Protein-Protein Interactions in Contact Activation of Blood Coagulation

BINDING OF HIGH MOLECULAR WEIGHT KININOGEN AND THE 5-(IODOACETAMIDO)FLUORESCEIN-Labeled Kininogen Light Chain to Prekallikrein, Kallikrein, and the Separated Kallikrein Heavy and Light Chains

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Binding of the 5-(iodoacetamido)fluorescein (IAF)-labeled high molecular weight (HMW) kininogen light chain to prekallikrein and D-Phe-Phe-Arg-CH₂Cl-inactivated kallikrein was monitored by a 0.040 ± 0.002 increase in fluorescence anisotropy. Indistinguishable average dissociation constants and stoichiometries of 14 ± 3 nM and 1.1 ± 0.1 mol of prekallikrein/mol of IAF-light chain and 17 ± 3 nM and 0.9 ± 0.1 mol of kallikrein/mol of IAF-light chain were determined for these interactions at pH 7.4, 0.14 and 22 °C. Prekallikrein which had been reduced and alkylated in 6 M guanidine HCl lost the ability to increase the fluorescence anisotropy of the IAF-kininogen light chain, suggesting that the native tertiary structure was required for tight binding. The kallikrein heavy and light chains were separated on the basis of the affinity of the heavy chain for HMW-kininogen-Sepharose, after mild reduction and alkylation of kallikrein under nondenaturing conditions. Under these conditions, alkylation with iodo[14C]acetamide demonstrated that only limited chemical modification had occurred. Binding of the IAF-kininogen light chain to the isolated alkylated kallikrein heavy chain, when compared to prekallikrein and kallikrein, was characterized by an indistinguishable increase in fluorescence anisotropy, average dissociation constant of 14 ± 3 nM, and stoichiometry of 1.2 ± 0.1 mol of kallikrein heavy chain/mol of IAF-light chain. In contrast, no binding of the D-Phe-Phe-Arg-CH₂Cl-inactivated kallikrein light chain was detected at concentrations up to 500 nM. Furthermore, 300 nM kallikrein light chain did not affect IAF-kininogen light chain binding to prekallikrein, kallikrein, or the kallikrein heavy chain. The binding of monomeric single chain HMW-kininogen to prekallikrein, kallikrein, and the kallikrein heavy and light chains was studied using the IAF-kininogen light chain as a probe. Analysis of the competitive binding of HMW-kininogen gave average dissociation constants and stoichiometries of 12 ± 2 nM and 1.2 ± 0.1 mol of prekallikrein/mol of HMW-kininogen, 15 ± 2 nM and 1.3 ± 0.1 mol of kallikrein/mol of HMW-kininogen, 14 ± 3 nM and 1.4 ± 0.2 mol of kallikrein heavy chain/mol of HMW-kininogen, and no detectable effect of 300 nM kallikrein light chain on these interactions. We conclude that a specific, nonenzymatic interaction between sites located exclusively on the light chain of HMW-kininogen and the heavy chain of kallikrein or prekallikrein is responsible for the formation of 1:1 noncovalent complexes between these proteins.

Contact of normal human plasma with negatively charged surfaces initiates proteolytic reactions which activate the intrinsic blood coagulation cascade, a fibrinolytic pathway, and generate bradykinin (1). HMW-kininogen is a nonenzymatic glycoprotein essential for a rapid rate of contact activation. HMW-kininogen greatly increases the rates of surface-dependent proteolytic activations of factor XII, prekallikrein, and factor XI, both in plasma and in mixtures of the purified proteins (2-6). Factor XII, generated by these reactions, activates factor XI and thereby the blood coagulation system (7). Kallikrein activates plasminogen (8-10) and catalyzes the limited proteolysis of HMW-kininogen, releasing kinins (11-13). The mechanism of action of HMW-kininogen as a cofactor of contact activation involves the formation of tight noncovalent complexes with prekallikrein (14-18), factor XI (18-20), and the corresponding activated enzymes (15, 17, 21). HMW-kininogen is thought to mediate the binding of these proteins to surfaces, thereby enhancing the rates of reciprocal reactions with surface-bound factors XII and XII (22, 23). Limited proteolysis of HMW-kininogen by kallikrein results in greater adsorption of this protein to kaolin surfaces, as well as increased HMW-kininogen-mediated adsorption of prekallikrein and factor XI in plasma (24). In addition, it has been reported that HMW-kininogen decreases the rate of inhibition of factor XI, and kallikrein by some plasma protease inhibitors (21, 25-27).

Kallikrein-cleaved HMW-kininogen consists of two polypeptide chains joined by a disulfide bond (11-13, 28-30). Studies of the separated heavy and light chains of HMW-kininogen demonstrated that the light chain is responsible for essentially all of the activity of this protein measured by

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The abbreviations used are: HMW-kininogen, high molecular weight kininogen; IAF, 5-(iodoacetamido)fluorescein; FFRCK, D-Phe-Phe-Arg-CH₂Cl; SDS, sodium dodecyl sulfate; S-2302, H-D-Pro-Phe-Arp-H₂Cl; IAF-LC, IAF-labeled high molecular weight kininogen light chain; HEPES, 4-[2-hydroxyethyl]-1-piperazinethanesulfonic acid.
HMW-kininogen Interactions

coagulation assays (11, 28-30) and the associated ability to bind to prekallikrein (15, 18, 30), kallikrein (15, 26), factor XI (11, 19), factor X (21), and kaolin surfaces (23). Kallikrein also consists of two types of polypeptide chains covalently linked by disulfide bonds (8, 9) which can be separated after mild reduction and alkylation of the protein (31). The isolated alkylated heavy chain binds tightly to immobilized HMW-kininogen while the light chain, which contains the proteolytic enzyme active site, does not (31).

Quantitative studies of the noncovalent protein-protein interactions involving HMW-kininogen were undertaken as a necessary step in evaluating the role of these interactions in the mechanism of contact activation and control of factor XII-dependent reaction pathways. A fluorescent labeled derivative of the HMW-kininogen light chain was prepared as a probe of these interactions by reaction of a single nonessential protein thiol group with 5-(iodoacetamidofluorescein (IAF) and characterized (30, 32). The formation of a tight, noncovalent, 1:1 complex between prekallikrein and IAF-kininogen light chain was demonstrated from measurements of the increase in fluorescence anisotropy (30). In the present study, fluorescence anisotropy was used to measure equilibrium binding of the IAF-kininogen light chain and unlabeled HMW-kininogen to prekallikrein, kallikrein, and the isolated kallikrein heavy and light chains. We present evidence that the binding sites for these interactions are contained entirely on the kallikrein heavy chain and HMW-kininogen light chain and that the interaction between these sites is essentially independent of the additional protein structure present in kallikrein or prekallikrein and native HMW-kininogen.

EXPERIMENTAL PROCEDURES

**HMW-kininogen**—HMW-kininogen was purified from normal human plasma as described previously (11, 30). The protein was incubated with 5-10 μM D-Phe-Phe-ArgCH₂Cl (FFRCK) (generously supplied by Dr. Elliott Shaw, Friedrich Miescher Institute, Basel, Switzerland, or purchased from Calbiochem-Behring) to inactivate any traces of kallikrein as described below and then was chromatographed on SP-Sephadex in the absence of polybrene. HMW-kininogen from the leading portion of the protein peak eluting from this column migrated as a single band of protein on 10% SDS-polyacrylamide gels under reducing conditions (Fig. 1). Traces of cleaved HMW-kininogen were visible on overloaded gels. Two preparations with specific clotting activities of 12 units/mg were used in these studies. Concentrations were calculated using a molecular weight of 108,000 (11) and E₂₈₀nm = 7.01 (12).

**IAF-kininogen Light Chain**—The HMW-kininogen light chain was purified and labeled with IAF (Molecular Probes) as described previously (30) except that 10 mM benzamidine was included in the buffers used for labeling and dialysis, prior to gel filtration on Sephadex G-150. The IAF-kininogen light chain was stored at -70 °C in 0.1 M Tris-Cl buffer, 0.05 M NaCl, 1 mM EDTA, pH 7.4. Two preparations were used in this study with labeling ratios of 0.89 IAF/LC and 0.95 IAF/LC and specific clotting activities of 33 units/mg. Concentrations of the labeled protein and the labeling ratio were calculated from the absorbance at 495 nm and 280 nm using ε₄₉₅nm = 84,000 for IAF, ε₂₈₀nm/ε₄₉₅nm = 0.24 to correct the absorbance at 280 nm for the contribution from the dye, E₂₈₀nm = 6.4, and a molecular weight of 30,000 (30, 32).

**Prekallikrein**—Prekallikrein was purified by modification of previously described procedures (8, 30, 31). DEAE-Sephadex chromatography and separation of prekallikrein and factor XI on SP-Sephadex were carried out as before (8), except that Polybrene was omitted after the DEAE-Sephadex column. The pooled prekallikrein from the SP-Sephadex column was dialyzed against 0.1 M Na acetate buffer, 1 mM EDTA, 10 mM benzamidine, pH 5.5 (starting buffer), and applied to a column (1.5 X 12 cm) of HMW-kininogen coupled to Affi-Gel 15 (Bio-Rad) (4 mg coupled/ml of gel) (30). The column was washed with 10 column volumes of starting buffer, followed by the same buffer containing 0.5 M NaCl to elute residual factor XI and finally with this buffer containing 3 M NaSCN to elute prekallikrein. The prekallikrein was dialyzed against 20 mM Na phosphate buffer, 1 mM NaCl, 1 mM EDTA, pH 6.0, and chromatographed on benzamidine-agarose (Pierce Chemical Co.). The column (1.5 X 15 cm) was washed with at least 5 column volumes of the equilibration buffer and then with the same buffer without NaCl. Prekallikrein was eluted with 0.2 M benzamidine in the buffer without NaCl. The protein was dialyzed against 0.1 M Na phosphate buffer, 1 mM NaCl, 1 mM EDTA, pH 5.5, and passed through a column (1.5 X 14 cm) of soybean trypsin inhibitor coupled to Affi-Gel 10 (12 mg coupled/ml of gel) to remove traces of kallikrein. A single preparation of prekallikrein with a specific clotting activity of 26 units/mg was used for most of the experiments in this study. A second preparation of comparable purity was used for the titrations of IAF-kininogen light chain as a function of ionic strength. The purified protein ran as two closely spaced bands on 10% SDS-polyacrylamide gels under reducing conditions with no detectable contaminants (Fig. 1).

Reduced and alkylated prekallikrein was purified by dialysis of 700 μl of a 2.5 mg/ml solution against 0.1 M Tris-Cl, 1 mM EDTA, 6 M guanidine HCl, 1.0 mM dithiothreitol, pH 8.0, followed by addition of 3.5 M iodoacetamide and incubation at room temperature for 1 h. The reaction mixture was dialyzed exhaustively against 6 mM Na acetate buffer, 0.15 M NaCl, 1 mM EDTA, pH 5.3 (storage buffer), and centrifuged to remove precipitated material. The protein concentration was determined by the method of Lowry et al. (53) with bovine serum albumin as a standard. Reduced and alkylated prekallikrein was inactive in coagulation assays.

**Kallikrein**—Kallikrein was obtained either as a by-product of the prekallikrein purification procedure previously used (30) or by controlled activation of prekallikrein with a mixture of purified factors XII and XIII. The generation of a form of kallikrein in which the heavy chain is degraded (34) was minimized by avoiding prolonged incubation of kallikrein at high pH and inactivation of the enzyme with FFRCK (Fig. 1). Kallikrein which was bound to the soybean trypsin inhibitor-agarose column in the final step of the procedure described above was eluted with 0.1 M Na phosphate buffer, 1 mM EDTA, 0.5 M benzamidine, pH 5.5, and dialyzed against storage buffer. Kallikrein was inactivated with FFRCK by addition of one-tenth volume of 0.2 M Na phosphate buffer, pH 7.2, to raise the pH, followed by a 1.5-fold molar excess of FFRCK and incubation at room temperature for 1 h. The enzyme was less than 5% active as measured by the decrease in the initial rates of S-2230 (Kabi) hydrolysis.

Purified prekallikrein (0.2 mg/ml) was activated in 10 mM Tris-Cl buffer, 0.15 M NaCl, 1 mM EDTA, pH 8.1, by addition of sufficient factors XII and XIII to give a maximum in kallikrein activity in 10-20 min, as measured by the initial rates of S-2302 hydrolysis. The enzyme was inactivated by addition of a 2-fold molar excess of FFRCK. Factors XII/XIII were removed by chromatography on QAE-Sephadex (35).

A molecular weight of 82,000 (36) and E₂₈₀nm = 11.7 (30) were used for both prekallikrein and kallikrein. The extinction coefficient is

![Fig. 1. SDS-gel electrophoresis of representative preparations of the proteins used in this study. The reduced proteins were run on 10% SDS gels in the buffer system described by Laemmli (55), and the gels were stained with Coomassie Blue. HMW-kininogen (HMW-K) (20 μg), IAF-kininogen light chain (12 μg), prekallikrein (PK) (14 μg), FFRCK-inactivated kallikrein (KALL) (20 μg), kallikrein heavy chain (KALL-HC) (~2 μg), and kallikrein light chains (KALL-LC) (~5 μg) are shown.](image-url)
indistinguishable from another published value (37). The absorbance of kallikrein solutions in storage buffer containing 1 mg/ml polyethylene glycol 6000 was measured in acryl cysteines (Sarstedt) to minimize losses due to adsorption of this protein to surfaces. Kallikrein Heavy and Light Chains—The kallikrein heavy and light chains were prepared by mild reduction and alkylation of the purified protein followed by affinity chromatography on HMW-kininogen-Sepharose or HMW-kininogen-Affi-Gel 15 as described by van der Graaf et al. (31). To determine the extent of chemical modification which occurs during this procedure, iodol[I-14C]acetamide (Amersham batch 54, 53 mCi/mmol) was used for alkylation. The heavy and light chains, isolated by affinity chromatography, were run on 7.5% SDS-polyacrylamide gels as described by Weber et al. (38) prepared with N,N'-diallyl-tartardiamide as the cross-linker (39) and either stained for protein with Coomassie Blue R-250 or sliced into 1.2-mm sections. Each gel slice was dissolved in 250 μl of 4% periodic acid, 3.75 ml of 5 M NaCl and the amount of protein applied to the gel. Protein concentrations were determined by the method of Lowry et al. (33) with bovine serum albumin as a standard. The incorporation of iodol[I-14C]acetamide was calculated using the specific radioactivity given by the manufacturer and the amount of protein applied to the gel. Protein concentrations were determined by the method of Lowry et al. (33) with bovine serum albumin as a standard. Molecular weights of 52,000 for the heavy chain (8, 9) and an average of 35,000 (9, 31) for the two light chains were used. For the fluorescence anisotropy experiments, the kallikrein light chains were inactivated with FFRCK as described above for kallikrein and dialyzed against storage buffer.

Equilibrium Binding—The fluorescence anisotropy (A) and intensity (F) of the IAF-kininogen light chain were measured with an SLM 8000 fluorimeter as described previously (30). Titrations were performed by adding small aliquots of the protein solutions to the IAF-kininogen light chain in 0.1 M Tris-Cl buffer, 0.05 M NaCl, 1 mM EDTA, 1 mg/ml polyethylene glycol 6000, 2 μM FFRCK, pH 7.4, at 22°C in acrylic cuvettes. The anisotropy measurements were not corrected for scattering and background since this had no significant effect, within the error of the measurements (±0.002) at the highest sensitivity used.

Initial attempts to titrate the IAF-kininogen light chain with enzymatically active kallikrein showed a rapid increase in anisotropy followed by a slow loss of anisotropy on a time scale comparable to that required for the measurements (minutes). The rate of the decrease in anisotropy was enzyme concentration-dependent and inhibited by benzamidine, suggesting that proteolysis was responsible. Irreversible inactivation of kallikrein by reaction with the active site-selective inhibitor FFRCK (40) abolished this effect. Therefore, to prevent proteolysis during the course of the equilibrium binding measurements, active site-blocked kallikrein and kallikrein light chains were used. Stock solutions of the other proteins in storage buffer were mixed with an equal volume of Tris buffer, and 2 μM FFRCK was added to inactivate any traces of kallikrein. Under these conditions, all of the changes in anisotropy occurred rapidly, within the time required for measurement. The anisotropy and fluorescence intensity of the IAF-kininogen light chain measured at several levels of saturation were constant for at least 30 min. The absence of proteolysis was verified by SDS-gel electrophoresis of the proteins after incubation individually or in approximately equimolar combination for 1 h at concentrations (300–600 nM) similar to those used in the binding experiments (not shown). On the basis of these experiments, we have concluded that our results reflect rapidly established equilibrium binding, uncomplicated by proteolysis.

Analysis of Binding Data—Fluorescence anisotropy titrations of the IAF-kininogen light chain in the absence of HMW-kininogen were analyzed by nonlinear least squares computer fitting with ΔAmax, K0 and the concentration of binding sites varied to obtain the best three-parameter fit (41). The stoichiometry (n) determined from titrations of 60 nM IAF-kininogen light chain was assumed in fitting titrations with the same protein preparations at lower light chain concentrations (15 nM) where this value was less well defined.

The binding of HMW-kininogen was studied from its competitive effect on the binding of the IAF-kininogen light chain. These experiments were analyzed graphically using the equations described previously (30) for tight binding of a probe, A, and competitor, B, to C where the interaction of A with C can be measured independently.

\[ A + nC \xrightarrow{K_a} AC_n \]

\[ B + IC \xrightarrow{K_i} BC \]

\[ ([C]'\tau - [C]_T) = \frac{K_B}{K_i} \left( 1 - \frac{1}{[B]_T} \right) + \frac{1}{[B]_T} \]

\[ ([C]'\tau - [C]_I) = \frac{K_B}{K_i} \left( 1 - \frac{1}{[B]_I} \right) + \frac{1}{[B]_I} \]

K0 and K0 are dissociation constants and \( \alpha \) is the fractional saturation of A. \([C]'\tau - [C]_I\) is the difference in the total concentrations required to achieve the same value of \( \alpha \) at the same \([A]\), in the presence and absence of B. For the experiments described here, A = IAF-kininogen light chain, B = HMW-kininogen, and C = prekallikrein, kallikrein, or kallikrein heavy chain. \( \alpha \) was calculated directly from the change in IAF-kininogen light chain anisotropy (42) as \[ A - A_0/\Delta A_{max} - A_0 = \Delta A/\Delta A_{max} \]

Nonlinear least squares analysis of titrations of IAF-kininogen light chain with prekallikrein, for example, in the absence of HMW-kininogen gave KIAF-LPC (where PK represents prekallikrein), ΔAmax, and \( \alpha \) from which [prekallikrein]-T could be calculated for any value of ΔA. Data confined in the presence of HMW-kininogen plotted according to Equation 2, in conjunction with the measured value of KIAF-LPC, were used to obtain KHMW-KPK (where HMW-K represents kininogen) and I. Since this method involves differences between titration curves, these plots could be made linear by varying the end points no more than ±0.002, changes which were within the range of values obtained from independent measurements. The dissociation constant and stoichiometry were determined from the best calculated fit to the untransformed data.

Perrin Plots—The anisotropy was measured at 25°C in the Tris buffer given above with succrose added to vary the viscosity, which was read from tabulated values. Acrylic or siliconized quartz cuvettes were used with no significant differences in the measured anisotropies. These measurements were corrected for scattering by subtraction of a blank. The anisotropy of the IAF-kininogen light chain-prekallikrein complex as a function of viscosity was obtained from the end points of titrations in buffers containing succrose. The apparent rotational correlation times were calculated as \( \phi = V_a/kT \), where \( V_a \) was calculated from the slope of the Perrin plot assuming a fluorescence lifetime for IAF of 4 ns (43). \( V_a = (M_0/N_0) (\theta + \delta) \) was used to calculate the hydrated molecular volume for a protein with molecular weight \( (M) \) and partial specific volume \( (\delta) \), where \( N_0 \) is Avagadro's number and \( \theta \) is the partial specific volume of water. An average degree of hydration (δ) of 0.35 g of H2O/g of protein was assumed. For the IAF-light chain, δ = 0.660 ml/g and \( M_0 = 50,500 \) (52) were used.

RESULTS

IAF-kininogen Light Chain Binding—Titration of the IAF-kininogen light chain at a concentration of 60 nM with prekallikrein or kallikrein resulted in indistinguishable increases in fluorescence anisotropy as shown in Fig. 2. Nonlinear least squares analysis of these titrations gave dissociation constants of 18.6 and 18.9 nM and stoichiometries of 1.0 and 0.9 for prekallikrein and kallikrein, respectively. Three preparations of kallikrein were used in this study with no detectable differences in the binding behavior; two were obtained as byproducts of prekallikrein purification and one by activation of purified prekallikrein as described under "Experimental Procedures." The enzyme was selectively inactivated at the active site with FFRCK, and the titrations were carried out in the presence of the inhibitor to prevent proteolysis and allow the measurement of equilibrium binding (see "Experimental Procedures"). The single preparation of prekallikrein used for most of the experiments in this study gave similar results to those obtained previously (K0 = 17 ± 3 nM) with different protein preparations and in the absence of FFRCK (30). Reduction and alkylation of prekallikrein in 6 M guani-
dine HCl resulted in loss of its ability to bind the IAF-kininogen light chain (Fig. 2).

The disulfide-linked heavy and light polypeptide chains of kallikrein were separated after mild reduction and alkylation of the protein under nondenaturing conditions by affinity chromatography on HMW-kininogen coupled to Sepharose (31). The amino acid compositions of kallikrein and the isolated heavy and light chains are listed in Table I. The number of groups alkylated during the procedure used to separate the polypeptide chains was determined using iodo[14C]acetamide as the alkylating reagent. On the basis of the radioactivity migrating in the position of the isolated heavy chain on a 7.5% SDS-polyacrylamide gel (Fig. 3) and the amount of protein applied to the gel, an incorporation of 0.6 mol of iodoacetamide/mol of kallikrein heavy chain was calculated. A similar experiment for the kallikrein light chain resulted in an estimate of 0.8 mol of iodoacetamide/mol of kallikrein light chain. The isolated heavy chain which bound to HMW-kininogen-Sepharose also bound tightly to the IAF-kininogen light chain, resulting in an increase in anisotropy similar to that observed with prekallikrein and kallikrein (Fig. 2). From this titration, a $K_d$ of 20 nM and stoichiometry of 1.3 mol of heavy chain/mol of IAF-kininogen light chain were obtained. In contrast, the kallikrein light chain, which contains the enzyme active site, did not bind to HMW-kininogen-Sepharose and produced no significant change in anisotropy ($<0.002$) at concentrations up to 500 nM, consistent with no tight binding.

Addition of single chain HMW-kininogen to the IAF-kininogen light chain at concentrations up to 1000 nM did not result in significant ($<0.002$) changes in fluorescence anisotropy. The fluorescence intensity (Fig. 2B) was unchanged ($\pm5\%$) in all of these experiments, indicating no change in the fluorescence yield accompanying the increase in anisotropy. The average values of the dissociation constants and stoichiometries determined from titrations of the IAF-kininogen light chain with prekallikrein, kallikrein, and the kallikrein heavy chain are listed in Table II. The same average maximum anisotropy change of 0.040 $\pm 0.002$ was obtained for each of these proteins.

The dissociation constant for prekallikrein binding to the IAF-kininogen light chain increased with increasing ionic strength. Dissociation constants of 15 nM ($\mu 0.14$), 28 nM ($\mu 0.19$), 248 nM ($\mu 0.50$), and 431 nM ($\mu 0.68$) were obtained from single titrations in 0.1 M HEPES buffers with NaCl added to achieve the desired ionic strength at pH 7.4 and 22 °C. All of the dissociation constants summarized in Table II were determined at the same ionic strength ($\mu 0.14$).

Binding of HMW-kininogen to Prekallikrein and Kallikrein—The binding of unlabeled, single chain HMW-kininogen to prekallikrein and kallikrein was studied from its effect

TABLE I

Amino acid compositions of the isolated heavy and light chains of human plasma kallikrein

| Amino acid   | Heavy chain residues/100 | Light chain residues/100 | Kallikrein |
|--------------|--------------------------|--------------------------|------------|
| Lysine       | 6.06                     | 8.77                     | 7.34       |
| Histidine    | 2.61                     | 2.59                     | 3.05       |
| Arginine     | 4.96                     | 3.59                     | 4.36       |
| Aspartic acid| 8.36                     | 7.89                     | 8.27       |
| Threonine    | 8.22                     | 6.62                     | 7.69       |
| Serine       | 9.42                     | 8.50                     | 7.41       |
| Glutamic acid| 9.45                     | 11.55                    | 10.20      |
| Proline      | 5.14                     | 4.60                     | 5.19       |
| Glycine      | 8.67                     | 9.46                     | 8.42       |
| Alanine      | 4.14                     | 4.41                     | 4.22       |
| Half-cystine | 6.93                     | 2.78                     | ND*        |
| Valine       | 5.67                     | 5.85                     | 6.65       |
| Methionine   | 1.34                     | 1.00                     | 1.45       |
| Isoleucine   | 3.44                     | 9.13                     | 4.79       |
| Leucine      | 7.48                     | 7.10                     | 7.82       |
| Tyrosine     | 2.76                     | 3.18                     | 1.88       |
| Phenylalanine| 5.34                     | 3.01                     | 4.44       |
| Tryptophan   | ND                       | ND                       | ND         |

* ND, not determined.
on the binding of the IAF-kininogen light chain. To this end, the IAF-kininogen light chain was either titrated with prekallikrein (or kallikrein) in the presence of fixed levels of HMW-kininogen, or displacement of the IAF-kininogen light chain from complexes with prekallikrein (or kallikrein) by titration with HMW-kininogen was measured. A possibility which would have complicated the interpretation of the binding data was that human HMW-kininogen self-associates (44). This was investigated by sedimentation equilibrium using an Airfuge and methods described previously (32). Sedimentation of HMW-kininogen to equilibrium at a concentration of 20 μM in the buffer used for the binding studies (including 5 mg/ml Dextran T40) gave a linear plot of log protein fluorescence against radial distance squared and an apparent molecular weight of 30,500 (assuming the partial specific volume of bovine HMW-kininogen (45)). This result was consistent with a previous estimate of the molecular weight under non-denaturing conditions of 108,000 (11), values of 84,400 ± 4,800 and 92,600 ± 9,300 under denaturing conditions (30), and a monomeric structure. Using fluorescein fluorescence to measure the distribution of IAF-kininogen light chain at equilibrium after sedimentation of 2 μM IAF-kininogen light chain in the absence and presence of 7 μM HMW-kininogen gave linear log plots and apparent molecular weights of 28,000 ± 1,200 and 31,000 ± 1,600, respectively, in agreement with the molecular weight of 30,500 ± 1,600 determined previously (32). These results indicate no detectable interaction between HMW-kininogen and itself or the IAF-kininogen light chain at concentrations higher than those used in the fluorescence anisotropy experiments. Therefore, the data obtained for HMW-kininogen reflect equilibrium binding properties of the monomeric, single polypeptide chain form of the protein.

Figs. 4A and 5A show titrations of 15 nM IAF-kininogen light chain with either prekallikrein or kallikrein in the absence of HMW-kininogen and in the presence of four different concentrations of HMW-kininogen ranging from 43.8 to 250 nM. The fluorescence intensity was constant (±5%) during these titrations (not shown). At increasing concentrations of HMW-kininogen, increasing concentrations of prekallikrein or kallikrein were required to achieve the same level of IAF-kininogen light chain saturation (α), consistent with competitive binding of HMW-kininogen and the IAF-kininogen light chain to prekallikrein or kallikrein. These titrations were
analyzed graphically as described under “Experimental Procedures.” When the prekallikrein titrations at different levels of HMW-kininogen were plotted according to Equation 1, a family of straight lines was obtained (Fig. 4B) which intersect on the abscissa at a point corresponding to a ratio of the dissociation constants of approximately 1. This indicates that prekallikrein has essentially the same affinity for the IAF-kininogen light chain and HMW-kininogen. The slopes and intercepts of the lines representing the best fit to each titration gave estimates of $K_d$ ranging from 10.7 to 12.5 nM for prekallikrein binding to HMW-kininogen with stoichiometries of 1.0 to 1.2 mol of prekallikrein/mol of HMW-kininogen. A satisfactory fit to all of the titrations was obtained with the curves calculated by assuming a single set of parameters with $K_{HMW,K,Kall}$ = 12.1 nM and $l$ = 1.1, as shown by the solid lines in Fig. 4A. Fig. 5B shows the results obtained for kallikrein plotted in the same way. Again, the dissociation constants for kallikrein binding to HMW-kininogen obtained from these plots ranged from 13.5 to 16.1 nM and were indistinguishable from the $K_d$ for IAF-kininogen light chain binding (14.7 nM). The best fit to all of the data shown in Fig. 5A was obtained with $K_{HMW,K,Kall}$ (where Kall represents kallikrein) = 14.5 nM and $l$ = 1.2.

Binding of HMW-kininogen was also studied by measuring displacement of the IAF-kininogen light chain from complexes with either prekallikrein or kallikrein by addition of single chain HMW-kininogen. Fig. 6A shows the results obtained when HMW-kininogen was added to the IAF-kininogen light chain in the absence and presence of three different concentrations of prekallikrein. Addition of HMW-kininogen in the presence of prekallikrein resulted in displacement of the IAF-kininogen light chain as measured by the decrease in fluorescence anisotropy. Addition of HMW-kininogen up to 1000 nM in the absence of prekallikrein produced a negligible effect on the anisotropy (Fig. 6A) or fluorescence intensity (not shown). A straight line was obtained when the data were plotted according to Equation 2 under “Experimental Procedures,” as shown in the inset. From the slope and intercept of this line and the $K_d$ determined for prekallikrein binding to the IAF-kininogen light chain, a $K_d$ of 12.4 nM and stoichiometry of 1.3 were calculated for prekallikrein binding to HMW-kininogen. Comparison of the experimental data with the titration curves calculated using these parameters indicates a good fit (Fig. 6A). The results of similar experiments using kallikrein instead of prekallikrein are shown in Fig. 6B. Analysis of these titrations (inset) gave a dissociation constant for kallikrein binding to HMW-kininogen of 15.2 nM with a stoichiometry of 1.3 as best fit to all of the experimental data. The dissociation constants for HMW-kininogen binding derived from these experiments in which the HMW-kininogen concentration was varied at fixed concentrations of prekallikrein or kallikrein agree with the estimates obtained from the preceding experiments in which the prekallikrein or kallikrein concentrations were varied at fixed levels of HMW-kininogen. The average dissociation constants and stoichiometries obtained for HMW-kininogen are listed in Table II.

Interaction of HMW-kininogen with the Kallikrein Heavy and Light Chains—The binding of the isolated kallikrein heavy chain to HMW-kininogen was studied by measuring the decrease in the anisotropy of mixtures of the IAF-kininogen light chain and kallikrein heavy chain, upon addition of HMW-kininogen. Analysis of the displacement titrations shown in Fig. 7 gave a best estimate of 14.4 nM for the dissociation constant of the kallikrein heavy chain–HMW-kininogen complex indistinguishable from the average value obtained for binding to the IAF-kininogen light chain (14 ± 3 nM).

The possibility of a kallikrein light chain interaction which was not detected in direct titrations of the IAF-kininogen light chain was investigated. The kallikrein light chain at a concentration of 300 nM did not have a measurable effect on titrations of the IAF-kininogen light chain with prekallikrein, kallikrein, or the kallikrein heavy chain. Similarly, 300 nM kallikrein light chain did not affect the displacement of the IAF-kininogen light chain from prekallikrein, kallikrein, or the kallikrein heavy chain by HMW-kininogen. The dissociation constants obtained from these experiments are listed in Table II.

Characterization of the Fluorescence Anisotropy Change—The initial anisotropy of the IAF-kininogen light chain of 0.093 at 25 °C was lower than that calculated from the Perrin equation for a spherical protein of the same molecular weight with the fluorescein rigidly attached and prompted us to characterize this signal in more detail. The anisotropy of the IAF-kininogen light chain was the same when measured be-
Fig. 6. Displacement of IAF-light chain bound to prekallikrein (A) or kallikrein (B) by HMW-kininogen. A, the change in anisotropy expressed as $\alpha$ for IAF-light chain (15 nM) titrated with HMW-kininogen (HMWK) in the absence (△) and presence of prekallikrein (PK) at concentrations of 38.8 nM (▲), 77.5 nM (○), and 129.2 nM (●). The curves were calculated using $K_{\text{IAF-LC,PK}} = 12.2$ nM, $n = 1.0$, obtained from a titration in the absence of HMW-kininogen and $K_{\text{HMW-K,PK}} = 12.4$ and $l = 1.3$ obtained from the plot shown in the inset. B, HMW-kininogen titrations of 15 nM IAF-light chain in the presence of kallikrein at concentrations of 39.8 nM (▲), 79.7 nM (○), and 132.8 nM (●). The curves were calculated using $K_{\text{IAF-LC,K}} = 14.9$, assuming $n = 0.9$, obtained from the reference titration and $K_{\text{HMW-K,K}} = 15.2$ and $l = 1.3$ obtained from the plot shown in the inset. KALL, kallikrein.

fore and immediately after chromatography on Sephadex G-25, excluding the possibility that the low anisotropy was due to the presence of free dye or fluorescent labeled low molecular weight impurities. Visual inspection of SDS gels of the IAF-kininogen light chain under ultraviolet light confirmed the absence of significant levels of fluorescent labeled contaminants.

DISCUSSION

In this study we compared the equilibrium binding of prekallikrein and kallikrein to the fluorescent labeled HMW-kininogen light chain and native HMW-kininogen. Binding of the separated kallikrein heavy and light chains was also studied to evaluate the contribution of these components to the interactions. All of our results can be accounted for by a model in which a specific interaction characterized by a dissociation constant in the range of 10–20 nM occurs between binding sites located on the light chain of HMW-kininogen.
and the heavy chain of kallikrein or prekallikrein. Analysis of fluorescence anisotropy titrations of the IAF-kininogen light chain with prekallikrein, kallikrein, or the kallikrein heavy chain indicates that they all bind with indistinguishable average dissociation constants in the range of 13–17 nM (see Table II). Under the same conditions, binding of the kallikrein light chain could not be detected in direct titrations of the IAF-kininogen light chain, nor did it significantly affect the binding of prekallikrein, kallikrein, or the heavy chain. These results are consistent with weak or nonexistent binding of the kallikrein light chain. They do not exclude the possibility that the kallikrein heavy and light chains interact with each other. The kallikrein heavy chain used in these studies was isolated on the basis of its affinity for HMW-kininogen-Sepharose after mild reduction and alkylation of kallikrein. The results of an experiment using iodo[14C]acetamide for alkylation demonstrated that limited chemical modification occurred during the procedure and furthermore suggested that the heavy and light chains of kallikrein may be joined by a single disulfide bond. Prekallikrein which was completely reduced and alkylated under denaturing conditions did not bind the IAF-kininogen light chain, providing evidence that the native tertiary structure is required for this interaction. SDS-gel electrophoresis of the proteins used in all of these experiments indicated that the purity of the proteins was unlikely to be a source of error in comparisons of the binding affinity. The experimentally determined stoichiometries support the existence of 1:1 complexes and the absence of significant amounts of inactive (nonbinding) protein. However, other factors affecting the determination of protein concentration, including the molecular weights assumed, may be additional sources of uncertainty. It is clear from these considerations and the estimated error in the dissociation constants that subtle differences in affinity between these proteins would not be detected in our experiments.

Indistinguishable average dissociation constants of 12 ± 2 nM, 15 ± 2 nM, and 14 ± 3 nM were derived for the binding of native single chain HMW-kininogen to prekallikrein, kallikrein, and the kallikrein heavy chain from experiments in which the IAF-kininogen light chain was used as a probe. These experiments, in agreement with qualitative results obtained by affinity chromatography (31), indicate that the kallikrein heavy chain contains the entire binding site responsible for the high affinity, nonenzymatic interaction between HMW-kininogen and prekallikrein or kallikrein. Clearly there are two different binding sites for native HMW-kininogen on kallikrein since the enzyme active site is contained on the kallikrein light chain and it must also bind HMW-kininogen. The equivalence of the dissociation constants we determined for FFRCK-inactivated kallikrein and the isolated heavy chain and the absence of detectable binding of the FFRCK-inactivated kallikrein light chain demonstrate that binding of HMW-kininogen at the modified enzyme active site does not contribute to the interactions we have measured. The results of a previous study (31) indicate that properties of both sites are required for the full activity of kallikrein in blood coagulation. The isolated kallikrein light chain substantially retains the enzyme activity of kallikrein against a synthetic tripeptide substrate, some natural protein substrates, and inhibitors (27, 31, 48). However, its function as a component of the contact system, as measured by activity in a kaolin- or sulfatide-initiated plasma coagulation assay, is 145–180-fold less than that of kallikrein (31). It seems likely that this large difference reflects the importance of the noncovalent interaction between kallikrein and HMW-kininogen, conferred by the presence of the disulfide-bonded heavy chain, in the reactions of contact activation. The observation that HMW-kininogen light chain and single chain HMW-kininogen bind prekallikrein and kallikrein with essentially the same affinity is correlated with retention of essentially all of the clotting activity of HMW-kininogen by the isolated light chain (11, 28–30). These observations are of interest with regard to the kallikrein-catalyzed release of bradykinin from HMW-kininogen. Association of the enzyme (kallikrein) with its substrate (HMW-kininogen) and possibly the products (two-chain HMW-kininogens) at a nonenzymatic site may affect this reaction. This interaction may contribute to the greater rate of HMW-kininogen cleavage by intact kallikrein as compared to its isolated light chain (31) and the difference between high molecular weight kininogen and low molecular weight kininogen as kallikrein substrates (49). Consistent with this idea, a decreased rate of HMW-kininogen cleavage has recently been demonstrated for a form of kallikrein in which the heavy chain has undergone limited proteolysis (50). While the information currently available indicates an important role for the noncovalent interactions involving HMW-kininogen, no quantitative correlation between the binding we observe and a biochemical function for HMW-kininogen has yet been demonstrated.

If the dissociation constant of 12 nM is employed for the binding of prekallikrein to HMW-kininogen in plasma, in the absence of other interactions and at the concentrations of prekallikrein (610 nM) and HMW-kininogen (740 nM) found in normal human plasma (1), 93% of the prekallikrein and 77% of the HMW-kininogen would be in a 1:1 complex. Such tight binding is consistent with the original demonstration of this complex by gel filtration of normal plasma, or mixtures of the purified proteins (14), and the stoichiometric binding observed by immunoelectrophoresis (15, 17). The dissociation constant determined in this study is similar to a previously published value of 29 nM for the HMW-kininogen-prekallikrein complex adsorbed to the surface of a plastic tube (18). The values obtained for kallikrein are significantly different from the dissociation constants reported for the complexes of kallikrein and HMW-kininogen (750 nM) or its alkylated light chain.
chain (140 nm) inferred from studies of the effect of these proteins on the rate of reaction with protease inhibitors (25, 26). These estimates were obtained from experiments at a higher ionic strength than that used in our studies. The ionic strength dependence of prekallikrein binding to the IAF-kininogen light chain suggests that binding of kallikrein to HMW-kininogen and its light chain will also become weaker with increasing ionic strength. With the data currently available, a direct comparison of these estimates of the dissociation constants is not possible. However, no effect of HMW-kininogen on the rate of kallikrein inactivation by Cl inhibitor was observed in another study (at a different temperature and ionic strength) where the formation of a complex was demonstrated by an independent method (48). Further investigation will be required to clearly establish the relationship between the nonenzymatic binding we have measured and the effect of HMW-kininogen on the rate of kallikrein inactivation.

Under our conditions, proteolytic conversion of prekallikrein to kallikrein did not result in a different affinity for single chain HMW-kininogen. Other proteolytic reactions may affect the affinity between these proteins and HMW-kininogen. In this study, the active site of kallikrein was blocked by covalent reaction with a tripeptide chloromethyl ketone, preventing cleavage of HMW-kininogen. Since single chain HMW-kininogen is a natural substrate for enzymatic degradation of HMW-kininogen by kallikrein, we would expect the kallikrein-HMW-kininogen complex to be short-lived in the absence of inhibitors, with HMW-kininogen being rapidly converted to two-chain forms. The affinity of these forms for prekallikrein and kallikrein may be different and has not been directly determined. On the other hand, the activity of HMW-kininogen measured by coagulation assays is unchanged by this limited proteolysis (11, 28, 29), suggesting that the binding of prekallikrein and kallikrein may not change. Other factors may influence the interaction between these proteins. The binding of HMW-kininogen to activating negatively charged surfaces could certainly affect the binding of prekallikrein and kallikrein, especially since the light chain portion of HMW-kininogen contains both the binding site for these proteins and the surface binding region (23).

The increase in IAF-kininogen light chain fluorescence anisotropy used in this study as a measure of protein complex formation was characterized in greater detail. The nonlinear Perrin plots obtained for the IAF-kininogen light chain and its complex with prekallikrein are consistent with the presence of more than one motion contributing to the depolarization of the fluorescence of the covalently attached dye. These motions may include rapid rotation of the dye near its point of attachment, less rapid motion of flexible protein substructures, and slow rotation of the whole protein. In this situation, the slope of the Perrin plot approached at low viscosity may be a valid measure of the depolarization due to rotation of the whole protein (51). However, the estimated rotational correlation time (\( \phi \)) for the IAF-kininogen light chain of 3.2 ns represents considerably faster motion than the calculated \( \phi \) of 11 ns for rotation of a hydrated spherical protein of equivalent molecular weight. Furthermore, measured rotational correlation times for globular proteins are typically longer than the calculated values for spheres (52). The \( \phi \) of 7 ns estimated for the prekallikrein-IAF-kininogen light chain complex corresponds to slower motion compared to the free protein, but still much faster than expected for rotation of a spherical 1:1 complex. This indicates that the depolarization of the fluorescence of the IAF-kininogen light chain, free and complexed with prekallikrein, is dominated by motions faster than rotation of the whole protein. The increase in anisotropy we observe on complex formation may not be simply a function of overall slower rotation of the complex, but may involve a change in the motion of a flexible protein segment to which the dye is attached. This is consistent with the anisotropy of the IAF-kininogen light chain being lower than that calculated from the Perrin equation for a spherical protein of the same molecular weight with the fluorescein rigidly attached. It may also explain why the same anisotropy was obtained for complexes formed with prekallikrein or the kallikrein heavy chain with respective molecular weights of 112,000 and 82,000. These results do not affect the validity of the anisotropy signal as a measure of the protein interactions.

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