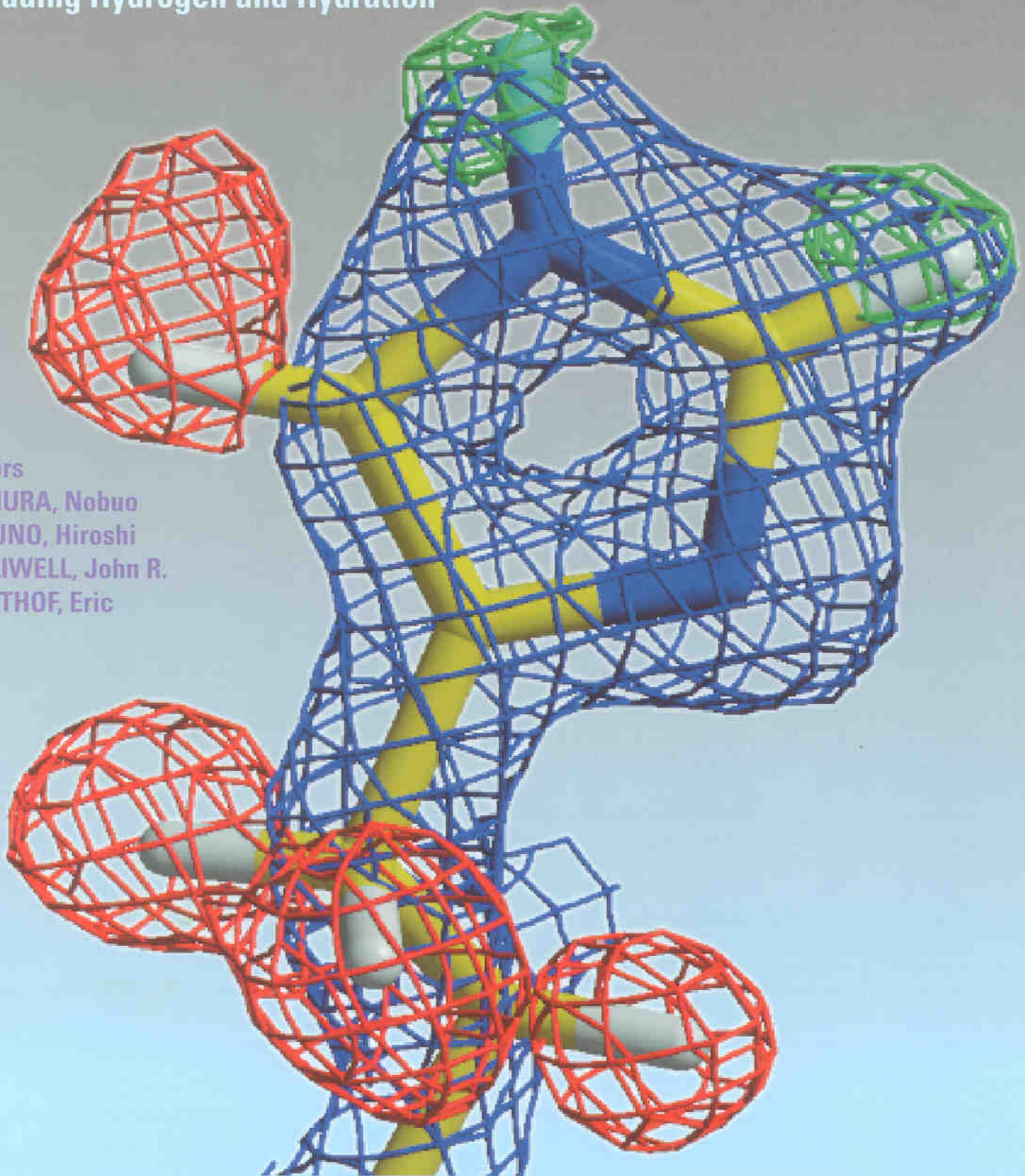# Hydrogen- and Hydration-Sensitive Structural Biology

Invited papers presented in the Symposium on 'Development of New Structural Biology Including Hydrogen and Hydration'

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Solvent Structure in the Active Site of Human Kallikrein 1

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Keywords

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Summary

The human kallikrein (hK) family of serine proteases comprises 15 members, 12 of which were identified only within the past decade. Although characterization is far from complete, the various members of this family appear to span the range from degradative to regulatory type proteases, and are involved in a variety of physiological processes and diseases. Human kallikrein 1 (hK1; also known as tissue kallikrein) cleaves various prohormones and bioactive peptides and plays a major role in blood pressure regulation, inflammation, and heart disease. As part of this function, hK1 exhibits unique dual-substrate specificity, and is able to hydrolyze low molecular weight kininogen between both Arg-Ser and Met-Lys sequences. The active site of *apo* hK1 exhibits structural features intermediate between that of *apo* and *pro* forms of known kallikrein structures and bound peptide ligands appear to contribute to the formation of a catalytically-competent active site structure. In response to the binding of peptide ligands, the S2 to S2' pockets demonstrate a variety of conformational changes, including the displacement of an extensive solvent network. The solvent network within the S2 to S2' pockets of the apo form suggests how novel inhibitors might be designed.

Introduction

The kallikreins (derived from the Greek word "kallikreas" which means pancreas, and historically a rich source of these proteases [1]) are a family of closely-related serine proteases that are distributed in a wide variety of tissues and biological fluids. The human kallikrein family has gained attention in recent years due to the fact that most of its members appear to be differentially expressed in normal versus cancerous tissues, and this may prove to be a useful diagnostic in certain diseased states. For example, prostate specific antigen (PSA, or hK3) and hK2 (sometimes also referred to as "glandular kallikrein") are considered as the most useful biomarkers known for prostate cancer [2,3]. Other kallikreins have been proposed as diagnostic markers for breast (hK3, hK6) and ovarian cancers (hK6, hK9, hK10 and hK11) [4-8]. More recently, several studies have shown that hK6 may play a key role in the regulation of myelin turnover, and in demyelinating disease [9-14] as well as the degradation of β-amyloid, or turnover of amyloid precursor protein [6,15]. Mouse K8 has been implicated in neuronal function (kindling epileptogenesis) [16,17]. Thus,

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the functional and biophysical properties of the kallikreins are of substantial interest.

The most extensively studied member of the kallikrein family is "tissue kallikrein" or hK1. This kallikrein is known to cleave various prohormones and bioactive peptides including kininogen, proinsulin, prorenin, and procollagenase [18] and plays a major role in inflammation and heart disease. The prohormone kininogen is synthesized in the liver and comprises two components: high molecular weight kininogen (120 kDa) and low molecular weight kininogen (68 kDa). Lysyl-bradykinin, or kallidin, is a decapeptide produced by the proteolytic action of hK1 upon low molecular weight kininogen via cleavage between two specific bonds involving Met-Lys and Arg-Ser sequences, and this dual specificity of hK1 is a unique functional property of this kallikrein [19]. Lysyl-bradykinin is a vasoactive peptide that lowers blood pressure and plays an important role in blood pressure regulation [20-22]. The actions of lysl-bradykinin are opposed by angiotensin II, a vaso-constrictive peptide produced from the proteolytic cleavage of angiotensinogen I by angiotensin converting enzyme (ACE) [23,24].

The use of the kallikreins as cancer biomarkers, particularly K2 and K3, suggests a possible role in tumor maintenance or metastasis. Inhibition of such kallikreins may prove useful in preventing or controlling such cancers. With regard to neurological disease, selective inhibition of K6 has been shown to delay the severity and onset of inflammatory CNS demyelinating disease in the mouse [4]. Although the function of the majority of the kallikreins remains to be elucidated, it is clear that it will be of great importance to develop inhibitors targeted against specific members of the human kallikrein family. Although structural data for the kallikreins would be extremely useful in the development of such inhibitors, the available structural data for the human kallikreins is, unfortunately, limited. At the present time only two examples of human kallikrein structures, hK1 and hK6, are available from the structural databank [25-27]. The available hK1 structure is of the mature active form in the absence of any bound inhibitor (*i.e.* the *apo* form of the enzyme). The *apo* hK1 structure exhibits features that indicate that substrate binding serves to order the active site into a catalytically-competent stereochemistry, and involves desolvation of an extensive solvent network within the S2 to S2' binding pockets. Small molecules that mimic this *apo* solvent network may therefore function as a novel type of inhibitor.

1. Active site stereochemistry of *apo* hK1

Human kallikrein 1 exhibits the characteristic His57/Asp102/Ser195 "catalytic triad" typical of the serine protease family. The typical rotamer for Ser195 in serine proteases is *gauche*+ ($\chi_1 = -60°$); this orientation promotes an electrostatic interaction of the side chain hydroxyl group with His57 of the catalytic triad (promoting an increase in nucleophilicity) and simultaneously orients the side chain in the direction of an appropriately bound peptide substrate for nucleophilic attack. However, the catalytic Ser195 in the *apo* hK1 structure exhibits two different conformations (*gauche*+ and *gauche*–), but neither is appropriately juxtaposed for function. In the *gauche*+ orientation Ser195 does not hydrogen bond with the adjacent His57, rather, solvent is the primary hydrogen bonding partner. In the *gauche*– orientation Ser195 hydrogen bonds with the adjacent His57, but is inappropriately oriented for nucleophilic attack (Fig. 1).

The structure of hK6 has been solved for both the inactive *pro*-form as well as the mature active form with bound benzamidine inhibitor [25,26]. In the *pro*-hK6 structure Ser195 adopts a *gauche*- orientation (similar to one of the orientations observed in the hK1 *apo* form), whereas, in the mature active form with bound benzamidine the *gauche*+ rotamer (hydrogen-bonded to His57) of Ser195 is observed. The recombinant hK1 protein utilized in the x-ray structure determination was shown to be equivalent in enzymatic activity to naturally-derived hK1 [27]. Thus, it was concluded that the binding of substrate contributes to in an induced-fit conformational change in the region of the active site Ser195 that results in a catalytically-competent stereochemistry [27].

Figure 1 Relaxed stereo diagram of the active site of *apo* hK1 (PDB 1SPJ) illustrating the alternative conformations of the active site Ser195 and the local solvent structure. The *gauche*-rotamer ($\chi_1 = +60°$) is shown in the lower panel, and the *gauche*+ rotamer ($\chi_1 = -60°$) is shown in the upper panel. Neither orientation in the *apo* structure appears catalytically competent.
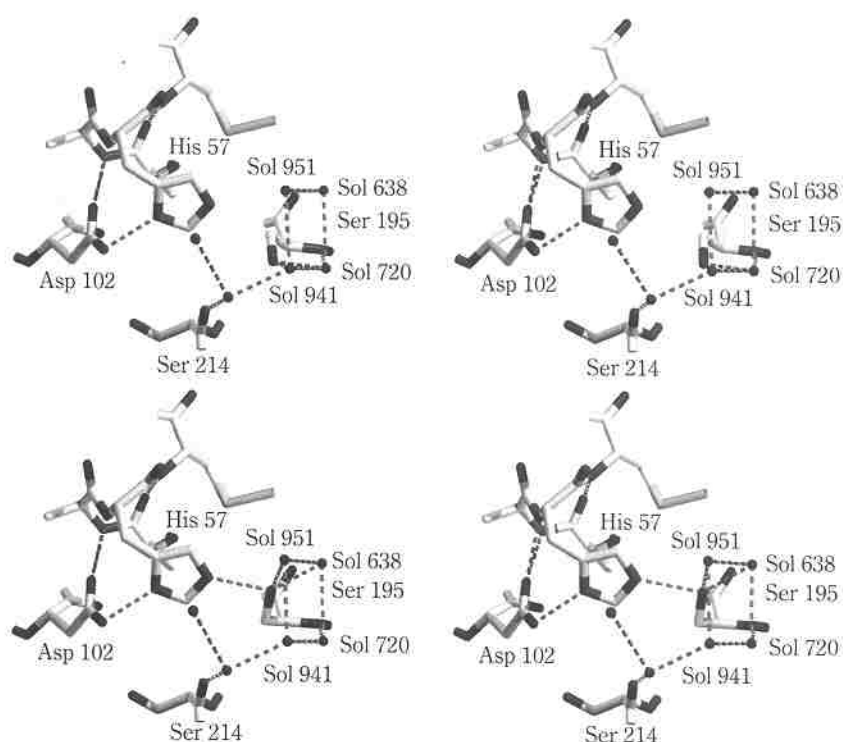
2. S2 to S2' binding pocket stereochemistry

Interactions within the S2 to S2' pockets of hK1 are primary determinants of Met-Lys bond cleavage specificity, and interactions within the S1' to S3' pockets have been identified as important contributors to efficient hydrolysis of short peptide substrates [28]. Furthermore, approximately 80% of the binding energy of peptide substrates and inhibitors to human K1 is contributed by interactions within the S2 to S1' pockets [29]. These and other studies identify interactions within the S2 to S2' pockets as forming essential structural determinants of substrate specificity and catalytic efficiency for human K1.

2-1. The S2 binding pocket

The S2 pocket of both human and porcine K1 comprises a hydrophobic cleft formed by the side chains of residues Trp 215 and Tyr 99 [30]. This hydrophobic cleft forms the basis of the preference of human and porcine K1 for substrates with hydrophobic P2 residues [28,30]. While the Trp 215 side chain exhibits a characteristically conserved rotamer orientation in all K1 structures (porcine K1/benzamidine complex (2PKA); porcine K1/BPTI complex (2KAI); porcine K1/hirustasin complex (1HIA); and the present report), the Tyr 99 side chain exhibits a variety of conformations in the different K1 structures. In the human *apo* K1 structure the cleft between Trp 215 and Tyr 99 in human *apo* K1 is broader by approximately 0.7Å in comparison to the porcine K1 structures. In addition to being broader, the human *apo* K1 structure exhibits an intervening solvent molecule (Sol 939, B factor of 27.7Å^2, Fig. 2) between these side chains. This solvent is positioned centrally with regard to the aromatic ring of the side chain Trp 215 and at a distance of 3.2Å normal to the center of this ring, and is therefore optimally positioned to hydrogen bond with the π electron cloud of the aromatic ring of Trp 215. This hydrated open form of the S2 pocket has not previously been observed for any kallikrein structure. A comparison of the human *apo* K1 and inhibited porcine K1 structures shows that this solvent, as well as neighboring solvent 398, are excluded and the Trp 215 and Tyr 99 residues move closer to each other, forming the characteristic hydrophobic S2 pocket upon substrate binding.

2-2. The S1 binding pocket

The S1 pocket is formed by the main chain atoms of residue positions 214-217 and 189-195, and includes interactions by the side chains of positions 189, 190, 195, 216, and 226. The S1 pocket of the human *apo* K1 structure contains a string of solvent molecules (solvent 710, 943, 785, and 691, respectively), that extend from the proximity of Ser 195 to Asp 189. These solvent molecules form a contiguous hydrogen bonding network, with solvent 691 residing at the "bottom" of the S1 pocket and hydrogen bonding to Asp 189 (Fig. 3). A comparison of the human *apo* K1 structure with the various porcine K1 structures indicates that solvents 710 and 943 are displaced by the hydrophobic side chain C^β, C^γ, and C^δ atoms of the P1 side chain Arg (1HIA) or Lys (2KAI) residues. A similar comparison of the structure of bovine β-trypsin in complex with a mutant form of BPTI containing a Met at the P1 positions (3BTM[31]), also indicates that solvent 710 and 943 (but neither 785 nor 691) would be displaced by this side chain. Neither solvent molecule 710 or 943 is an exclusive hydrogen bonding partner for any protein atom, thus their displacement by a hydrophobic side chain carbon atom does not result in an unsatisfied hydrogen bonding partner within the S1 pocket. Solvent 785 is replaced by Lys N^ζ (2KAI) or Arg $N^{\eta 1}$ (1HIA) and solvent 691 is replaced by Arg $N^{\eta 2}$ (1HIA). Solvent 785 serves as the exclusive hydrogen bonding partner of the main chain carbonyl of position Thr 190. Being replaced by either the P1 Lys N^ζ or Arg $N^{\eta 1}$ group thus maintains important hydrogen bonding interactions within the S1 pocket. As with a P1 Arg bound in the S1 pocket, Sol 691 also is displaced when Lys binds (2KAI). Thus, the displaced solvent within the S1 pocket provides a "road map" for the locations of the side chain atoms of the P1 residue.

2-3. The S1' binding pocket

In the human *apo* K1 structure the S1' pocket is occupied by solvent molecules 854 and 789 that extend from the proximity of a bound P1' C^β outward, respectively. In the absence of a bound peptide inhibitor the side chain of Gln 41 adopts a χ_2 angle of ~ −60°.
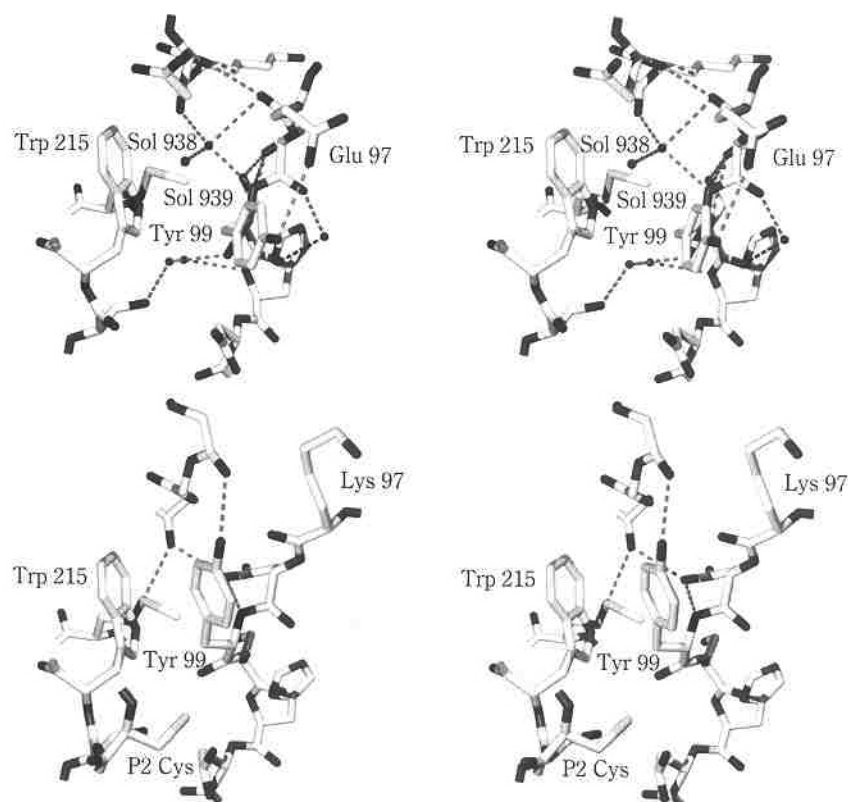

Figure 2 Relaxed stereo diagram of the S2 site of *apo* hK1 (upper panel) and porcine K1 in complex with bovine pancreatic trypsin inhibitor (2KAI; lower panel). The location of the inhibitor P2 residue (Cys) is indicated. Binding of the peptide inhibitor results in a rotation of the Tyr99 side chain, forming a hydrophobic S2 pocket with Trp215, and displacing solvents 398 and 399.

However, in the porcine K1/hirustasin complex the Gln 41 side chain adopts a χ_2 angle of ~180°, "flipping up" and orienting lengthwise along the edge of the S1' pocket (Fig. 4). The rotation of the Gln 41 side chain appears necessary to avoid a steric clash with the main chain carbonyl oxygen of a bound P2' residue. Consequently the His 35 side chain must rotate out of the way of the repositioned Gln 41 side chain, and the His side chain rotates 120°, from the *gauche+* rotamer to *trans*. Inspection of the porcine K1/Hirustasin complex (1HIA) with an Ile side chain in the P1' position, shows that the side chain $C^{\gamma 1}$ and C^{δ} atoms follow the solvent 854 and 789 channel in human *apo* K1. The reoriented Gln 41 side chain provides *van der Waals* contact surface along the side of the S1' pocket for a bound P1' aliphatic side chain. Since the structural changes of Gln 41 appear to arise from the presence of a bound P2' main chain carbonyl, there is an apparent synergy between the binding of the P2' main chain and the P1' side chain, thus substrates lacking a P2' residue may exhibit reduced affinity for the P1' residue.

2-4. The S2' binding pocket

The S2' pocket is a hydrophobic cleft formed by residue positions Phe 40, Phe 151, and Gly 193. These residues are essentially juxtaposed when comparing an overlay of human *apo* K1 with either porcine K1/benzamidine complex (2PKA; empty S2' pocket), porcine K1/BPTI complex (2KAI; Arg in the P2' position), or porcine K1/hirustasin complex (1HIA; Arg in the P2' position). There are two solvent molecules located in the S2' pocket in human *apo* K1 (925, and 931) and these are displaced by the terminal guanidino group of the P2' Arg residue (see Fig. 4). Thus, the structural alterations upon the binding of a P2' residue primarily involve displacement of these solvent groups.
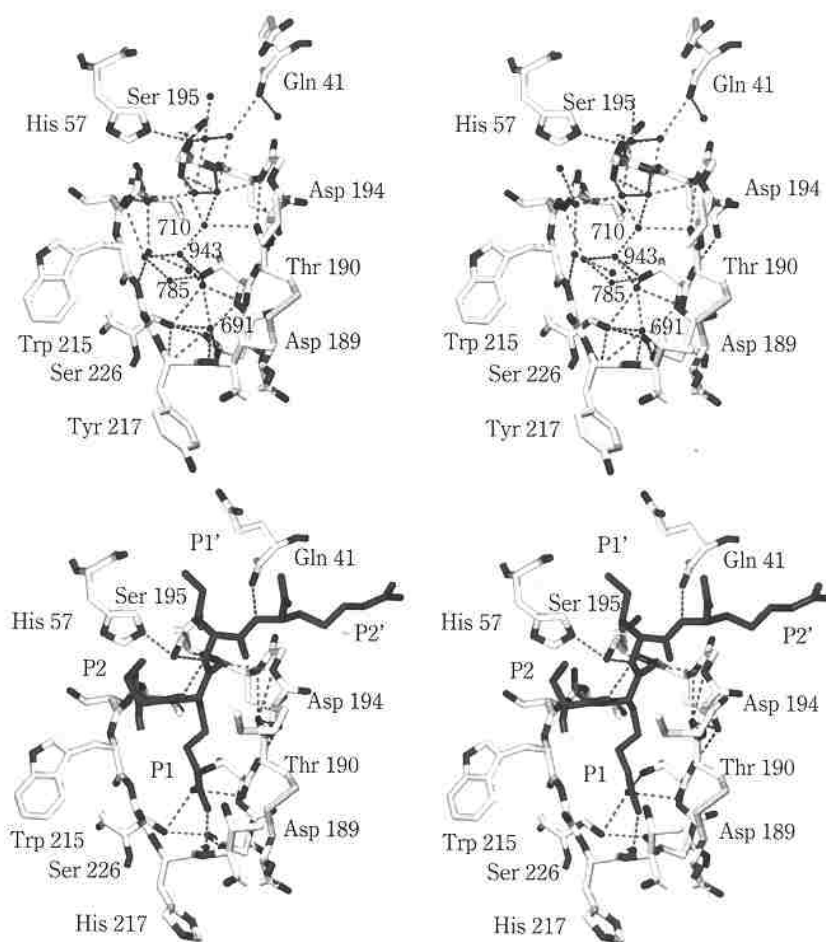

Figure 3 Relaxed stereo diagram of the S1 site of *apo* hK1 (upper panel) and porcine K1 in complex with hirustasin (1HIA; lower panel). The P2 to P2' residues of the hirustasin inhibitor are indicated in dark grey shading. The solvent structure within the S1 binding pocket in *apo* hK1 is indicated, and specific solvent displaced by the bound P1 side chain atoms are identified.

3. Displaced solvent network

The solvent displaced upon binding of a peptide inhibitor/substrate within the S2 to S2' pockets of hK1 fall into three general categories: 1) solvent displaced by bound main chain groups, 2) solvent displaced by the side chain groups of the bound peptide, and 3) solvent displaced as a direct consequence of structural changes induced by bound peptide. Table 1 lists the set of displaced solvent within the S2 to S2' sites upon peptide binding. A total of seven solvent groups are displaced by peptide P2 to P2' main chain atoms (*i.e.* approximately 2 solvent per residue). The number of solvent displaced by the side chain groups of these residues is dependent upon the particular amino acids, however, a total of 10 solvent are identified that can be displaced by side chains bound within the S2 to S2' pockets, with a P1 arginine displacing the greatest number (four total). Finally, two solvent are displaced due to rearrangements within the S2 pocket upon peptide binding. A tetra peptide substrate or inhibitor bound within the S2 to S2' pockets can therefore potentially displace up to 19 ordered solvent molecules in the human K1 structure. Related solvent networks, or "canals", postulated to be displaced upon substrate binding, have been observed in high-resolution structures of bovine trypsin and porcine elastase [32,33], although neither are as extensive as in hK1. The release of a localized solvent from a protein binding pocket is associated with an entropic gain of approximately 2.1 kcal/mol [34]. Thus, the release of 19 solvent molecules in hK1 by a bound peptide inhibitor/substrate represents a substantial contribution to the overall binding free-energy.

4. Design of a novel inhibitor based upon the solvent network within the S2 to S2' pockets

Fig. 5 illustrates an overlay of the solvent structure within the *apo* hK1 S2 to S2' binding pockets with the P2 to P2' region of the hirustatin inhibitor as bound within the porcine K1 structure (1HIA). While the P2 to P2' peptide displaces these solvent groups it does not substitute polar groups within identical three-dimensional juxtapositions. This results in adjustments within the structure so as to optimize hydrogen-bonding and *van der Waals* interactions with the peptide, and are presumably associated with the induced-fit conformational changes that result in a catalytically-competent hK1 structure. We furthermore hypothesize that if a small molecule were designed to mimic this solvent structure

Figure 4   Relaxed stereo diagram of the S1' site of *apo* hK1 (upper panel) and porcine K1 in complex with hirustasin (1HIA; lower panel). The P2 to P2' residues of the hirustasin inhibitor are indicated in dark grey shading. The solvent structure within, and adjacent to, the S1' binding pocket in *apo* hK1 is indicated, and specific solvent displaced by the bound P1' side chain atoms are identified.
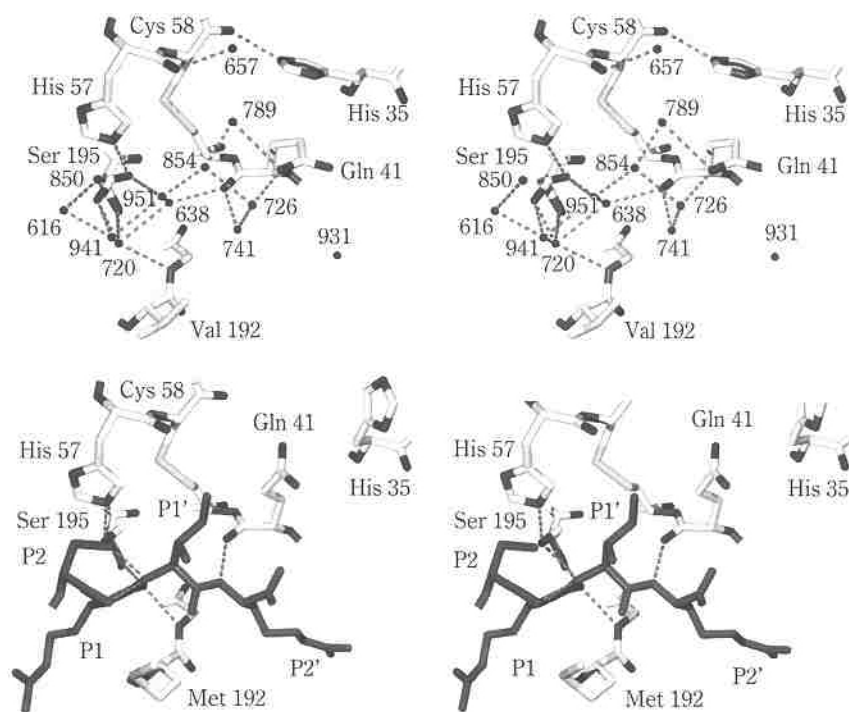
Table 1 Solvent displaced upon peptide binding in the S2 to S2' sites in human K1

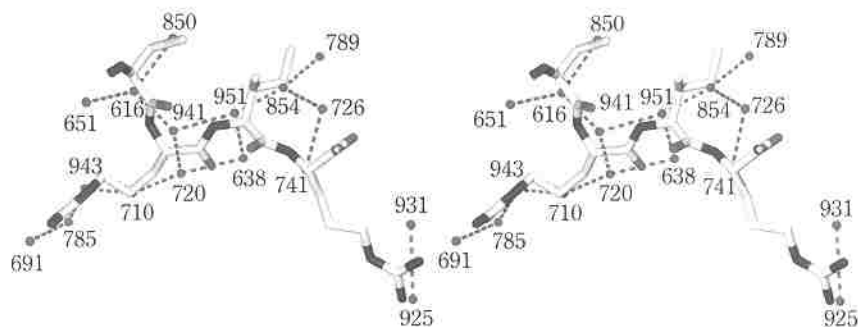| Peptide group	Displaced solvent (side chain atoms involved)
P2 main chain	616, 651
P1 main chain	638, 720, 941
P1' main chain	638
P2' main chain	726, 741
P2 side chain	850 (γ)
P1 side chain	710 (β, γ), 943 (δ, ε, ζ), 691 (ζ, η), 785 (ζ, η)
P1' side chain	854 (β, γ), 789 (δ, ε), 657 (ζ, η)
P2' side chain	925 (ζ, η), 931 (ζ, η)
Formation of S2 pocket	938, 939

Figure 5   Relaxed stereo diagram of the solvent structure within the S2 to S2' binding pockets of *apo* hK1 overlaid with the P2 to P2' residues from the porcine K1/hirustasin complex. The P2 to P2' peptide displaces this solvent structure, but does not exactly juxtapose polar groups, thus resulting in an induced fit upon binding that promotes a kinetically-competent structure. (page 6)

within the S2 to S2' binding pockets of the *apo* hK1 structure it would selectively bind to, and stabilize, the *apo* hK1 structure. Since this structure is not catalytically competent, such a molecule would be an effective inhibitor of hK1. This would represent a novel type of inhibitor, however, it remains to be seen if such a molecule can be constructed.

Acknowledgements

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Kallikrein 1 Active Site Solvent Structure

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