain II, SCP, factor XIIIa, and human kininogens are quoted from Sasagawa et al. (1982), Aoki et al. (1986), Emori et al. (1986), Takagi et al. (1986), Ichinose et al. (1986) and Takahashi et al. (1986), and Ohkubo et al. (1984) and Takagaki et al. (1985), respectively.

to be a strong β-turn-forming region. The side chains of aspartic acid and/or asparagine residues represent calcium-coordinating ligands at positions $+X$, $+Y$, and $+Z$. The sequence in this part of the EF hand site also shows the presence of a conserved glycine residue at position 6. It is an essential residue that allows the peptide backbone to undergo a large change in direction in this part of the EF loop and permits the proper folding of ligands around the metal. Positions 8 and 10 of the EF loop region are conserved as a hydrophobic site that offers a natural extension to the hydrophobic region of the carboxyl-terminal α-helical region. The side chain length of a glutamic acid or aspartic acid residue at position 12 ($-Z$) may represent a critical factor in the ability of calcium to induce part of the carboxyl-terminal helix. Figure 6 shows that in human kininogens, Asp is located in position 1, Asn in position 3, a highly hydrophobic residue (Phe) in position 8, and Asp in position 12. Particularities are the Lys residue in position 5 and the Asp residue in position 6. Although position 6 in more than 80 sequences of Ca^{2+}-binding loops is occupied by a Gly residue, it was recently reported that domain II of SCP, whose position 6 is occupied by Asp, has Ca^{2+}-binding capacity (Takagi et al., 1986) (Figure 6). Concerning position 5, it has not been reported how the substitution in the position of a Lys residue affects the metal-binding capacity. Furthermore, the sequence Asp-Gly in positions 3 and 4 is very conserved in the EF hand site of the Ca^{2+}-specific binding domain, for example, calmodulin domains I, II, III, and IV (Dedman et al., 1977; Sasagawa et al., 1982). It has also been observed that the EF hand site containing the Asx-X sequence in positions 3 and 4 would be able to bind both Ca^{2+} and Mg^{2+} as shown in domains III and IV of skeletal muscle troponin C (Nagy & Gergely, 1979; Leavis et al., 1980) and domains III and IV of cardiac muscle troponin C (Holroyde et al., 1979). Accordingly, the sequence Asp-X at positions 3 and 4 of kininogens supports the data that domain 1 of kininogens could also bind Mg^{2+} as well as Ca^{2+}. However, because the affinity constant of kininogens measured by Ca^{2+} titration is 0.33 ± 0.09 mM, which is very low as compared with those of ordinary EF hand sites such as calmodulin (Haiech et al., 1981) and troponin C (Potter & Gergely, 1975) (10^{-5} to $\sim 10^{-8}$ M), it is speculated that the amino acid substitutions observed in positions 5 and 6 might affect the Ca^{2+}-binding affinity of the heavy chain.

We also predicted the secondary structure of the amino-terminal portion of the heavy chain according to the method of Chou and Fasman (1974) and confirmed the similarity of the secondary structures between the EF hand and this amino-terminal portion. However, the length of β-turn in the center loop of kininogens was shorter than that of ordinary EF loop region. It might also affect the metal-binding affinity of kininogens.

On the other hand, it is interesting that the Ca^{2+}-binding properties of kininogens are similar to those of factor XIIIa, which is a plasma protein as well as kininogens; namely the affinity constants of both proteins are low, and the number of Ca^{2+}-binding sites is one.

On the basis of our present data, it is reasonable to think that the amino-terminal portion of domain 1 is a possible Ca^{2+}-binding site.

Furthermore, we investigated the relevant sequences of the three proteins, bovine kininogens (Kitamura et al., 1983; Nawa et al. 1983), rat T-kininogen (Furuto-Kato et al., 1985), and rat α_1-major acute phase protein (α_1MAP) (Anderson & Heath, 1985). As shown in Figure 7, these three proteins might be able to bind Ca^{2+} judging from the sequence homology.

Recently, the binding of factor XI to platelets has been shown to be dependent on Zn^{2+} and Ca^{2+}, and HMW kini-

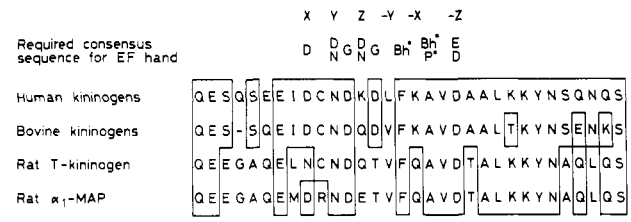

FIGURE 7: Comparison of the amino-terminal amino acid sequences of human kininogens, bovine kininogens, rat T-kininogen, and rat α_1-major acute phase protein (α_1MAP). The sequences are positioned to show maximal homology. Residues homologous with those of human kininogens are boxed. Consensus Ca^{2+}-binding center loop was quoted from Gariepy and Hodges (1983) and Haiech and Sallantin (1985). *X*, *Y*, *Z*, *−Y*, *−X*, and *−Z* refer to vertices of the calcium coordination octahedron. Bh* and P* indicate bulky hydrophobic residue and positively charged residue, respectively. The sequences of human kininogens, bovine kininogens, rat T-kininogen, and rat α_1MAP are quoted from Ohkubo et al. (1984) and Takagaki et al. (1985), Kitamura et al. (1983) and Nawa et al. (1983), Furuto-Kato et al. (1985), and Anderson and Heath (1985), respectively.

nogen has also been shown to act synergistically with Ca^{2+} to enhance the ability of low concentrations of Zn^{2+} to promote factor XI binding (Greengard et al., 1986). Although the physiological role of the metal binding of kininogens remains unknown, the function may play an important role in the binding of factor XI to platelets.

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Registry No. Ca, 7440-70-2; Mg, 7439-95-4; Zn, 7440-66-6.

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Fragmentation of Rabbit Skeletal Muscle Calsequestrin: Spectral and Ion Binding Properties of the Carboxyl-Terminal Region†

Mamoru Ohnishi‡ and Reinhart A. F. Reithmeier*
Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7
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ABSTRACT: Rabbit skeletal muscle calsequestrin was fragmented by using trypsin in the presence and absence of calcium. Calcium ion was found to protect calsequestrin from proteolysis, and the peptides produced in the presence of calcium were stable to further digestion. Peptides produced in the presence or absence of calcium had a decreased helical content but maintained their ability to bind calcium. The amino acid sequence of a 59-residue carboxyl-terminal tryptic peptide was determined by automated Edman degradation and carboxypeptidase Y digestion of carboxyl-terminal tryptic, chymotryptic, and cyanogen bromide peptides. This peptide is highly acidic (Asp + Glu = 42%, Lys + Arg = 0), and it bound a total of 15 calcium ions per mole of peptide (K_d = 8.5 mM). The intrinsic tryptophan fluorescence of the peptide was enhanced by 10% upon binding Ca^{2+} with the dissociation constant of 1 mM. Analyses of the circular dichroism spectra of the peptide showed that it was primarily in a random-coil conformation with little helical (2%) and moderate β-structure (25%) regardless of the calcium concentration. This peptide also bound 7 mol of terbium per mole of peptide with high affinity (K_d = 7.5 μM).

Calsequestrin is a calcium binding protein found in the terminal cisternae of sarcoplasmic reticulum (MacLennan & Wong, 1971). It has an important physiological function in that it sequesters calcium ions within the sarcoplasmic reticulum when muscle is relaxed [see MacLennan et al. (1983) for a review]. Calsequestrin binds 40–50 mol of calcium per mole of protein with a dissociation constant of 1 mM under physiological conditions (MacLennan & Wong, 1971; Ikemoto et al., 1972, 1974; Ostwald & MacLennan, 1974). Calsequestrin is also found in heart muscle (Cala & Jones, 1983; Campbell et al., 1983), where it likely plays a crucial role in sequestrating calcium in cardiac sarcoplasmic reticulum. Physicochemical and biochemical properties of calsequestrin have been extensively examined by many workers, i.e., circular dichroism (Ikemoto et al., 1972, 1974; Ostwald & MacLennan, 1974; Cozens & Reithmeier, 1984), fluorescence spectroscopy (Ikemoto et al., 1972, 1974; Ohnishi & Reithmeier, 1987), ultracentrifugation (Cozens & Reithmeier, 1984), NMR spectroscopy (Aaron et al., 1984), Raman spectroscopy (Williams & Beeler, 1986), and crystallization (Maurer et al., 1985; Williams & Beeler, 1986).

One of the unique properties of calsequestrin is that it contains high amounts of acidic amino acid residues (MacLennan & Wong, 1971). The calcium binding mechanism of calsequestrin is not yet understood. Many calcium binding proteins have been isolated, and their calcium binding mechanisms have been elucidated, i.e., EF hand (Kretsinger & Nockolds, 1973; Reid & Hodges, 1980), γ-carboxyglutamic acid containing proteins (Furie et al., 1979; Bajaj et al., 1982), β-hydroxyaspartic acid containing proteins (Drakenberg et al., 1983), and phosphoproteins (Lee et al., 1977; Bennick et al., 1981). However, the calcium binding mechanism of calse-

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* Address correspondence to this author at the Membrane Biology Group, Department of Medicine, University of Toronto, Toronto, Ontario, Canada M5S 1A8. Alberta Heritage Foundation for Medical Research scholar.
‡ Alberta Heritage Foundation for Medical Research postdoctoral fellow.

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