Pure & Appl. Chem., Vol. 66, No. 1, pp. 27–34, 1994.
Printed in Great Britain.

Structural biology of high molecular weight kininogen

Robert W. Colman

Sol Sherry Thrombosis Research Center, Temple University School of Medicine, Philadelphia, PA, USA.

Abstract

High molecular weight kininogen is a multifunctional plasma protein. After cleavage of bradykinin, the light chain contains a zymogen binding domain (D6), which binds prekallikrein, and a surface binding domain (D5). Using peptides, monoclonal antibodies and deletion mutagenesis, two subdomains, HK-rich and HGK-rich, have been defined as necessary for binding to anionic surfaces and coagulant activity. The heavy chain contains D5, responsible for calpain inhibition. Using affinity labeling, we have shown that the motif QVVAG is necessary as a binding domain on HK, but appears to require additional inhibitory sequences to interact with the thiol in the active site of calpain.

INTRODUCTION

Kininogens are plasma proteins which are the natural substrates of plasma and tissue kallikrein and yield, on hydrolysis, the nonapeptide, bradykinin. In 1967, two forms of purified human plasma kininogen (kinin precursors) were described by Jacobsen and Kriz: (ref. 1) high molecular weight kininogen (HK) and low molecular weight kininogen (LK). HK is an α-globulin with a plasma concentration of 80 μg/ml. (ref. 2) LK is a β-globulin with a plasma concentration of approximately 160 μg/ml (ref. 3). In 1974, an individual, Mrs. Mayme Williams, who required an elective operation, was found to have prolonged *in vitro* coagulation time. Extensive investigation established that she lacked both HK and LK (ref. 4). The role of kininogens has been extended by investigation of this individual's plasma and cells, which have revealed much about the biological role of this molecule.

GENETICS OF HIGH MOLECULAR WEIGHT KININOGEN

Both HK and LK are coded for by the same single copy gene (ref. 5). The gene is 27 kb long and consists of 11 exons in which the primary transcripts are differentally spliced to yield two different RNAs. Low molecular weight kininogen contains the first 9 exons plus a small part of the 10th including the codons for bradykinin and 12 additional amino acids. It is then spliced to the 11th exon. HK contains the same 9 exons plus all of the long 10th exon. After translation and removal of the signal peptide and glycosylation of about 40% by weight, HK has a molecular weight of 120 kDa and LK of 68 kDa.

We localized the gene by *in situ* hybridization of a metaphase chromosome spread to chromosome 3q26q to terminal (ref. 6). Nearby are the genes for the evolutionarily related cystatins, histidine-rich glycoprotein and α2HS glycoprotein.

We have also recently characterized the mutation in Ms. Williams, who lacked both HK and LK (ref.7). Northern analysis, reverse transcriptase and PCR showed a normal pattern of the two mRNAs present in her post mortem liver, and Southern analysis failed to show a gross defect with 20 restriction enzymes. Primers flanking each exon were used for PCR amplification of normal and Williams gene. Each amplified exon fragment was sequenced. A C to T transition was located at nucleotide 587 downstream from the start codon ATG, resulting in a CGA (Arg)-TGA (stop)

mutation in exon 5 of the kininogen gene. Since the stop mutation is localized before the differential splice site (in exon 10), the synthesis of both LK and HK proteins are affected.

The mutation eliminates the recognition site of the restriction enzyme Csp45-I. Results of Csp45-I enzyme digestion of the PCR amplified exon 5 fragments (252 bp) of the patient, 3 daughters (with 50% functional HK), and 1 granddaughter (normal functional HK) reveal that the patient is homozygous (1 band: 252 bp) for the mutation, while the 3 daughters are heterozygous (3 bands: 252, 134 and 118 bp). The coagulant activities confirm that the inheritance is autosomal recessive. This family represents the first documented mutation defined as responsible for total kininogen deficiency.

DOMAIN STRUCTURE OF HIGH MOLECULAR WEIGHT KININOGEN

The mature form of HK protein contains 626 amino acids (Fig. 1). On cleavage by kallikrein, the nonapeptide bradykinin is released from D4 corresponding to the beginning of the 10th exon. The cleaved protein contains an NH2-terminal heavy chain (362 amino acids) bound to a COOH-terminal light chain (255 amino acids) by a single interchain disulfide bridge. The heavy chain consists of three large domains, D1, D2, D3, each derived evolutionarily from the more primitive cystatin by gene duplication and corresponding to the first 9 exons, and is identical in HK and LK.

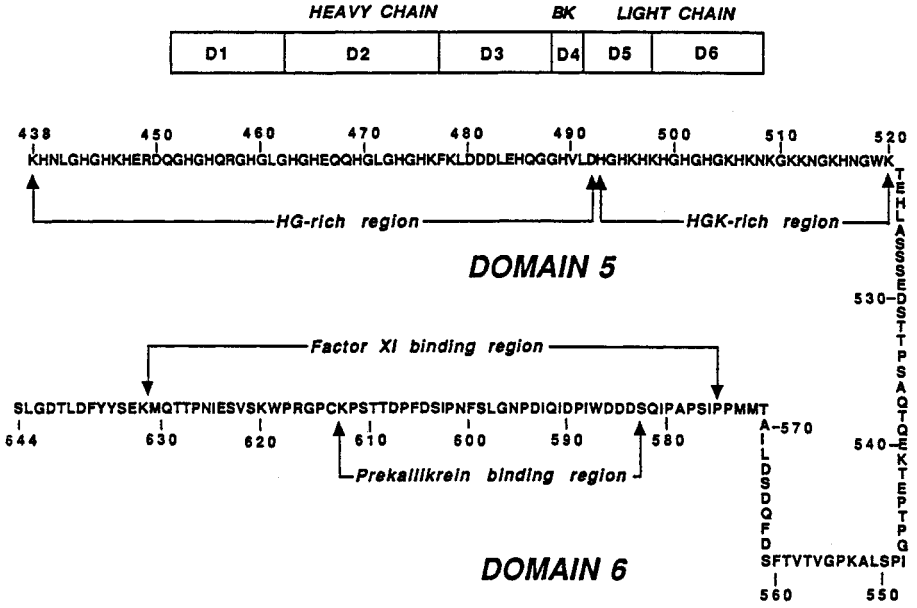

Fig. 1. Structure of HK LC. Top: Six domains of HK. Bottom: Amino acid sequence of HK LC (domain 5 and domain 6). The HG-rich and HGK-rich regions in domain 5, and the prekallikrein and factor XI binding regions in domain 6, are marked. Reproduced with permission from reference 18.

LK has a unique small (4 kDa) light chain of unknown function. The light chain of HK (56 kDa) contains the procoagulant activity which depends in part on its ability to associate with the zymogens, prekallikrein (PK), (ref. 8) through residues 556 to 595 of HK, or FXI, through residues 556-613 (D6). The coagulant activity of HK also depends on the binding of cleaved HK to hydrophilic surfaces. This function is thought to be mediated through its histidine-glycine-rich region (residues 407-498) (D5).

PROTEOLYTIC CLEAVAGE OF HIGH MOLECULAR WEIGHT KININOGEN

Plasma kallikrein cleaves HK in a three-step sequential manner (ref. 9). The first two cleavages, at Lys380-Arg381 and Arg389-Ser390, yield a kinin-free protein (HKa) composed of two disulfide-linked 64,000 and 56,000 chains and a nonapeptide, Bk. The third cleavage results in a

stable kinin-free protein composed of two disulfide-linked 64,000 and 45,000 chains and liberates a small 7,000 peptide. These cleavages lead to major conformational changes as detected by circular dichroism (ref. 10). Studies with human HK show that cleaved forms, HKa, binds to a greater extent to an activating surface (ref. 11), indicating that HK exists as a procofactor that can be activated by cleavage with kallikrein. FXIa cleaves HK initially, at the site of the third cleavage by kallikrein, resulting in two fragments (a heavy chain of 75,000 and a light chain of 46,000) (ref. 12) followed by two cleavages to release Bk. Prolonged exposure of HK to FXIa results in an extensive proteolysis of the HK light chain to produce a degraded form of HK (HKi) with a loss of coagulant cofactor activity (ref. 12).

SURFACE BINDING DOMAIN OF HIGH MOLECULAR WEIGHT KININOGEN

To obtain additional data regarding the composition of the surface binding site of HK (D5), we further characterized MAb C11C1, an antibody developed in our laboratory, (ref. 13) which recognizes specifically the light chain of HK and inhibits coagulant activity. We found that an IgG fraction of MAb C11C1 inhibited the binding of ^{125}I-HK to an anionic surface, kaolin, 57% at a final concentration of 30 mM. Furthermore, this was specific since the MAb 2B5 to the heavy chain failed to inhibit the coagulant activity. This finding confirms that the light chain is responsible for the coagulant activity. The action of MAb C11C1 is selective since there was no interference with ^{125}I-FXI binding to HK and no changes in the rate of cleavage of HK by plasma kallikrein.

Since MAb C11C1 selectively inhibited the binding of HK to kaolin, we then defined the epitope in native HK light chain (Fig. 2) which was recognized (ref. 14). When HK (lane 1) was digested sequentially with plasma kallikrein and factor XIa, a series of polypeptides resulted including the largely intact heavy chain is seen along with a small amount of undigested light chain (1) as well as main fragments l_1 and l_2. Purification by affinity chromatography on MAb C11C1 bound to agarose followed by molecular filtration yielded l_2, a polypeptide of 7.3 kDa.

When the peptide was subjected to amino acid analysis, it proved to be composed of predominantly His and Gly. Moreover, the peptide did not adsorb to the Immobilon-P-membrane normally used for sequencing. Therefore, it was covalently coupled to aminophenyl-glass fiber paper to obtain the

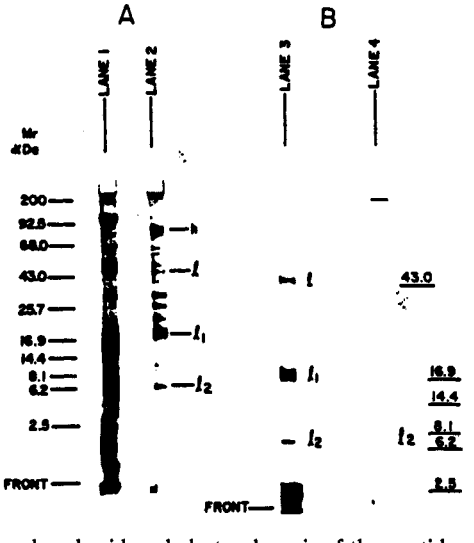

Fig. 2. Sodium dodecyl sulfate polacrylamide gel electrophoresis of the peptides purified using C11C1 affinity column. The 15% gels were stained with silver after fixation by glutaraldehyde. Lanes 1 and 2 were run for 2.5 h under reduced conditions. Lane 1 represents the partially cleaved starting material (120 kDa). Lane 2 shows the starting material after cleavage by human plasma kallikrein and FXIa. The molecular weight standards for lanes 1 and 2 are indicated on the left side. The third and fourth lanes were run for 3 h under nonreduced conditions. Lane 3 represents the different-sized peptides containing the epitope recognized by C11C1. Lane 4 represents the purified l_2 fragment. The molecular weight standards for lanes 3 and 4 are indicated on the right. Reproduced with permission from reference 14.

N-terminal sequence. The first 6 amino acids were HGLGHG; however, despite an adequate yield, no sequence was obtained from steps 7 through 17. Since the covalent coupling is to free COOH groups, the presence of a Glu in position 8 would prevent the further sequencing since it would be the part of the covalent attachment. Based on the Mr and the N-terminal analysis, the sequence was determined as 459-516 and did not overlap with the sequence 556-613 responsible for binding FXI.

To further define the requirements for interaction of human HK with negatively-charged surfaces, we selected and synthesized HGLGHGH because it is a highly conserved sequence found in human as well as in bovine HK, and because it forms the N-terminal amino acid sequence of the 7.3 kDa peptide containing 57 amino acids. HGLGHGH is repeated twice (residues 441-447 and 451-457) within the second of the three homologous regions.

We then investigated the effect of HGLGHGH and two unrelated control peptides (RGYSLG and HHLGGHKQAGDV) upon the binding of cleaved ^{125}I-HK to kaolin (Fig. 3). At a concentration of 5000 μM, HGLGHGH blocked the specific binding of ^{125}I-HK to kaolin 50%. The control peptides had no effect.

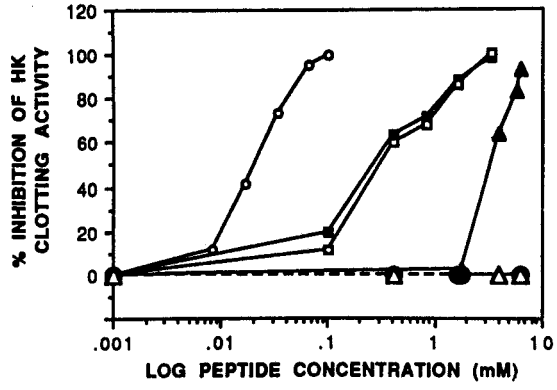

Fig. 3. Effect of the peptides derived from the histidine-rich region upon the coagulant activity of HK. The HK (0.06 mM) was incubated at 37°C for 8 min in the presence of different concentrations of either unrelated control peptides RGYSLG (●–●) and HGLGGAKQAGDV (Δ–Δ) or the three histidine-rich-region-derived peptides HGLGHGH (▲–▲), HGLGHGHEQQHGLGHGH (□–□), no zinc; and (■–■), plus zinc), and l_2 fragment (o- - o). Calcium (6 μM) was added to initiate the HK coagulant assay. Each value is the mean of duplicate assays. Reproduced with permission from reference 14.

We therefore synthesized another peptide of intermediate length - a heptadecapeptide. The feature of this peptide derived from the light chain of HK is that the heptapeptide is repeated twice with a glutamic acid-glutamine-glutamine sequence intervening. The HEQQH is a canonic sequence similar to that of thermolysin for zinc binding. This 17-mer inhibited the binding of ^{125}I-HK to kaolin 50% at 2300 μM but in the presence of zinc this declined to 630 μM. The 73 kDa peptide, l_2, inhibited at 380 μM.

Thus, we have defined a heptadecapeptide which binds zinc and comprises much of the surface binding domain of HK. However, additional residues contained in a 57 amino acid sequence may also comprise areas important for interaction with anionic surfaces.

We then examined the surface binding domain amino acids more closely (Fig. 4). When this sequence is compared to bovine and rat, we find significant sequence homologies. The residues 443-475 HG-rich region is 55% homologous and especially in the heptadecapeptide region. After an area of lower homology missing in the bovine protein, a second area rich in HG and lysine (K) is 61% homologous. We therefore designed deletion mutants to examine both of these regions as well as the domain 6 region which binds factor XI and prekallikrein.

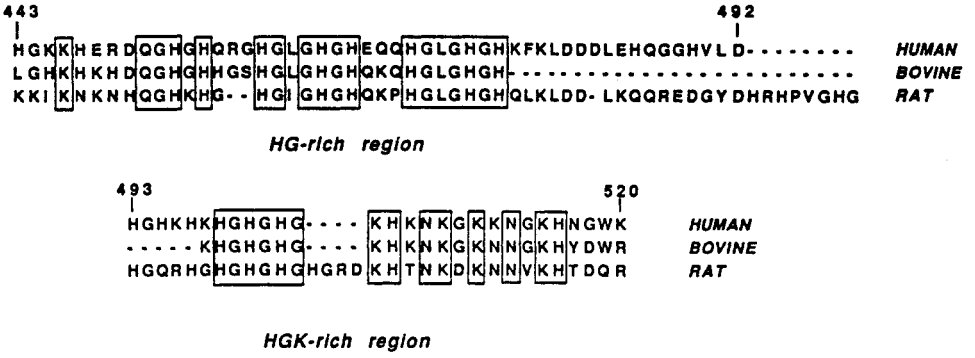

Fig. 4. Comparison of HG-rich and HGK-rich regions of human HK LC with bovine and rat HK. The HG-rich and HGK-rich regions of human HK LC were compared with the corresponding sequences in bovine and rat HK. The residues are numbered to identify the position in the human HK LC. Identical amino acid residues in all three species are boxed. Gaps are introduced to obtain maximum homology. Reproduced with permission from reference 18.

We have generated various deletion mutants of HK LC and the stuctures of these recombinant fragments expressed in *E. coli* (Fig. 5). All the recombinant fragments contain glutathione S-transferase (GST) at their N-terminus. The rHK LC contains residues Lys438-Ser644 of HK comprising the HG-rich region, HGK-rich region, and domain 6 (the zymogen binding region). The deletion mutants His459-Ser644, Glu466-Ser644, Leu-483-Ser644, and His 493-Ser644 lack residues 438-458, 438-465, 438-482, and 438-492 (in the HK-rich region), respectively, but contain domain 6 at the C-terminus. The fragment His493-Ser644 thus contains HGK-rich region, domain 6, but not HG-rich region. The recombinant fragment Ser583-Ser644 lacks residues 438-582 (entire domain 5 and part of domain 6). The recombinant fragment Lys438-Asp492 contains HK-rich region but lacks HGK-rich region and domain 6, while the fragment Lys438-Ser531 contains both HG-rich and HGK-rich regions (entire domain 5), and lacks entire domain 6. The deletion mutant D493-520 HK LC contains HG-rich region and entire domain 6, and lacks HGK-rich region.

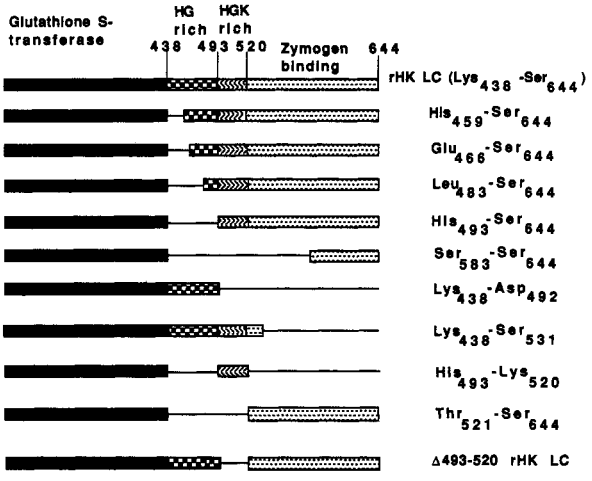

Fig. 5. The schematic structures of the HK LC deletion mutants. Reproduced with permission from reference 18.

Each mutant was tested for its ability to inhibit the binding of ^{125}I-HK (Table I). The inhibition displayed by each fragment was concentration-dependent and the IC50 values are shown. While unlabeled HKa inhibited the 125I-HKa binding to kaolin most efficiently (IC50 of 5 μM), various recombinant fragments also inhibited although with less efficiency (IC50 of 37.8-74.4 μM). The smallest fragment with inhibitory activity (His493-Ser644) is an order of magnitude less effective than unlabeled HKa.

TABLE I: IC50 VALUES OF RECOMBINANT HK LC FRAGMENTS

Polypeptide	IC_{50} (μM)
rHK LC	37.8 ± 9.3
His_{459}-Ser_{644}	55.3 ± 10.2
Glu_{466}-Ser_{644}	56.4 ± 5.5
Leu_{483}-Ser_{644}	52.4 ± 9.2
His_{493}-Ser_{644}	74.4 ± 8.1
Lys_{438}-Ser_{531}	42.6 ± 2.3
Lys_{438}-Asp_{492}	49.1 + 2.4
His_{493}-Lys_{520}	52.9 ± 3.3
Thr_{521}-Ser_{644}	N.D.
Ser_{583}-Ser_{644}	N.D.
GST N.D.	
HKa	5.0*
I_2 fragment	380*

Reproduced with permission from Ref. 18.
N.D.: Not determinable since the inhibition at 100 μM was less than 25%.
The values are mean ± SD of three separate experiments.

In order to further examine the results obtained by competition experiments, two recombinant fragments lacking the C-terminus of HK LC (Lys438-Asp492 and Lys438-Ser531) were produced. The fragments His493-Ser644, Lys438-Ser531, and Ser583-Ser644 were radiolabeled by Iodogen method. In order to rule out the presence of a surface binding subdomain in residues N-terminal to 493-His, recombinant fragment Lys438-Asp492 was also radiolabeled. These fragments were then used in a kaolin binding assay. Radiolabeled HK LC and fragment Lys438-Ser531 and His493-Ser644 bound to kaolin, displacing fibrinogen in a concentration-dependent manner, indicating that the surface binding components are present on these proteins. The Ser583-Ser644 fragment, lacking the entire domain 5, did not bind to kaolin in a concentration-dependent manner, indicating the deficiency of a surface binding component. Consistent with our peptide data, Lys438-Asp492 fragment also bound to kaolin in a concentration-dependent manner. This result confirmed the presence of a second anionic surface binding subdomain in the HG-rich region (Lys438-Asp492) of HK LC.

In order to determine the specificity of radiolabeled recombinant fragment binding to kaolin, unlabeled HKa was included in the assay at increasing molar ratios. Except for the Ser583-Ser644 fragment, all the radiolabeled recombinant fragments were displaced by unlabeled HKa, indicating that the binding of these fragments to kaolin is specific and that they all contain at least one anionic surface binding subdomain. The presence of more than one subdomain does not seem to increase the binding either additively or synergistically (Lys438-Ser531 fragment and rHK LC). The binding of Ser583-Ser644 fragment was low and nonspecific, and hence was not displaced with unlabeled HKa. These results are in agreement with the direct binding studies.

If there were two subdomains in domain 5 of HK LC, then the presence of either surface binding subdomain and zymogen binding region in any HK LC recombinant fragment could result in coagulant activity. This possibility was tested by constructing a deletion mutant of HK LC lacking residues 493-520 (HGK-rich region). Thus, if there is a surface binding subdomain in the HG-rich region (Lys438-Asp492) of HK LC, then this fragment lacking residues 493-520 should have coagulant activity. Similarly, the His493-Ser644 fragment should have coagulant activity if residues 493-520 (HGK-rich region) contain a surface binding subdomain. The coagulant activity of various recombinant fragments was assayed (Table II). As shown, rHK LC, His493-Ser644 fragment and D493-520 HK LC have similar coagulant specific activities, while the other HK LC fragments Lys438-Asp492, Lys438-Ser531, and Ser583-Ser644 did not have clotting activity. The coagulant activity is a result of HK LC binding both to anionic surfaces and the zymogens. Fragments Lys438-Asp492 and Lys438-Ser531 have only one or both anionic surface binding subdomains but not the zymogen binding region, while the Ser583-Ser644 fragment contains only zymogen binding

TABLE II: COAGULANT ACTIVITY OF DELETION MUTANTS

Polypeptide	Surface Binding Domain HG	HGK	Zymogen Binding Domain	Coagulant Activity U/μ mole
HKa (Purified from plasma)	+	+	+	1.51
HK LC (Reduced and alkylated)	+	+	+	1.40*
rHK LC	+	+	+	0.76**
rHK LC (As GST fusion protein)	+	+	+	1.20
Lys_{438}-Asp_{492}	+	-	-	0
His_{493}-Lys_{520}	-	+	-	0
Thr_{521}-Ser_{644}	-	-	+	0
His_{493}-Ser_{644}	-	+	+	0.96
Lys_{438}-Ser_{531}	-	+	-	0
Δ493-520 HK LC	+	-	+	0.87
Ser_{583}-Ser_{644}	-	-	+	0

Reproduced with permission from Ref. 18.

subdomain. The recombinant fragment D493-520 HK LC contains Lys438-Asp492 (HG-rich surface binding subdomain) fused to Thr521-Ser644 (zymogen binding subdomain) and hence has coagulant activity, while the surface binding HGK-rich subdomain C-terminus to 493-His is responsible for coagulant activity of the His493-Ser644 fragment.

These results confirm the presence of two anionic surface binding subdomains, one in the HG-rich region and the other in the HGK-rich region in HK LC.

HK—A CYSTEINE PROTEASE INHIBITOR

We will now focus on the heavy chain of HK. The heavy chain consists of three large domains, D1, D2, and D3, each derived evolutionarily from the more primitive cystatin by gene duplication and corresponding to the first 9 exons, and is identical in HK and low molecular weight kininogen (LK). Domains 2 and 3 both inhibit papain and have the conserved QVVAG motif. We demonstrated that the platelet calcium-activated thiol protease, calpain, was potently inhibited by human HK and less so by LK and α2-macroglobulin (ref. 15). In a detailed investigation of the kinetics of the inhibition, we demonstrated that HK was a tight binding inhibitor with a Ki of 500 pM (ref. 16). This implied a close fit between HK and platelet calpain. Only domain 2 inhibits calpain.

We therefore turned to the importance of specific amino acids in the HK heavy chain for the inhibition of platelet calpain. The sequence QVVAG (Gln-Val-Val-Ala-Gly) in the heavy chain of kininogen is thought to be important in its inhibitory action. Moreover, the addition of the nitrosulfopyridyl ring results in an adduct perpendicular to the rest of the structure in possible position to react with the active site of calpain.

Therefore, a series of peptides was synthesized by Dr. Rei Matsueda which have this sequence with Cys substituted for alanine and nitrosulfopyridine linked to the sulfhydryl group (ref. 17). This activated SH group can react covalently with the SH group at the active site of calpain. Although QVVAG itself is not inhibitory, these peptides proved to be a potent affinity label or irreversible inhibitor of calpain. Presumably, QVVAG provided a binding region and the nitrosulfopyridine could react with the SH in the active site. The most specific turned out to be P1, FQVVC(PyS)G. (ref. 19).

When P1 is in molar excess to calpain, the reaction obeys pseudo-first order kinetics. When the rate constant is plotted as a function of inhibitor concentration, the slope is the second order rate constant, about $6 \times 10^3 M^{-1}s^{-1}$.

We are currently modeling D2, which inhibits calpain, to find inhibitory sites other than QVVAG.

REFERENCES

1. Jacobsen, S. and Kriz, M. *Br. J. Pharmacol.*, **29**: 25 (1967).
2. Schmaier, A.H., Zuckerberg, A., Silverman, C., Kuchibhotla, J., Tuszynski, G.P. and Colman, R.W. *J. Clin. Invest.*, **71**: 1477-1489 (1983).
3. Muller Esterl, W., Vohle Timmermann, M., Boos, B. and Dittman, B. (1982) *Biochim. Biophys. Acta.*, **706**: 145-152.
4. Colman, R.W., Bagdasarian, A., Talamo, R.C., Scott, C.F., Seavey, M., Guimaraes, J.A., Pierce, J.V. and Kaplan, A.P. *J. Clin. Invest.*, **56**: 1650-1662 (1975).
5. Kitamura, N., Kitagawa, H., Fukushima, D., Takagaki, Y., Miyata, T. and Nakanishi, S. *J. Biol. Chem.*, **260**: 8610-8617 (1985).
6. Cheung, P.P., Cannizzaro, L.A. and Colman, R.W. *Cytogenet. Cell Genet.*, **59**: 24-26 (1992).
7. Cheung, P.P., Kunapuli, S.P., Wachtfogel, Y.T., Scott, C.F. and Colman, R.W. *Blood.* **78**: 391a (1991). (Abstract)
8. Mandle, R.J., Colman, R.W. and Kaplan, A.P. *Proc. Natl. Acad. Sci. U. S. A.* **73**: 4179-4183 (1976).
9. Mori, K. and Nagasawa, S. *J. Biochem.*, **89**: 1465-1473 (1981).
10. Villanueva, G.B., Leung, L., Bradford, H. and Colman, R.W. *Biochem. Biophys. Res. Commun.*, **158**: 72-79 (1989).
11. Scott, C.F., Silver, L.D., Schapira, M. and Colman, R.W. *J. Clin. Invest.*, **73**: 954-962 (1984).
12. Scott, C.F., Silver, L.D., Purdon, A.D. and Colman, R.W. *J. Biol. Chem.*, **260**: 10856-10863 (1985).
13. Schmaier, A.H., Schutsky, D., Farber, A., Silver, L.D., Bradford, H.N. and Colman, R.W. *J. Biol. Chem.*, **262**: 1405-1411 (1987).
14. DeLa Cadena, R.A. and Colman, R.W. *Prot. Sci.*, **1**: 151-160 (1992).
15. Schmaier, A.H., Bradford, H., Silver, L.D., Farber, A., Scott, C.F., Schutsky, D. and Colman, R.W. *J. Clin. Invest.*, **77**: 1565-1573 (1986).
16. Bradford, H.N., Schmaier, A.H. and Colman, R.W. *Biochem. J.*, **270**: 83-90 (1990).
17. Matsueda, R., Umeyama, H., Puri, R.N., Bradford, H.N. and Colman, R.W. Chem. Lett., 191-194 (1990).
18. Kunapuli, S.P., DeLa Cadena, R.A., and Colman, R.W. *J. Biol. Chem.*, **268**: 2486-2492 (1993).
19. Puri, R.N., Matsueda, R., Umeyama, H., Bradford, H.N. and Colman,R.W. *Eur. J. Biochem.*, **214**: 233-241 (1993).