Direct Evidence for Multifacial Contacts between High Molecular Weight Kininogen and Plasma Prekallikrein[†]

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ABSTRACT: HK31 (S565-K595) has previously been shown to encompass the binding domain for plasma prekallikrein (PK) within domain 6 of high molecular weight kininogen (HK). The complementary binding domain for HK within PK is mapped to PK56 (F56-G86), in the apple 1 domain, and to PK266 (K266-C295), in the apple 4 domain. Isothermal titration calorimetry was used to directly monitor binding among HK31, PK56, and PK266. Either PK peptide binds to HK31 in 1:1 stoichiometry, regardless of whether a binary complex is first formed between PK266 and HK31 or between PK56 and HK31. Binding of the alternate PK peptide into a ternary complex is facilitated nearly 2-fold. The ternary complex consists of 1:1:1 HK31:PK56:PK266. Furthermore, binary and ternary complex formation is entropically driven and thermodynamically favored, suggesting that the conformational changes accompany binding. Fluorescence emission spectroscopy revealed that binding of PK56 caused a limited decrease in intrinsic tryptophan fluorescence emission intensity of HK31 while binding of PK266 to HK31 or the complex of HK31/PK56 had no such effect. We conclude that the two PK peptides bind to the HK peptide at different sites. The binding between HK and PK is likely due to conformational changes which serve to juxtapose the PK binding domain within HK with the HK binding site involving two spatial proximity segments.

High molecular weight kininogen (HK),[1] a single-chain plasma glycoprotein, participates in contact activation reactions through specific interactions with prekallikrein (PK) and factor XI (Colman, 1984; Dela Cadena et al., 1993; Wachtfogel et al., 1993; Baglia et al., 1990). In plasma, HK circulates in a stoichiometric complex with either PK or factor XI, but not both proteins (Mandle et al., 1976; Thompson et al., 1977). Our previous results showed that the binding domain sequences within HK for either of these latter two proteins are likely to be alternatively expressed on the surface of HK (You et al., 1991). HK binding to PK in plasma serves to promote surface-dependent activation of the zymogen and to position HK for efficient cleavage by the resulting enzyme, kallikrein (Bock et al., 1985).

The binding site sequence within HK for PK has been mapped to S565-K595 of domain 6 of the light chain of HK, and a synthetic peptide encompassing this sequence (HK31; Table 1) displays the same binding affinity for PK (Vogel et al., 1990) as does intact, native HK (Tait & Fujikawa, 1986, 1987). HK31 assumes a folded structure in solution that is stabilized by long-range interactions between N- and C-terminal residues (Scarsdale & Harris, 1990). HK31 contains essential features necessary for binding PK, such as an ordered N-terminus containing a short helix segment. N- or C-terminal truncations of this sequence cause detrimental perturbations in structure which result in peptides that possess diminished binding capacity (You et al., 1991). For example, deleting the first four N-terminal residues (W569-K595) decreases binding 4-fold (You et al., 1991; Vogel et al., 1990), probably because HK31 and HK27 assume different structures in solution (You et al., 1991, 1993). However, the shortest chain length peptide that retains binding activity for PK is D568-T591 (Lin et al., 1996); loss of additional residues dramatically decreased binding.

PK, a single-chain plasma glycoprotein, contains four homologous, unique 90-residue repeat "apple" domains (Chung et al., 1986). The binding site for HK within PK has been mapped to two discontinuous segments of the native protein (Herwald et al., 1993; Page & Colman, 1991; Page et al., 1994), and synthetic peptides encompassing the PK56 of the apple 1 domain and PK266 of the apple 4 domain bind to HK31 with micromolar affinities (Page et al., 1994).

We have now examined complex formation between HK31, PK56, and PK266. Isothermal titration calorimetry was used to show conclusively that a ternary complex can be formed in 1:1:1 stoichiometry between HK31, PK56, and PK266. Binding of the three molecules is entropically driven (with near-zero enthalpic changes), showing that conformation changes accompany binding. The results of fluorescence emission spectroscopy confirmed that the two PK peptides bind to HK31 in independent fashion. Extension of these

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[1] Abbreviations: HK, high molecular weight kininogen; HK31, synthetic S565-K595 peptide in domain 6 of the light chain of HK; HPLC, high-performance liquid chromatography; ITC, isothermal titration calorimetry; PK, prekallikrein; PK56, synthetic F56-G86 in the apple 1 domain of plasma prekallikrein; PK266, synthetic K266-C295 in the apple 4 domain of plasma prekallikrein. Single-letter abbreviations are used to denote all amino acids.

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Table 1: Sequences of PK56, PK266, and HK31[a]

peptide	sequence
PK56	60 70 80 Ac-F G C*F L K D S V T G T L P K V H R T G A V S G H S L K Q C*G-NH_2
PK266	270 280 290 Ac-K R T L P E P C*H S K I Y P G V D F G G E E L N V T F V K G C*-NH_2
HK31	565 575 585 595 Ac-S D D D W I P D I Q T D P N G L S F N P I S D F P D T T S P K-NH_2

[a] "*" denotes −S−S− linkage between two cysteines.

results to the native proteins suggests that complex formation between HK and PK is accompanied by conformational changes which serve to bring the distal binding segments of PK within close spatial proximity to the binding domain of HK.

MATERIALS AND METHODS

Peptide Synthesis. All peptides were synthesized by solid-phase methods, purified by preparative reverse-phase HPLC, and characterized by analytical reverse-phase HPLC and amino acid analysis (You et al., 1991, 1993; Scarsdale & Harris, 1990; Page & Colman, 1994). All synthesis work was done at Commonwealth Biotechnologies, Inc., Richmond, VA. The sequences of the HK- and PK-based peptides prepared for this study are shown in Table 1.

Isothermal Titration Calorimetry (ITC). ITC was used to directly assess solution-phase binding between HK31 and PK56, between HK31 and PK266, and among HK31, PK56, and PK266. All experiments were done using an Omega titration calorimeter (Microcal, Inc.) as previously described (You et al., 1993; Page et al., 1994; Tyler-Cross et al., 1993, 1994). Briefly, to assess binding between pairs of peptides, HK31 was placed in the calorimeter cell at an initial concentration of 0.25 mM in 30 mM phosphate buffer, pH 7.0, and either PK266 (initial concentration, 2.5 mM) or PK56 (initial concentration 3.96 mM) was placed in the dropping syringe. Twenty injections of the PK peptides were made into the rapidly mixing (400 rpm) solution of HK31, maintained at 30 °C. Each 10 mL injection of PK peptide was followed by a 2 min equilibration period. The resulting binding isotherm was corrected by subtraction for the heat of mixing and/or dilution obtained by injection of ligand (PK peptide) into buffer alone (in the absence of HK31).

ITC was also used to determine whether a ternary complex forms between HK31, PK56 and PK266. In these experiments, the binary complex between HK31 and PK266, or between HK31 and PK56, was first formed following the ITC protocol described above. Once saturation binding was achieved, 200 mL of the solution in the calorimeter cell was removed. The dropping syringe was then loaded with the alternative third PK peptide, which was then added to the calorimeter cell following the same ITC protocol. Two binding isotherms were thus obtained; the first reflects complex formation between HK31 and one of the PK peptides, and the second reflects ternary complex formation between the binary complex and the respective third PK peptide. Both isotherms were corrected by subtraction for heat of dilution and/or mixing due to injection of the appropriate ligand into buffer solution alone.

We again used ITC to assess complex formation when all three peptides were present prior to complex formation. Here, PK56 and PK266 were placed in the calorimeter dropping syringe at initial concentrations of 2.5 mM, and HK31 was placed in the calorimeter cuvette, at an initial concentration of 0.25 mM. The experiment was then done as previously described.

Fluorescence Emission Spectroscopy. Intrinsic Trp fluorescence emission and pH dependent fluorescence emission spectroscopies were performed essentially as previously described (You et al., 1991, 1993; Scarsdale & Harris, 1990). All experiments were done at ambient room temperature (λ_{excit} = 295 nm, λ_{emiss} = 366 nm, 0.5 cm pathlength cell) usually with 50 μM peptide in 50 mM Na_3PO_4, buffer, pH 7.2. The emission spectra of the complexes formed between HK31 and PK56 or between HK31 and PK266 were determined using 12 μM HK31 and increasing concentrations (up to 0.5 mM) PK56 or PK266.

RESULTS

Primary Structure of Synthesized Peptides. Table 1 illustrates the peptides prepared for this study which are referenced to the position number of the native protein (HK or PK) upon which they are based. HK31 has been extensively studied (Tait & Fujikawa, 1986, 1987) and has been shown to wholly encompass the prekallikrein binding domain within kininogen. PK56 and PK266 were shown previously to incorporate portions of the kininogen binding domain within kallikrein (Page & Colman, 1991; Herwald et al., 1993; Page et al., 1994).

Complex Formation between HK31 and PK56 and between HK31 and PK266. We have previously shown the utility of using ITC to assess the thermodynamic parameters mediating solution-phase binding between short-chain peptides and their ligands (You et al., 1993; Page et al., 1994; Tyler-Cross et al., 1993, 1994). Table 2 shows that either PK56 or PK266 can bind to HK31 to form a binary complex in 1:1 stoichiometry. PK266 appears to make a slightly tighter complex (K_d = 74 μM) than PK56 (K_d = 146 μM), but both binding reactions are entropically driven (+ΔS values), yielding essentially energetically equivalent complexes (ΔG^b = −5 kcal/mol for the PK266/HK31 complex and −6 kcal/mol for the PK56/HK31 complex). We also titrated PK56 with PK266 to see if a binary complex formed between the two PK peptides. A flat isotherm was obtained, indicating the lack of complex formation.

Tertiary Complex Formation between HK31, PK56, and PK266. ITC was also used to directly demonstrate ternary complex formation between HK31, HK56, and HK266. PK56 was found to bind to the preformed HK31/PK266 binary complex in 1:1 stoichiometry (making the ternary complex stoichiometry 1:1:1), and the K_d for binding PK56 (68 μM) is 2-fold better than that for complex formation

Table 2: Thermodynamics of Binding Between HK31, PK56, and PK266[a]

binding pair	K_d (μM)	ΔH (kcal/mol)	ΔS (eu)	N	ΔG (kcal/mol)[b]
HK31 + PK266	74.2	−0.14	+18.4	1.06	−5.75
HK31 + PK56	131.1 ± 20.8	+4.5 ± 1.6	+26.4 ± 3.5	1.32 ± 0.67	−5.39
HK31/PK266 + PK56	67.9 ± 0.07	+1.6 ± 0.7	+25.1 ± 2.6	1.39 ± 0.57	−5.78
HK31/PK56 + PK266	41.8 ± 5.65	+0.78 ± 0.35	+22.9 ± 1.6	1.34 ± 0.08	−6.07
HK31 + PK56/PK266	K_{D1} = 33.7	+8.43	+51.4	1.44	−6.20
	K_{D2} = 33.5	−13.4	−28.8	1.27	−6.22

[a] All experiments were done at 30 °C in 30 mM phosphate buffer, pH 7.01. For these experiments, HK31 was placed in the calorimeter cell at 0.25 mM, and either PK266 (2.5 mM) or PK266 (3.96 mM) was placed in the dropping syringe. Once the binary complexes were formed (*i.e.*, HK31/PK266 and HK31/PK56), the indicated alternate PK peptide was added to the calorimeter cell. For the last experiment in the table, PK56 and PK266 were placed in the dropping syringe at 2.5 mM and HK31 was placed in the calorimeter cuvette at 0.25 mM. All isotherms were corrected by subtraction for heat of mixing and dilution following injection of ligand(s) into buffer alone. Where two experiments were done, the average values (±1 SD) are shown. [b] $\Delta G = -RT$ 1n K; calculated from the average K_d value.

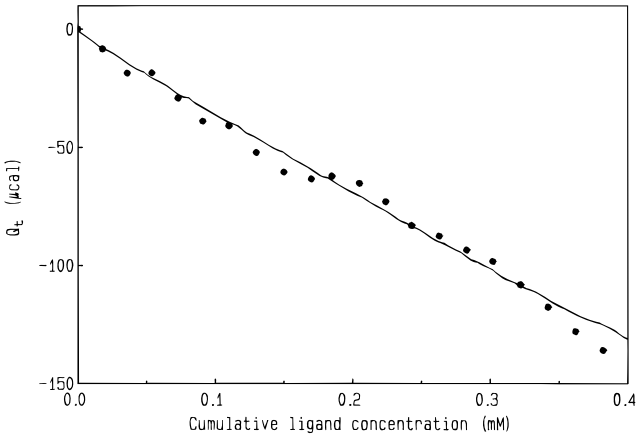

FIGURE 1: Determination of the binding isotherm of PK56 and PK266. PK56 and PK266 were placed in the calorimeter dropping syringe at initial concentrations of 2.5 mM, and HK31 was placed in the calorimeter cuvette at an initial concentration of 0.25 mM. The experiment was then done as described in Materials and Methods. The binding isotherm was fit using a two-site equation.

between HK31 and PK56 in the absence of PK266 (Table 2). Similarly, PK266 was found to bind to the preformed HK31/PK56 binary complex in 1:1 stoichiometry, and its K_d (= 46 μM) is nearly 2-fold better than that for binding between HK31 and PK266 in the absence of PK56 (Table 2). Hence, binding of either PK peptide to HK31 facilitates binding of the second PK peptide. Binding of the third molecule to either binary complex is also entropically driven, leading to energetically equivalent ternary complexes (ΔG^b = −6 kcal/mol).

We then assessed complex formation when all three peptides were introduced at the same time. If PK56 and PK266 were to bind to HK31 in independent fashion, we might expect that the binding isotherm would fit a two-independent site binding equation, and two association constants would be obtained that mirrored the constants obtained in the earlier experiments. If, however, binding of one peptide precluded binding of the other, we would expect the resulting binding isotherm to conform to a single binding site equation and to reflect the binding mode of only one of the peptides.

Determination of Binding Isotherm. The binding isotherm was fit using a two-site equation, indicating that both PK56 and PK266 bind simultaneously to HK31 (Figure 1). The dissociation constants obtained for the two binding sites, K_{d1} = 33.7 μM and K_{d2} = 33.5 μM, respectively (Table 2), are entirely consistent with the constants obtained when each peptide was titrated individually. However, the mode of binding for one of the binding sites was now found to be exothermically driven (ΔH = −13.4 kcal/mol; S = −28.8 eu), while that of the second was again found to be endothermically driven (ΔH = +8.4 kcal/mol; ΔS = +51.4 eu).

We cannot ascribe either binding event with a particular peptide, but the stoichiometry of binding in the ternary complex was found to be 1:1 between PK56 and HK31, and 1:1 between PK266 and HK31, yielding an overall stoichiometry of the complex of 1:1:1. As one might expect with a curve-fitting algorithm, the data could also be made to conform to a single binding site equation. However, here, the standard deviation of fit was higher than that obtained for the two-site equation fit, and the macroscopic dissociation constant obtained (87 μM) was higher than that obtained using the two-site fit. Hence, we conclude that the peptides form a ternary complex involving two independent binding sites on HK31, and the binding of PK56 effects a conformational change in HK31, PK56, or both molecules.

Characterization of the Solution Structures of the Peptides. HK31 contains a single Trp residue located near the N-terminus, and changes in the intrinsic Trp fluorescence emission spectrum thus reflect structural perturbations of the hydrophobic microenvironment which encompasses W569 (Scarsdale & Harris, 1990; You et al., 1991, 1993). The fluorescence emission spectra of HK31 are the same at pH 7.0 and 7.2 or at 23 and 30 °C. Because there are no Trp residues in either PK56 or PK266, complexation of either of these peptides with HK31 might be expected to affect the intrinsic emission spectra if the binding locus on HK31 involves the Trp residue in the N-terminal region of the peptide. Binding of PK56 by HK31 caused a limited decrease in emission intensity, while binding of PK266 had no effect (Figure 2a). Thus, binding of PK56 causes a change in the microenvironment encompassing the Trp residue in HK31; binding of PK266 does not obviously affect this domain. Furthermore, once PK56 is bound, addition of very high concentrations of PK266 does not displace PK56 (Figure 2b). We can conclude that PK56 and PK266 bind to HK31 at non-overlapping sites.

The following equation was used to estimate the binding constant of HK31 and PK56, where a concentration of PK56 which gave 50% of maximal binding was used.

$$K_d = \frac{[\text{HK31}]\,[\text{PK56}]}{[\text{HK31}\cdot\text{PK56}]} = [\text{PK56}]$$
$$= [\text{PK56}]_o - [\text{HK31}\cdot\text{PK56}]$$

where HK31·PK56 is the binary complex and $[PK56]_o$ is

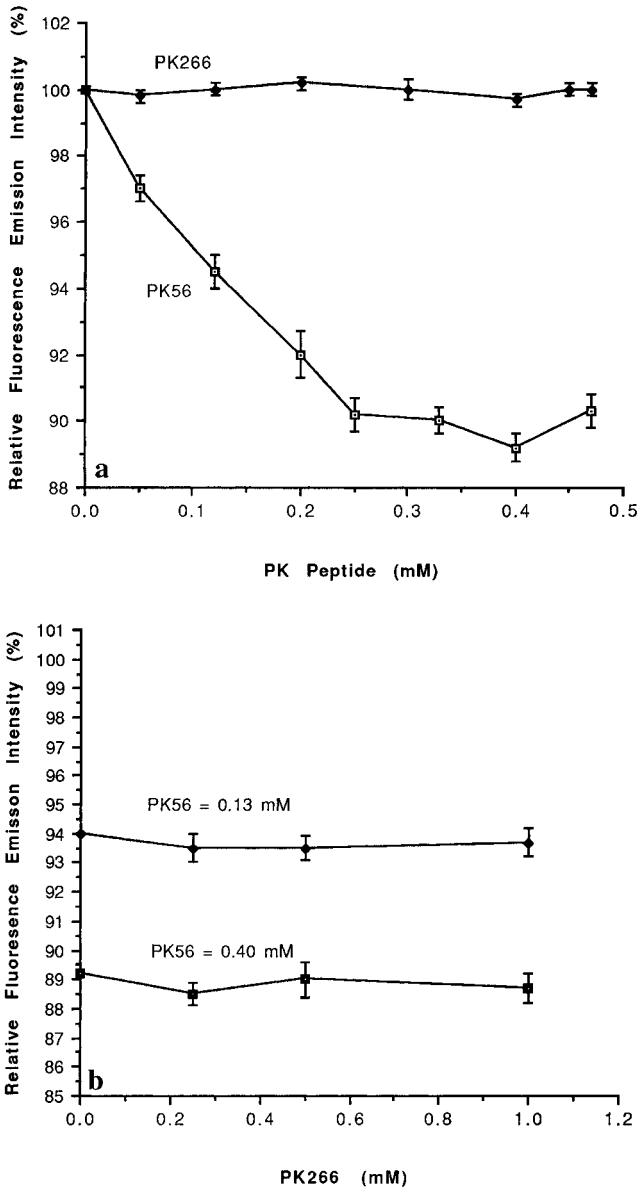

FIGURE 2: Effect of PK56 (□) and PK266 (●) on relative Trp fluorescence emission intensity. The concentration of HK31 is 12 μM. (a) Peptide solutions were made in a buffer containing 50 μM sodium phosphate, pH 7.2. The excitation wavelength is 295 nm; the emission wavelength is 365 nm. No emission maximum wavelength shift took place after mixing HK31 with PK56. (b) Adding PK266 to the mixture of HK31 and PK56 at the two concentrations shown did not change the emission intensity. The results are the mean of three experiments performed in triplicate. The bars are SEM.

the initial concentration of PK56. Therefore, $K_d = 100\ \mu M - 6\ \mu M = 94\ \mu M$. This value is in good agreement with the K_d from Table 2 of 131 μM which is obtained from ITC.

DISCUSSION

HK31, or a shorter-chain HK peptide, D567-K591, significantly inhibits the intrinsic pathway of fibrinolysis (Lin et al., 1996; Motta et al., 1995), by inhibiting the binding of PK to cell-bound HK and its activation to kallikrein. Understanding more about the nature of binding between HK and PK may lead to the design of a powerful inhibitor for fibrinolysis. The complementary binding domain for HK within PK appears to be composed of two discontinuous segments (Page & Colman, 1991; Page et al., 1994), and this discontinuous domain is adequately represented by synthetic peptides (PK56 and PK266) which retain binding affinity for HK (Page et al., 1994). Thus, we thought it likely that HK31 contains two functionally separate regions that interact (bind) with the complementary peptide domain peptides of PK.

ITC was used to directly quantify solution-phase binding between HK31, PK56, and PK266 and test the above hypothesis. In the binary complexes, HK31/PK56 or HK31/PK266, binding between molecules shows a 1:1 stoichiometry and is mediated by conformational changes (positive ΔS values) occurring in HK31 or the PK peptides or both. Usually, these conformational changes are driven by exclusion of water from the interface between molecules, and we can envision that binding of either PK peptide involves numerous intermolecular contacts.

The exciting finding was that a ternary complex does indeed form with the second PK peptide, and that ternary complex formation is facilitated in either case by formation of the binary complex. While the overall stoichiometry in the ternary complex is 1:1:1, it does not appear to be an ordered event; that is, binding of either PK56 or PK266 can occur first, but complex formation with the second PK peptide results in energetically equivalent complexes regardless of the order of binding of the PK peptides. Surprisingly, when the ternary complex was found in the presence of all three molecules, at least one binding mode was enthalpically driven. This is different from when the binary complexes were preformed followed by addition of the alternative PK peptide. Here, both binding events were entropically driven.

Perhaps complex formation between HK and PK is more accurately mimicked in the experiment where all three components were present simultaneously. This would suggest that the first contact between HK and PK is a simple binding interaction (enthalpically driven), while the second binding event involves contacts that result from more subtle changes which accompany conformational alterations in the proteins. On the basis of the results of the fluorescence emission experiments (as discussed below), we would suggest that the endothermic binding event involves PK56, while PK266 is involved in the exothermic binding interaction. Alternatively, we might simply consider that when all three components are present simultaneously, at least one mode of interaction between the peptides is different from when the binary complexes are preformed. Regardless, although the PK peptides cannot bind to each other, they clearly bind to HK peptide when presented simultaneously.

Fluorescence emission spectroscopy was then used to confirm the independent nature of HK31 binding to PK56 and PK266. We have now shown that upon binding to PK56, unlike binding to PK266, HK31 experiences an environmental change at the N-terminal region of the peptide. The estimated K_d of HK31 and PK56 (94 μM) from fluorescence emission study is close to ITC analysis (131 μM). Because binding of PK266 did not cause observable changes in fluorescence emission of either HK31 or HK31/PK56, we conclude that PK266 must bind to a site that does not overlap with PK56 on HK31. This conclusion is consistent with the ITC data and indicates that HK31 would bind PK56 and PK266 simultaneously, thus mimicking the contacts that might be made between native HK and the distal binding sites for HK within PK.

Thus, we have shown that HK31 possesses two binding regions, complimentary to the discontinuous binding segments of PK. We do not know whether either of the conformation changes exhibited by these peptides upon complex formation has physiological significance, but this finding is consistent with our hypothesis that a conformational change within HK is required to express either the PK binding domain or the factor XI binding domain on the surface of the HK protein (You et al., 1993). Expression of one domain conformer of HK over the other might be under the control of some unknown "physiological switch" (You et al., 1993; Page et al., 1994).

Extension of the results obtained with these synthetic peptides to the native proteins suggests that complex formation between HK and PK is accompanied by conformational changes which serve to bring the distal binding segments of PK within close spatial proximity to the binding domain of HK. Factor XI shows extensive sequence and conformational homology with PK. However, the only binding domain for HK is PK56 in the factor XI apple 1 domain; apple 4 domain of factor XI does not contain a binding site for HK, in contrast to PK. However, while PK has four disulfide loops in apple 4, factor XI has only three plus an unpaired thiol that is involved in dimer formation. It is likely that these major changes in the primary sequence and/or the interaction between the two apple 4 domains in factor XI, a natural dimer, disrupt the potential binding site in FXI apple 4 for HK.

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