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Human High Molecular Weight Kininogen as a Thiol Proteinase Inhibitor: Presence of the Entire Inhibition Capacity in the Native Form of Heavy Chain†

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ABSTRACT: High molecular weight (HMW) kininogen was purified from fresh human plasma by two successive column chromatographies on DEAE-Sephadex A-50 and Zn-chelate Sepharose 4B. The purified HMW kininogen appeared to be a single band on sodium dodecyl sulfate (SDS)–polyacrylamide disc gel electrophoresis in both the presence and absence of β-mercaptoethanol. However, it gave two bands on nonreduced SDS–polyacrylamide slab gel electrophoresis, a major band of dimeric form (M_r 200 000, ca. 95%) and a minor band of monomeric form (M_r 105 000, ca. 5%). Under reduced conditions, the dimeric form was converted stoichiometrically to a monomeric form (M_r 110 000), and the monomeric form observed under nonreduced conditions (M_r 105 000) was converted to a heavy chain (M_r 60 000) and a light chain (M_r 50 000). The formation of a dimer of HMW kininogen was also confirmed by an immunoblotting experiment. This unique property of intact HMW kininogen to form a dimer was further utilized in studies on the kininogens and their derivatives as thiol proteinase inhibitors. The purified HMW kininogen strongly inhibited the caseinolytic activities of calpain I, calpain II, and papain but not those of trypsin, chymotrypsin, and thermolysin, indicating that it was a group-specific inhibitor for thiol proteinases. When HMW kininogen was reduced with 0.14 or 1.4 M β-mercaptoethanol, its inhibitory activity was partially or mostly inactivated, but on subsequent air oxidation its activity was almost completely recovered. In addition, kinin-free and fragment 1,2 free HMW kininogen showed higher inhibitory activity than the intact HMW kininogen. The heavy chain exhibited the highest activity and bound with 2 mol of papain per mole of the heavy chain. These results and the sequence data previously reported on low molecular weight (LMW) kininogen [Ohkubo, I., Kurachi, K., Takasawa, T., Shiokawa, H., & Sasaki, M. (1984) *Biochemistry 23*, 5691–5697] clearly demonstrated that two reactive sites for thiol proteinase inhibitor activity are located in the heavy chain of HMW and LMW kininogens and that the light chain and fragment 1,2 moieties of the kininogens interfere with the inhibitory activity of the heavy chain by steric hindrance.

High molecular weight (HMW)[1] kininogen has been reported to play two important physiological roles in liberating a vasoactive nonapeptide, bradykinin (Werle et al., 1948), and in functioning as a cofactor in the contact phase of the intrinsic blood coagulation cascade (Davie et al., 1975, 1979; Heimark et al., 1980). In the plasma, LMW kininogen, like HMW kininogen, is known to be a kinin precursor, but it does not accelerate coagulation, because it has no histidine-rich fragment (fragment 1,2) (Kato et al., 1981). HMW kininogen is composed of heavy and light chains held together with a disulfide bond, a bradykinin, and a histidine-rich fragment. On the other hand, LMW kininogen is composed of heavy and light chains linked by a disulfide bond and a bradykinin moiety. The bradykinin moieties and heavy chains of the two kininogens are identical, but their individual light chains are quite different in amino acid composition and size (Kitamura et al., 1983; Nawa et al., 1983).

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[1] Abbreviations: HMW kininogen, high molecular weight kininogen; LMW kininogen, low molecular weight kininogen; TPI, thiol proteinase inhibitor; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; Polybrene, hexadimethrine bromide; EDTA, ethylenediaminetetraacetic acid; Bz-L-Arg-pNA, *N*-benzoyl-L-arginine-*p*-nitroanilide; HRPO, horseradish peroxidase; SCM, reduced and S-carboxymethylated; β-ME, β-mercaptoethanol; Tris, tris(hydroxymethyl)-aminomethane; IgG, immunoglobulin G.

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Recently, we have reported that α_2-thiol proteinase inhibitor (α_2TPI) is identical with LMW kininogen on the basis of the amino acid sequence deduced from cDNA isolated for α_2TPI (Ohkubo et al., 1984) and also confirmed that LMW kininogen can actually inhibit a thiol proteinase, ficin. We further predicted that LMW kininogen may have two reactive sites per molecule, each reactive site possibly located on each internal repeat sequence found in the heavy chain. On the basis of these data, it has been speculated that HMW kininogen, which includes the same heavy chain as LMW kininogen, should also have similar inhibitory activity toward thiol proteinases. More recently, Sueyoshi et al. (1985) and Müller-Esterl et al. (1985) have reported the affinity of HMW kininogen for thiol proteinases such as papain and cathepsins B, H, and L.

In the present study, we describe the essential structure required for the inhibitory activity located in the heavy chain of HMW kininogen and the kinetics of the inhibitory reaction assigned to these sites.

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 separated from outdated citrated blood supplied from the Aichi Red Cross Blood Center, Nagoya, Japan. Human HMW kininogen deficient plasma was purchased from George King Bio-Medical, Inc., Salem, NH. Human kidneys were obtained at autopsies in this medical school. Actin (activated cephaloplastin reagent) was obtained from Dade Diagnostics, Inc., Miami, FL. Papain, trypsin, chymotrypsin, thermolysin, aprotinin, and PMSF (phenylmethanesulfonyl fluoride) were purchased from Sigma Chemical Co., St. Louis, MO. The chromogenic substrate Bz-L-Arg-pNA and Lys-bradykinin were obtained from Protein Research Foundation, Osaka, Japan. Casein was from Difco Laboratories, Detroit, MI. DEAE-Sephadex A-50, Red Sepharose, and the PD-10 column were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Zn-chelate gel was prepared as described previously (Porath et al., 1975). All other chemicals were of analytical grade.

Methods

Purification of High Molecular Weight Kininogen. High molecular weight kininogen (HMW kininogen) was isolated from fresh citrated human plasma within 12 h by two-step column chromatographies on DEAE-Sephadex A-50 (Kerbiriou & Griffin, 1979) and Zn-chelate Sepharose 4B (Ohkubo et al., 1980). All purification procedures were performed at room temperature to avoid cold activation in the presence of proteinase inhibitors (Armstrong & Mills, 1965; Gjonnaess, 1972).

Human plasma (650 mL) was dialyzed against buffer A (121 mM Tris and 44 mM succinic acid, pH 7.7, containing 1 mM EDTA, 1 mM benzamidine, 1 mM ϵ-aminocaproic acid, 0.1 mM PMSF, 50 μg/mL Polybrene, 100 units/L aprotinin, 0.02% NaN_3, and 100 mM NaCl). The dialyzed sample was applied to a column of DEAE-Sephadex A-50 (10 × 20 cm) previously equilibrated with the same buffer. The column was washed extensively with the buffer, and then HMW kininogen was eluted with buffer B (193 mM Tris and 75 mM succinic acid, pH 7.4, containing 1 mM EDTA, 1 mM benzamidine, 1 mM ϵ-aminocaproic acid, 0.1 mM PMSF, 50 μg/mL Polybrene, 100 units/L aprotinin, 0.02% NaN_3, and 200 mM NaCl) at a flow rate of 1000 mL/h. Fractions containing HMW kininogen were pooled and dialyzed against buffer C (25 mM Tris-HCl buffered saline, pH 8.0, containing 1 mM benzamidine, 1 mM ϵ-aminocaproic acid, 0.5 mM EDTA, 0.1 mM PMSF, 100 units/L aprotinin, and 0.01% NaN_3). The dialyzed sample was applied to a Zn-chelate Sepharose 4B column (3.6 × 20 cm) equilibrated with buffer C. The column was washed extensively with the same buffer at a flow rate of 400 mL/h. HMW kininogen was then eluted with a linear gradient of 0–50 mM L-histidine in the same buffer.

Purification of Kinin-Free HMW Kininogen, Kinin-Free and Fragment 1,2 Free HMW Kininogen, LMW Kininogen, and Kinin-Free LMW Kininogen (α_2TPI). Kinin-free HMW kininogen was purified from outdated human plasma by the same procedure as native HMW kininogen. Kinin-free and fragment 1,2 free HMW kininogen was also purified from outdated human plasma by an improved procedure utilizing DEAE-cellulose, DEAE-Cellulofine, Blue Sepharose, Sephadex G-200, 1st Red Sepharose, and 2nd Red Sepharose (unpublished results).

Low molecular weight kininogen (LMW kininogen) was purified from fresh citrated human plasma by a newly developed procedure employing DEAE-Sephadex A-50, ammonium sulfate precipitation, DEAE-Sephacel, Red Sepharose, hydroxylapatite, and butyl-Toyopearl (unpublished results).

Kinin-free LMW kininogens, which correspond to α_2TPI_1 and α_2TPI_2, were purified as described previously (Ohkubo et al., 1984). All samples migrated as a homogeneous single band on SDS–polyacrylamide gel electrophoresis.

Preparations of Heavy Chain, SCM Heavy and Light Chains, SCM HMW Kininogen, and SCM LMW Kininogen. Native heavy chain was prepared from kinin-free LMW kininogen (α_2TPI). A sample of 10 mg of kinin-free LMW kininogen was incubated for 30 min at room temperature in 3 mL of 30 mM phosphate buffer, pH 7.2, containing 14 mM β-ME. The protein was chromatographed on a Sephacryl S-200 column in the above buffer. Fractions containing the heavy chain were pooled and dialyzed against the same buffer without β-ME. The dialysis was continued for 24 h at 4 °C for mild air oxidation with three exchanges of the outer solution. The amino terminus of the heavy chain was confirmed to be blocked, and the sequence of the carboxy-terminal region was determined to be -Ile-Ser-Leu-Met by the method of Gilles et al. (1983) using carboxypeptidase Y.

Reduced and S-carboxymethylated (SCM) HMW kininogen, SCM LMW kininogen, and SCM heavy and light chains were prepared according to the method of Kerbiriou and Griffin (1979). All preparations gave single bands on SDS–polyacrylamide gel electrophoresis in the presence of β-ME.

Preparations of Calpain I and Calpain II. Calpain I was purified to apparent homogeneity from the cytosol fraction of human erythrocytes by the method of Hatanaka et al. (1983). The specific activity of the purified calpain I was 120 units/mg of protein.

Calpain II was purified from human kidneys by a procedure involving column chromatographies on DEAE-cellulose, Sephacryl S-300, DEAE-Bio-Gel A, and Red Sepharose (unpublished results). The purified calpain II preparation gave three bands of M_r 75 000, 25 400, and 21 600 on SDS–polyacrylamide slab gel electrophoresis. The main band of M_r 75 000 corresponded to the high molecular weight subunit containing the active site of calpain, and two minor bands of M_r 25 400 and 21 600 might have been those of cleavage products of the low molecular weight subunit (M_r 30 000). The specific activity of the purified calpain II was 117 units/mg

of protein. One unit of the enzyme was defined as the amount of enzyme that hydrolyzed casein resulting in an increase of 1.0 of the absorbance at 280 nm for 1 h (Ishiura et al., 1978).

Preparation of Human Urinary Kallikrein. Urinary kallikrein was purified from pooled fresh urine from healthy subjects by the method of Ole-MoiYoi et al. (1979). The purified urinary kallikrein migrated as a single band on SDS–polyacrylamide disc gel electrophoresis, and its specific activity was 0.58 units/μL of solution.

Assay of Clotting Activity of HMW Kininogen. Clotting activity of HMW kininogen was determined by the two-stage assay for actin-activated partial thromboplastin time using HMW kininogen deficient plasma (Griffin & Cochrane, 1976). One unit of clotting activity is defined as the activity present in 1 mL of standard normal plasma.

Assay of Thiol Proteinase Inhibitor Activity. The routine inhibition assay for HMW kininogen was performed by using papain as a target proteinase and Bz-L-Arg-pNA as a substrate by the method described previously (Sasaki et al., 1981). One unit of activity was defined as the amount of inhibitor that inactivated papain to hydrolyze 0.5 μmol of Bz-L-Arg-pNA min^{-1} mL^{-1} at 30 °C (Ohkubo et al., 1984).

Inhibition of caseinolytic activity of calpain I, calpain II, papain, trypsin, chymotrypsin, and thermolysin was measured by using 1.3 units of each enzyme and increasing amounts of HMW kininogen by the method of Ishiura et al. (1978).

In a series of inhibition assays with reduced HMW and LMW kininogens and other derivatives, activated papain without added reducing reagent was used to avoid the effect of the reagent on disulfide bonds in the kininogen molecules. Prior to the experiment, papain was activated with 1.4 M β-ME for 10 min at room temperature. The residual β-ME was then removed by passing the reaction mixture through a PD-10 column twice. All the buffers used in this experiment were bubbled with N_2 gas for 60 min. The activated papain (1.24 nmol) and kininogen or its derivatives (0.45 nmol) dissolved in 1225 μL of 30 mM sodium phosphate buffer, pH 7.2, containing 1 mM EDTA, were placed in a cuvette (5 × 10 × 40 mm) in a Hitachi 228 spectrophotometer. The mixture was preincubated for 1 min at 30 °C, and the reaction was started by adding of 275 μL of substrate solution (3 mM Bz-L-Arg-pNA). The increase of the absorbance at 410 nm was recorded for 3 min. The amount of papain used was adjusted to give a difference absorption in 3 min of about 0.125 at 410 nm, and the amounts of HMW and LMW kininogens were used that caused about 50% inhibition of the papain activity.

Kinin Bioassays. Kinin activity was assayed by using isolated rat uterus (Shimuta et al., 1981). The uteri were isolated from virgin rats, weighing 185–220 g, that had received a subcutaneous injection of 10 μg of estradiol benzoate/100 g of body weight 24 h before sacrifice.

Polyacrylamide Gel Electrophoresis. Slab gel electrophoresis was carried out on 10% polyacrylamide gels in 25 mM Tris, 192 mM glycine, pH 8.3, and 0.1% SDS by the method of Laemmli (1970), and disc gel electrophoresis was done on 6% polyacrylamide gels either in 100 mM Tris–phosphate buffer, pH 7.0, containing 0.1% SDS, by the method of Weber and Osborn (1969) or in 25 mM Tris and 192 mM glycine, pH 8.3, by the method of Davis (1964). Proteins in the gels were stained with Coomassie brilliant blue R-250.

Immunoblotting. Proteins were transferred from SDS–polyacrylamide slab gel to a nitrocellulose membrane electrophoretically by the method of Lin and Kasamatsu (1983). The proteins were reacted with affinity-purified rabbit anti-LMW kininogen antibody, followed by treatment with HRPO-conjugated goat anti-rabbit IgG. Color development was achieved by adding the substrate solution (0.01% 2,7-diaminofluorene, 5% ethanol, and 6 mM H_2O_2 solution in 20 mM Tris-HCl buffer, pH 7.6).

Cleavage of HMW Kininogen with Human Urinary Kallikrein. HMW kininogen (final concentration, 22 μg/mL) was incubated at 37 °C with human urinary kallikrein (17.4 units/mL) in 2.2 mL of 20 mM Tris-HCl buffer, pH 7.5. Samples of 150 μL were taken out at intervals and mixed with 1.5 μL of aprotinin solution (1 mg/mL). Then samples of 100 μL were used for bioassay of kinin, which was liberated during the incubation, and other samples of 50 μL were subjected to SDS–polyacrylamide slab gel electrophoresis to examine the cleavage of HMW kininogen by kallikrein.

Reduction and Reoxidation of HMW Kininogen. HMW kininogen was incubated with 0.14 M or 1.4 M β-ME for 30 min at room temperature under N_2 gas, and the reduced samples were passed through a PD-10 column twice to remove residual β-ME. Half the eluate was used as reduced HMW kininogen, and the other half was dialyzed against 20 mM Tris-HCl buffer, pH 7.5, for 24 h at 4 °C to achieve mild air oxidation. The inhibitory activities of both reduced and reduced–reoxidized HMW kininogens were determined by using activated papain with no additional reducing reagent (see Assay of Thiol Proteinase Inhibitor Activity). The process of reoxidation of the reduced form was followed by SDS–polyacrylamide slab gel electrophoresis as the conversion of the monomeric form (M_r 110000) to the dimer (M_r 200000).

Results and Discussion

Purification and Some Characteristics of Human HMW Kininogen. HMW kininogen was purified 1463-fold from fresh citrated human plasma with a yield of 33% as clotting factor. Its specific activities as clotting factor and thiol proteinase inhibitor were 24 units/mg of protein and 41 units/mg of protein, respectively. The purified HMW kininogen gave a single band on polyacrylamide disc gel electrophoresis in the absence of SDS (Figure 1A). The purified protein also gave single bands of M_r 105 000 and 110 000, respectively, when analyzed by SDS–polyacrylamide disc gel electrophoresis in the absence (Figure 1B) and presence (Figure 1C) of β-ME. However, the purified HMW kininogen gave two bands on nonreduced SDS–polyacrylamide slab gel electrophoresis, a main band of M_r 200 000 (ca. 95%) and a minor band of M_r 105 000 (ca. 5%) (Figure 2, lane A). The same sample gave three bands under reduced conditions, a main band of M_r 110 000 and two faint minor bands of M_r 60 000 and 50 000 (Figure 2, lane A′). HMW kininogen digested with kallikrein for 5 and 30 min contained a larger amount of protein of M_r 105 000 under nonreduced conditions (Figure 2, lanes B and C), and in the presence of 1% β-ME, the amounts of the proteins of M_r 60 000 and 50 000 increased with the incubation time in parallel with the increase of Lys-bradykinin release (Figure 2, lanes B′ and C′). Immunoblotting analysis of these bands showed that both bands reacted well with the anti-LMW kininogen antibody (Figure 3). These data indicate that the single-chain molecule of HMW kininogen migrates as a dimeric form, and the two-chain molecule (nicked or kinin-released HMW kininogen) moves as a monomeric form on nonreduced SDS–polyacrylamide slab gel electrophoresis. Furthermore, the data provided a very convenient method for differentiating the intact HMW kininogen from its derivatives such as reduced form and nicked or kinin-released form. The HMW kininogen preparation that we have obtained usually contained approximately 5% of a nicked or kinin-free HMW

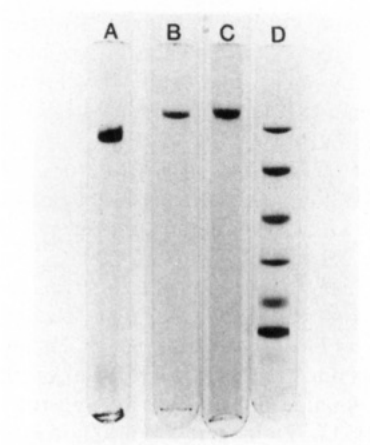


FIGURE 1: Polyacrylamide disc gel electrophoresis of HMW kininogen. Electrophoresis was carried out on a 6% polyacrylamide gel in 25 mM Tris and 192 mM glycine, pH 8.3 (gel A), or on a 6% polyacrylamide gel in 100 mM Tris–phosphate buffer, pH 7.0, containing 0.1% SDS (gels B, C, and D). For gels A and B, electrophoresis was carried out in the absence of reducing reagent, and for gel C, in the presence of 1% β-ME. Gel D included standard proteins: phosphorylase *b* (M_r 94 000), albumin (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), trypsin inhibitor (M_r 20 100), and α-lactalbumin (M_r 14 400). The gels were stained for proteins with Coomassie brilliant blue R-250. The apparent molecular weights for HMW kininogen estimated from gels B and C were 105 000 and 110 000, respectively.

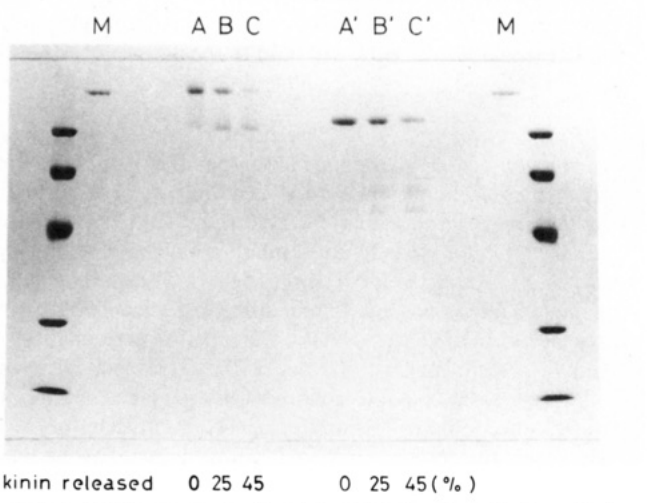


FIGURE 2: SDS–polyacrylamide slab gel electrophoresis of purified HMW kininogen compared with HMW kininogen treated with kallikrein. Electrophoresis was carried out on a 10% polyacrylamide gel in 25 mM Tris and 192 mM glycine, pH 8.3, containing 0.1% SDS. The purified HMW kininogen used for Figure 1 was applied to lanes A and A′, and HMW kininogen treated with kallikrein for 5 and 30 min (for cleavage conditions, see Methods) was applied to lanes B and B′ and lanes C and C′. α_2-Macroglobulin as a reference with a large subunit molecular weight (M_r 185 000) was applied to lane M. The same standard proteins as for Figure 1 were applied to the two side lanes. Standard proteins and lanes A′, B′, C′, and M contained 1% β-ME, whereas lanes A, B, and C contained no reducing reagent.

kininogen as described above. At the present time, it is not clear whether the single-chain molecule is converted proteolytically to the two-chain molecule during purification steps or whether a small amount of nicked HMW kininogen is ordinarily present in the blood as a catabolic intermediate (or intermediates).

Inhibitory Activity of HMW Kininogen. The inhibitory activity of HMW kininogen was examined by employing calpain I, calpain II, papain, trypsin, chymotrypsin, and thermolysin. The inhibition curves showed that 50% of the caseinolytic activities of calpain I (1.32 units), calpain II (1.32 units), and papain (0.26 units; equivalent to 1.32 casein units) were inhibited by 6.0, 3.0, and 4.0 μg of HMW kininogen, respectively, but that the activities of trypsin, chymotrypsin, and thermolysin were not inhibited by HMW kininogen (Figure 4). Although the HMW kininogen preparation contained approximately 5% of the two-chain molecule, it is reasonable to think that intact HMW kininogen has thiol proteinase inhibitor activity, since the inhibitory activity of this preparation was strong enough and almost comparable to that of α_2TPI (kinin-free LMW kininogen). Furthermore, the inhibitory activity of kinin-free HMW kininogen was lower than that of the HMW kininogen preparation as shown in Figures 6–8, indicating that kinin-free HMW kininogen cannot contribute to the increase in the total inhibition capacity of the HMW kininogen preparation. The data indicate that HMW kininogen is an active inhibitor itself for thiol proteinases and not a precursor of the inhibitor. A cytosolic inhibitor specific to calpains, namely, calpastatin, has been reported to inhibit calpain II more than calpain I (Murachi et al., 1981). HMW kininogen showed the same character as calpastatin as an inhibitor for calpains.

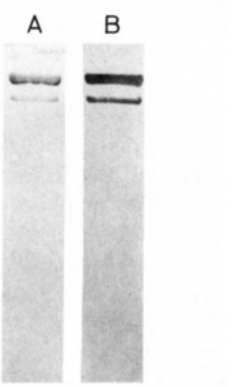


FIGURE 3: Immunoblotting of HMW kininogen by affinity-purified anti-LMW kininogen antibody. HMW kininogen was subjected to electrophoresis on a 10% polyacrylamide slab gel in the presence of 0.1% SDS under nonreduced conditions (A), transferred to a nitrocellulose membrane (B), and reacted with affinity-purified anti-LMW kininogen antibody. Gel A was stained with Coomassie brilliant blue R-250.

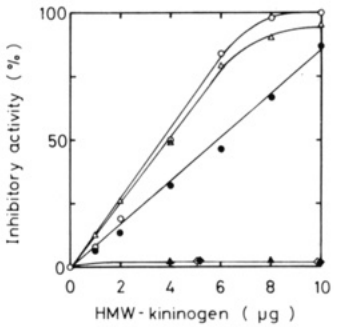


FIGURE 4: Inhibitory activity of HMW kininogen. The inhibitory activities of HMW kininogen toward calpain I (●), calpain II (○), papain (△), trypsin (♦), chymotrypsin (◇), and thermolysin (▲) were measured with casein as a substrate. The caseinolytic activities of these enzymes were adjusted to 1.3 units to give approximately 0.3 UV absorbance at 280 nm in the assay system employed for this experiment.

Reduction and Reoxidization of HMW Kininogen. HMW kininogen was reduced with 0.14 or 1.4 M β-ME, and the residual β-ME was removed by gel filtration on a PD-10 column. Part of the reduced sample was dialyzed against a large volume of 20 mM Tris-HCl buffer, pH 7.5, without any reducing reagent for 24 h at 4 °C to achieve mild reoxidation. The inhibitory activities of the reduced and reduced–reoxidized

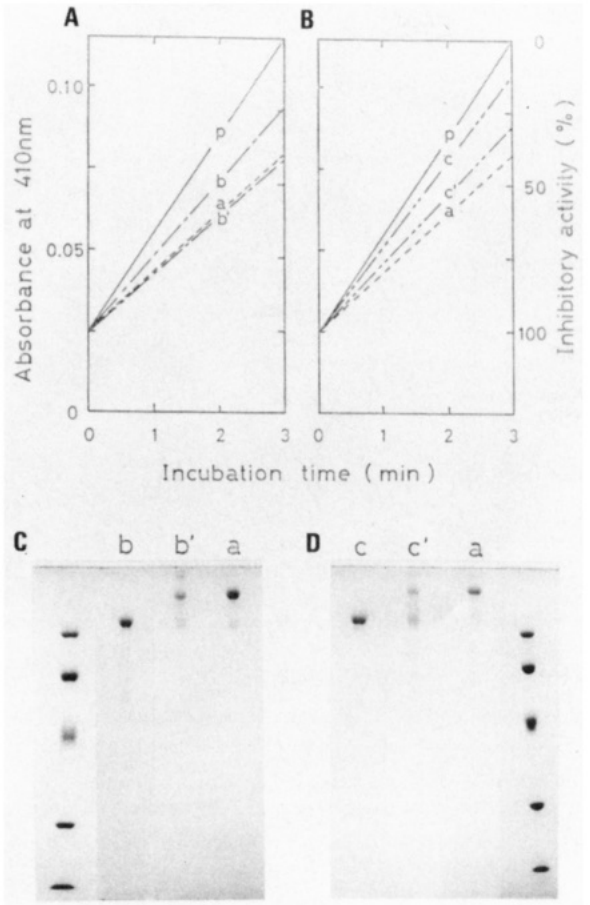


FIGURE 5: Reduction and reoxidation of HMW kininogen. Reduced and reduced–reoxidized HMW kininogens were preincubated with activated papain without any added reducing reagent in a cuvette. The reaction was started by adding the substrate of papain, Bz-L-Arg-pNA, and the increase in absorbance at 410 nm was monitored for 3 min in a Hitachi 228 spectrophotometer (for details, see Methods). (A and B) Inhibitions by reduced and reduced–reoxidized HMW kininogens. The hydrolysis rates of Bz-L-Arg-pNA by papain are shown: without any additives (p) or with native HMW kininogen (a), HMW kininogen reduced with 0.14 M β-ME (b), reoxidized HMW kininogen after reduction with 0.14 M β-ME (b'), HMW kininogen reduced with 1.4 M β-ME (c), or reoxidized HMW kininogen after reduction with 1.4 M β-ME (c'). (C and D) Electrophoretic patterns of reduced HMW kininogen and its reoxidized sample on SDS–polyacrylamide slab gel under nonreduced conditions. Symbols a, b, b', c, and c' indicate the same samples as for (A) and (B). The bands on the left in (C) and the right in (D) are those of the same standard proteins used in Figure 1.

samples were estimated by using activated papain as a target proteinase (see Methods). As shown in Figure 5A,B, native HMW kininogen inhibited 40% of the papain activity (Figure 5A-a,B-a), while the inhibitory activity of HMW kininogens reduced with 0.14 and 1.4 M β-ME decreased to 25% (Figure 5A-b) and 13% (Figure 5B-c), respectively. However, after mild reoxidation of the preparations their inhibitory activities were recovered to 41% (100% recovery) (Figure 5A-b') and 32% (82% recovery) (Figure 5B-c').

The electrophoretic profiles on nonreduced slab gel (Figure 5C,D) indicated that the reduced and reoxidized states of HMW kininogen corresponded to the monomeric and dimeric structures. As shown in Figure 5C-b, HMW kininogen reduced with 0.14 M β-ME shows the monomeric form (M_r 110000), and its reoxidized sample is converted to the dimeric form (M_r 200000) with small amounts of higher order oligomers (Figure 5C-b'). Likewise, HMW kininogen reduced with 1.4 M β-ME is composed of the monomeric form (Figure 5D-c), but its reoxidized sample is partly in the dimeric form

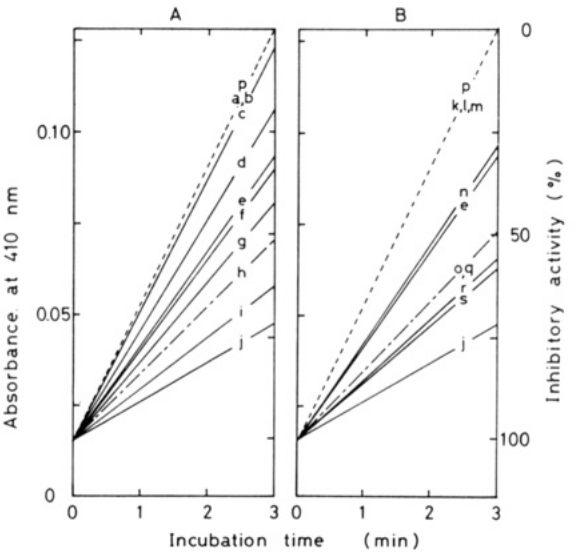
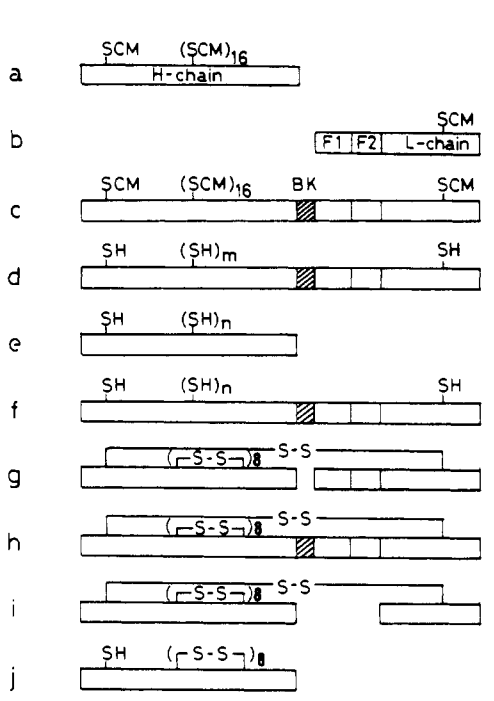


FIGURE 6: Inhibition of papain activity by HMW and LMW kininogens and their derivatives. HMW and LMW kininogens and their derivatives were preincubated with activated papain without any added reducing reagent in a cuvette. The reaction was started by adding the substrate of papain, Bz-L-Arg-pNA, and the increase in absorbance at 410 nm was monitored for 3 min. (A) Inhibitions by HMW kininogen and its derivatives. Rates of hydrolysis of Bz-L-Arg-pNA by papain are shown: without any additives (p) or in the presence of reduced and S-carboxymethylated (SCM) heavy chain, light chain, and HMW kininogen (a, b, and c), 1.4 M β-ME reduced HMW kininogen (d), 0.14 M β-ME reduced heavy chain and HMW kininogen (e and f), kinin-free HMW kininogen (g), native HMW kininogen (h), kinin-free and fragment 1,2 free HMW kininogen (i), or native heavy chain (j). (B) Inhibitions by LMW kininogen and its derivatives. Rates of hydrolysis of Bz-L-Arg-pNA by papain are shown: without any additives (p) or in the presence of reduced and S-carboxymethylated (SCM) heavy chain, light chain, and LMW kininogen (k, l, and m), LMW kininogens reduced with 1.4 and 0.14 M β-ME (n and o), native LMW kininogen (q), or kinin-free LMW kininogens, α_2TPI$_2$, and α_2TPI$_1$ (r and s). For conditions, see Methods.

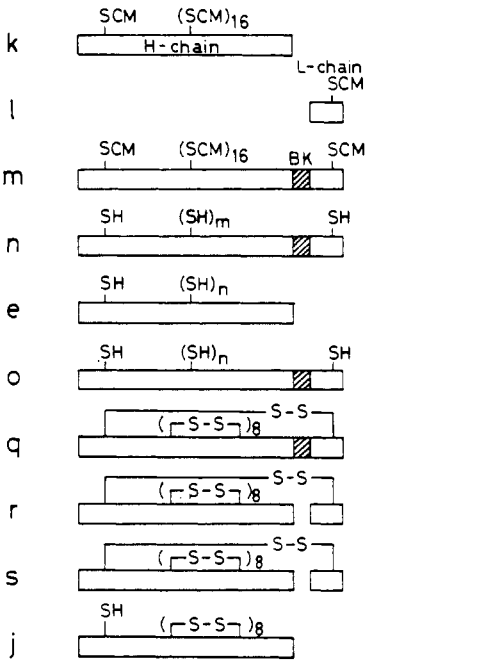
with about half the sample still in the monomeric form (Figure 5D-c'). These results indicate that this molecular interconversion is closely correlated with the inhibitory activity of HMW kininogen and that the tertiary structure supported by intramolecular disulfide bonds is necessary for the inhibitory activity of HMW kininogen. The results further indicate that the dimer is formed by an intermolecular disulfide bond (or bonds), since the dimer formation was observed only in the presence of SDS without a reducing reagent, the dimeric form disappeared completely on addition of β-ME, and the reduced monomeric HMW kininogen was reconverted to the dimeric form by mild air oxidation (Figure 5). In the similar experiment with LMW kininogen, the dimeric form was not observed, indicating that dimer formation depends on the particular property of the light chain or fragment 1,2 moiety of HMW kininogen.

Inhibitory Activities of Various Derivatives of HMW and LMW Kininogens. To characterize the essential structure for thiol proteinase inhibitor activity of HMW kininogen, several derivatives of the HMW kininogen molecule were prepared. LMW kininogen and its derivatives were also prepared for comparison. In this study, 1.24 nmol of papain (16.3 units/mg) and 0.45 nmol of each sample were used for the assay of inhibitory activity. The active site of papain was titrated with E-64. In this system, native HMW kininogen and LMW kininogen inhibited 52% and 50% of the papain activity (Figure 6). These inhibition capacities correspond to 1.4 molecules of papain per kininogen molecule. The inhibitory activities, binding ratios, and schematic representations of all the samples



		Inhibition (%)	Molar ratio (E/I)
a	SCM·H-chain	0	0
b	SCM·L-chain	0	0
c	SCM·HMW-KG	4.0	0.1
d	1.4M β-ME treated HMW-KG	19.0	0.5
e	0.14M β-ME treated H-chain	30.0	0.8
f	0.14M β-ME treated HMW-KG	33.6	0.9
g	Kinin-free HMW-KG	42.0	1.2
h	Native HMW-KG	52.0	1.4
i	Kinin- and fragment 1,2-free HMW-KG	62.3	1.7
j	Native H-chain	74.0	2.0

FIGURE 7: Schematic molecular structures and inhibitory activities of HMW kininogen and its derivatives. Percent inhibitions and binding ratios were determined graphically from Figure 6. BK = bradykinin.



		Inhibition (%)	Molar ratio (E/I)
k	SCM·H-chain	0	0
l	SCM·L-chain	0	0
m	SCM·LMW-KG	0	0
n	1.4M β-ME treated LMW-KG	27.5	0.7
e	0.14M β-ME treated H-chain	30.0	0.8
o	0.14M β-ME treated LMW-KG	50.0	1.4
q	Native LMW-KG	50.0	1.4
r	Kinin-free LMW-KG (α_2-TPI$_2$)	55.2	1.5
s	Kinin-free LMW-KG (α_2-TPI$_1$)	58.4	1.6
j	Native H-chain	74.0	2.0

FIGURE 8: Schematic molecular structures and inhibitory activities of LMW kininogen and its derivatives.

are summarized in Figures 7 and 8. As shown in Figures 7 and 8, 1.4 M β-ME reduced and carboxymethylated (SCM) heavy and light chains and SCM HMW kininogen did not show any detectable inhibitory activities, while the partially reduced (0.14 M β-ME treated) heavy chain and HMW kininogen retained 30% and 33.6% inhibitions, which corresponded to 0.8 and 0.9 molecules of papain per kininogen molecule, respectively. Derivatives of HMW and LMW kininogens generated by digestion with kallikrein generally exhibited higher inhibitory activities (55–62%) than the native kininogens (50–52%) with the one exception of kinin-free HMW kininogen (42%). The highest inhibition capacity (74%) with a full binding ratio (2.0) was observed in heavy chain that was free of kinin, fragment 1,2, and light chain. The stoichiometry (2.0 papain molecules bound to 1 molecule of heavy chain) agreed well with the predicted number of reactive sites located in the heavy chain of LMW kininogen (Ohkubo et al., 1984). The distinct high levels in the inhibitory activities of the derivatives, in particular, that of the heavy chain, clearly indicate that the light chain and fragment 1,2 moieties of kininogen molecules interfere with the inhibitory activity of heavy chain by steric hindrance. During inflammation, it is speculated that trifunctional HMW kininogen liberates a kinin and a fragment 1,2 sequentially by the action of kallikrein and is finally converted to a unifunctional protein with elevated proteinase inhibitor activity.

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Registry No. Proteinase inhibitor, 37205-61-1; calpain, 78990-62-2; papain, 9001-73-4; thiol proteinase, 37353-41-6; kallikrein, 9001-01-8.

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Control of the Redox Potential of Cytochrome *c* and Microscopic Dielectric Effects in Proteins†

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ABSTRACT: X-ray structural information provides the opportunity to explore quantitatively the relation between the microenvironments of heme proteins and their redox potentials. This can be done by considering the protein as a "solvent" for its redox center and calculating the difference between the electrostatic energy of the reduced and oxidized heme. Such calculations are presented here, applying the protein dipoles–Langevin dipoles (PDLD) model to cytochrome *c*. The calculations focus on an evaluation of the difference between the redox potentials of cytochrome *c* and the octapeptide–methionine complex formed by hydrolysis of cytochrome *c*. The corresponding difference (~7 kcal/mol) is accounted for by the PDLD calculations. It is found that the protein provides basically a low dielectric environment for the heme, which destabilizes the oxidized heme (relative to its energy in water). The effect of the charged propionic acids on the heme is examined in a preliminary way. It is found that the negative charges of these groups are in a hydrophilic rather than a hydrophobic environment and that the protein–water system provides an effective high dielectric constant for their interaction with the heme. The dual nature of the dielectric effect of the cytochrome (a low dielectric constant for the self-energy of the heme and a high dielectric constant for charge–charge interactions) is discussed. The findings of this work are consistent with the difference between the folding energies of the reduced and oxidized cytochrome *c*.

The oxidation–reduction (redox) potentials of cytochromes span a range of nearly 800 mV [for example, see Xavier et al. (1981), Margoliash & Schejter (1966), and Meyer & Kamen (1982)]. In thermodynamic terms, the variation in cytochrome redox potentials corresponds to a *range* of 17 kcal/mol in the free energy of oxidation of the ferrous heme prosthetic group. Since 17 kcal/mol amounts for more than

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