Crystal structure and biochemical characterization of human kallikrein 6 reveals a trypsin-like kallikrein is expressed in the central nervous system

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Running Title: Structure and biochemical properties of hK6
Summary

The human kallikreins are a large multi-gene family of closely-related serine-type proteases. In this regard, they are similar to the multi-gene kallikrein families characterized in the mouse and rat. There is a much more extensive body of knowledge regarding the function of mouse and rat kallikreins in comparison to the human kallikreins. Human kallikrein 6 has been proposed as the homologue to rat myelencephalon specific protease, an arginine-specific degradative-type protease abundantly expressed in the central nervous system and implicated in demyelinating disease. We present the x-ray crystal structure of mature, active recombinant human kallikrein 6 at 1.75 Å resolution. This high-resolution model provides the first three-dimensional view of one of the human kallikreins and one of only a few structures of serine proteases predominantly expressed in the central nervous system. Enzymatic data is presented that supports the identification of human kallikrein 6 as the functional homologue of rat myelencephalon specific protease and is corroborated by a molecular phylogenetic analysis. Furthermore, the x-ray data provides support for the characterization of human kallikrein 6 as a degradative protease with structural features more similar to trypsin than the regulatory kallikreins.
Introduction

Recent studies demonstrate that humans have a large multi-gene family of at least 15 different kallikreins (serine type proteases, abbreviated as KLK in reference to the gene, or hK in reference to the protein) (1). Similarly, the mouse and rat kallikrein gene families are characterized by a large number of closely related members that presumably arose due to gene duplication events (2-6). The different members of the mouse and rat kallikreins are characterized by a high degree of amino acid identity, but typically exhibit different preferences towards peptide substrates (7-12). Several human kallikreins have been identified as potentially useful diagnostic markers for breast (KLK3, KLK6), prostate (KLK2, KLK3), and ovarian (KLK6, KLK9, KLK10, KLK11) cancers as well as neurodegenerative diseases such as Alzheimers (KLK6) (1,13-17).

Myelencephalon specific protease (MSP)\(^1\) is a member of the rat kallikrein gene family that is abundantly expressed in the rodent CNS, and shown to be up-regulated in response to glutamate receptor-mediated excitotoxic injury (18). Potential human homologues to rat MSP

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\(^1\)Abbreviations: KLK, kallikrein; hK, human kallikrein protein; MSP, myelencephalon specific protease; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; MALDI, matrix-assisted laser desorption/ionization; TOF, time of flight; L-BAPNA, benzoyl L-arginine paranitranilide; TBS, tris buffered saline; PBS, phosphate buffered saline; PVDF, polyvinylidene difluoride; EDTA, ethylene diamine tetraacetice acid; DMSO, dimethyl sulfoxide; AMC, amino methyl coumarin; MBP, myelin basic protein; BPT, bovine pancreatic trypsin; PPK, porcine pancreatic kallikrein; PAM, percent accepted mutation; CFAD, complement factor D.
have also been identified (18) and have been alternatively named protease M (19), Zyme (20) and neurosin (21). Mouse homologues to MSP have been reported as brain and skin serine protease (BSSP) (22) and brain serine protease (BSP) (23). It has been postulated that MSP/protease M/neurosin may play a key role in the regulation of myelin turnover, and in demyelinating disease (18,24-27) including the development of multiple sclerosis lesions (25). Furthermore, this kallikrein may also play a role in the degradation of β-amyloid, or turnover of amyloid precursor protein (28,29). The kinetic properties of MSP have identified it as a degradative-type protease with broad specificity for cleavage after Arg residues (27). A potential human homologue to rat MSP has been identified (human kallikrein 6, or hK6), based upon amino acid sequence identity (69.1%) in comparison to the other human kallikreins (27,30). hK6 has the highest expression in the central nervous system, breast, kidney, and uterus, and may prove to be a useful biomarker for ovarian and breast cancers as well as Alzheimer’s disease (1).

The x-ray crystal structure and biochemical characterization of this enzyme will provide insight into its structure/function relationship and assist in the development of specific inhibitors. We report here biochemical data, phylogenetic analyses, and the x-ray crystal structure for mature active hK6 protein that support its identification as the human homologue to rat MSP and provides a structural interpretation for its catalytic features and autolytic regulation. This report provides the first structural data for one of the human kallikreins, and the x-ray structure determination of hK6 is an important step in elucidating structure/function relationships for this important class of human proteins.
Experimental Procedures

Expression, crystallization, and data collection. Mature active hK6 was expressed and purified from a baculovirus/insect cell line system essentially as described for rat myelencephalon-specific protease (MSP) (27), using a synthetic (Asp)_4Lys pro-sequence and activation by enterokinase. Purified active hK6 was concentrated to 20 mg/ml in 40 mM sodium acetate, 100 mM NaCl, and 20 mM benzamidine, pH 4.5. Crystallization conditions were identified using a hanging-drop sparse-matrix screen (31) of precipitants, salts, and pH conditions (Hampton Research, Laguna Niguel, CA). Diffraction quality crystals grew from 30% (w/v) PEG 4000, 0.2 M magnesium chloride hexahydrate, and 0.1 M Tris hydrochloride, pH 8.5 after two weeks incubation at 4 °C.

X-ray intensity data were collected at 103 K from a single crystal (0.5 × 0.2 × 0.05 mm) with a Rigaku imaging plate area detector R-Axis IIc using Cu-Kα radiation. Data were processed and scaled using DENZO and SCALEPACK (32,33). This crystal diffracted to at least 1.75 Å. The space group was tentatively identified as orthorhombic P2_12_12_1 with cell constants a = 39.1 Å, b = 62.1 Å, c = 85.8 Å. Based upon a molecular mass of approximately 29 kDa for hK6, a Matthews' coefficient V_m = 1.80 Å³/Da suggested a single molecule in the asymmetric unit (34).

Molecular Replacement and Structure Refinement. Initial phases were calculated by molecular-replacement using Atlantic salmon trypsin (PDB code 1A0J) as a search model and the Crystallography & NMR System (CNS) software package (35). The rotational search resulted in a single peak 8σ above the noise level, and a subsequent translational search in the P2_12_12_1 space
group of the correctly rotated model resulted in a single peak 4σ above the noise level. The R$_{cryst}$ was 47.3% after rigid body refinement of this initial solution.

A 3 Å $2F_{obs} - F_{calc}$ SIGMAA-weighted composite annealed omit map (5% of data omitted) was calculated and the structure was built and refined through alternating cycles using the graphic program O (36) and CNS. All refinements were performed by simulated annealing using a maximum likelihood target, and this cyclic procedure was repeated several times with gradual increase of the resolution to 1.75 Å. A random selection of 3% of the data was assigned for calculation of R$_{free}$ and was not included in the refinement. Solvent molecules were added at the last stage of refinement at stereochemically reasonable positions.

*Autolysis of hK6.* Autolysis of hK6 was evaluated using 16.5% Tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (tricine SDS-PAGE) (37) and activity assays with L-benzoyl-arginine paranitranilide (L-BAPNA). Mature hK6 in PBS, pH 7.31, was incubated at 37 °C and samples at time points of 0, 1, 2, 4, 6, 8, 10, and 24 hr were taken. Identical samples were evaluated using the L-BAPNA assay and 16.5% Tricine SDS-PAGE. Polypeptides resolved by the tricine SDS-PAGE were transferred by electroblotting to polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA) and then subjected to amino-terminal peptide sequencing on an Applied Biosystems Procise 492 model protein sequencer (Applied Biosystems, Foster City, CA).

*Determination of kinetic constants.* Substrate stock solutions of Tos-Gly-Pro-Arg-aminomethylcoumarin (AMC) and Tos-Gly-Pro-Lys-AMC (Bachem, King of Prussia, PA) were prepared in dimethyl sulfoxide (DMSO). Enzyme concentrations of 20 nM and 200 nM were
utilized in the hydrolysis of the Arg and Lys containing substrates, respectively. The assay mixture contained 50 mM Tris-HCl and 0.1 mM EDTA, pH 8.5, and the final concentration of DMSO was less than 5%. Assays were performed using substrate concentrations of 9.0µM to 2 mM, at 37 °C. Hydrolysis of the AMC substrates was monitored fluorometrically with an excitation wavelength of 380 nm and an emission wavelength of 460 nm on a Varian Cary Eclipse fluorescence spectrophotometer (Varian, Inc., Palo Alto, CA) and all data points were collected in triplicate. Steady-state kinetic constants $K_m$ and $k_{cat}$ were determined from averaged data sets of initial reaction rate versus substrate concentration by nonlinear fitting to the Michaelis-Menten equation using the Datafit software package (Oakdale Engineering, Oakdale, PA).

Digestion of myelin basic protein and extracellular matrix proteins by hK6. Rat myelin basic protein (MBP) isolated from spinal cord was added to hK6 at a 1000:1 mass ratio in 50 mM Tris and 100 mM NaCl, pH 8.0. This mixture was incubated at 37 °C and time points were taken at 10, 30, 60, 120, and 240 min. The MBP and degradative fragments were resolved using Tricine SDS-PAGE (16.5%). Laminin from basement membrane of Engelbreth-Holm-Swarm mouse sarcoma (Sigma Chemical Co., St. Louis, MO) was diluted in TBS, pH 7.5, to a concentration of 1 mg/ml. Active hK6 was added to a concentration of 4.2 µM (10:1 w/w ratio of laminin: hK6). The sample was incubated at 37 °C, and aliquots of the digestion mix were taken at 0, 1, and 24 h, resolved on 7.5% SDS-PAGE and visualized by Coomassie blue staining. Mouse fibronectin (Life Technologies, Rockville, MD) was used as provided as a stock solution of 1.0 mg/ml in 2.7 mM potassium chloride, and 10% glycerol, pH 7.3. Mature hK6 was added to a final
concentration of 4.2 µM (10:1 w/w ratio of fibronectin: hK6). The sample was incubated at 37 °C, and aliquots were taken and analyzed in a manner identical to that of the laminin digestion.

Phylogenetic analyses. A dataset of hK6-related proteins was collected and assembled from protein sequence databases (as of Sept. 2001) using FastA (38) and LookUp (39) within the Genetics Computer Group’s Wisconsin Package SeqLab interface (GCG, 2001). An Expectation Value of $10^{-4}$ was used as a list cut-off and all entries other than human, rat, and mouse were excluded. Redundancies, splicing variants, and other isoforms and were then sorted out leaving a dataset of thirty-three protein sequences (Table I). PileUp (40) with the BLOSUM30 matrix (41) was used to initially align the sequences, followed by considerable regional realignment and manual adjustment. The final aligned amino acid sequence dataset is available from the authors by request.

GCG’s ToFastA and Don Gilbert’s ReadSeq (1993) were used to create a PHYLIP (42) format dataset from the alignment, where columns of excessive homoplasy, as judged by similarity less than 15%, were excluded. Three phylogenetic inference methods were used on the resultant data matrix: 1) The maximum likelihood, quartet-puzzling program Tree-Puzzle (43) run with the JTT amino acid substitution model (44) and 1000 steps produced a maximum likelihood tree estimate with branch lengths and node support values. 2) Pair-wise distances were estimated with PHYLIP’s ProtDist PAM model (45) and least squares fit to an optimal globally rearranged tree by the PHYLIP Fitch algorithm with ten random additions. 3) The data matrix was bootstrapped 100 times by PHYLIP’s SeqBoot, ProtDist generated 100 PAM based pair-wise distance matrices, and then PHYLIP’s Neighbor neighbor-joining algorithm and Consense program provided bootstrap node support values. Majority rule, that is wherever two
or more of the three estimates agreed, provided the resolved clades on the final tree presented in Fig. 1. Final node supports values were calculated as the average between the Tree-Puzzle and bootstrapped neighbor-joining results wherever they agreed on a particular node; all values greater than 50% were printed at their respective node.
Results

Recombinant hK6 protein. The homogeneity of purified hK6 was evaluated using amino-terminal sequencing and MALDI-TOF mass spectrometry. Mass spectrometry revealed that the hK6 samples used for crystallization contained intact, glycosylated enzyme (Fig. 2). The major peak had a mass of 25,866 Da, which is a difference of +1366 Da from the mass calculated from the protein sequence. This extra mass corresponds to approximately six N-acetylglucosamine molecules. Furthermore, peaks corresponding to six different glycosylated forms were visible in the mass spectrum, with the average difference in mass between each form being ≈184 Da (corresponding to the mass of one hexose unit). Amino-terminal sequencing analysis yielded a single sequence of Leu-Val-His-Gly, representing the correct amino terminal sequence for mature hK6.

X-ray structure refinement. A total of 140 solvent molecules were added to the refined hK6 structure. One tentatively assigned solvent molecule exhibited octahedral coordination geometry with adjacent solvent molecules and short (~2.0Å) contact distances with these groups. This solvent was therefore assigned as a Mg$^{2+}$ ion (46). Unambiguous density was also visible within the active site region indicating the presence of a bound benzamidine inhibitor with terminal amine groups clearly defined. In the final refined structure 227 of the 229 amino acid residues are defined in the electron density map. The observed electron density is in full agreement with the amino acid sequence deduced from the cDNA sequence (20). The peptide backbone of hK6 could be traced unambiguously from its amino-terminal Ile16 to Gln243 (using the chymotrypsinogen numbering scheme (47)). C-terminal residues Ala244 and Lys245 lacked adequate electron density and were not built into the model. The side chain residues of Lys24,
Arg110, Gln239, and Gln243 are undefined in the electron density map and were therefore modeled as Ala residues. Asp150 was modeled in multiple rotamer conformations. Some of the loop regions, in particular the region from Trp215 to Pro225, required extensive rebuilding due to large differences from that of the search model. The model refined to acceptable values of stereochemistry and crystallographic residual (Table II).

*Digestion of myelin-related and extracellular matrix proteins by hK6.* Rat myelin basic protein (MBP) was extensively and rapidly degraded by hK6 (Fig. 3). Extended incubation resulted in a characteristic pattern of four lower molecular mass fragments. Rat plasma fibronectin was rapidly degraded by hK6 to yield a polypeptide with an apparent molecular mass of ≈200 kDa (Fig. 3). This polypeptide was subsequently degraded to numerous smaller fragments after extended incubation with hK6. Mouse laminin was likewise rapidly degraded by hK6, yielding an initial polypeptide with a mass of ≈140 kDa and numerous smaller peptide fragments (Fig. 3).

*Determination of steady-state kinetic constants.* Active hK6 exhibited characteristic Michaelis-Menten kinetics with all substrates. Kinetic constants for the hydrolysis of Tos-GlyProArg-AMC and Tos-GlyProLys-AMC are listed in table III. When compared to rMSP, hK6 has a somewhat reduced activity towards these substrates, and exhibits a general preference in $k_{cat}$ for Arg in the substrate P1 position relative to Lys.

*Autolysis of hK6.* Tricine SDS-PAGE revealed that hK6 undergoes autolysis (Fig. 4). Amino-terminal sequencing of the Tricine SDS-PAGE resolved autolysis fragments identified a peptide sequence corresponding to a single cleavage site between residues Arg76 and Glu77. Activity assays against L-BAPNA indicates that the autolytic event results in a corresponding loss of...
enzyme activity and that this autolytic inactivation follows a second order rate constant (data not shown).

*Phylogenetic analyses.* The three phylogenetic inference estimations consistently grouped certain clades, yet the resolution at the base of the tree remained obscure. Importantly, every analysis specifically associated human hK6 with the rodent MSPs, clearly indicating their orthologous relationship. This particular node on the tree had almost as much support as that grouping the rat and mouse MSP’s to each other, 83.5% and 89.5% respectively. Other orthologues between the human and rodent genes in the tree were as expected and range in support value from below 50% for the hK4 human and mouse homologues to 98% in the human and mouse hK7 system. Paralogous hK relationships in the tree, where they were resolved, had quite low support values, ranging from below 50% for those nodes associating hK7 with hK5 and hK4, to 62% between hK2 and hK13, up to 73.5% between hK9 and hK11. Conversely the support values for most of the classical trypsin homologues were quite high although the complement factor D (CFAD) system is only weakly supported, at 53%, as being trypsin’s nearest paralogue. hK10 appeared to have diverged the furthest from the common ancestor of all the hKs, although hK15 and hK4 were almost as divergent. All of the trypsins and CFADs diverged as much or greater from the common ancestor of all the sequences on the tree as did any of the hKs. In fact, the human CFAD had almost 0.7 substitutions per site along its length in its divergence from the last common ancestor of the dataset.
Discussion

*Human kallikrein 6 is functionally related to rat MSP.* Previously reported northern blot analysis of rat MSP and KLK6 have demonstrated a similar abundant expression in the brain in comparison to peripheral tissues (48). These studies also demonstrated tissue-specific expression in the spinal cord and medulla oblongata, and showed that the pattern of expression of MSP differed from that of tissue plasminogen activator. Rat MSP exhibits the highest amino acid identity (69.1%) with hK6, in comparison with the other human kallikreins; KLK6 has therefore been proposed as the human homologue of MSP (27). The present phylogenetic analysis strongly corroborates this assertion. In spite of basal resolution so poor that it is impossible to tell with any confidence just what the ancestral paralogous branching order of the kallikreins was, the orthology of hK6 and the rodent MSPs is obvious (with near 85% node support value). Future work in pursuing these basal relationships is being made through the use of a DNA alignment that corresponds to our aligned protein dataset. Much more sophisticated models of evolution are available for DNA than is for protein datasets, especially as implemented in PAUP*’s (49) maximum likelihood method. These sophisticated models may provide a greater evolutionary look-back time than the present study achieved and allow for the teasing of some order out of the original gene duplications that led to this large, complicated, and important gene family.

Rat MSP is characterized as a degradative protease, with greater catalytic efficiency for Arg versus Lys in the P1 position. It has been shown to rapidly degrade various myelin-associated and extracellular matrix proteins, and is autolytically regulated via cleavage after residue positions Arg74 and Arg81 (27). The results of the present study demonstrate that the characteristic digestive patterns exhibited by hK6 against MBP, laminin and fibronectin
substrates are virtually identical to those seen with rat MSP (27). Rat MSP and hK6 are also both inactivated by autolysis, however, the sites of autolysis are similar but not identical. Arg residues are present at position 74 in both hK6 and rat MSP, however, while rat MSP has an Arg at position 81, hK6 has a Glu. Conversely, while hK6 has an Arg at position 76, rat MSP has a Thr. Therefore, when considering the arginine preference of both enzymes, hK6 could autolytically cleave at positions 74 and 76, while rat MSP could cleave at positions 74 and 81. While positions 74 and 81 in rat MSP are autolytically cleaved, amino terminal sequencing of autolyzed hK6 identifies only an amino terminal starting at residue position 76. It may be the case that hydrolysis after arginine 76 occurs rapidly in hK6 and therefore subsequent hydrolysis after arginine 74 releases a dipeptide that would be lost during PAGE resolution. In any event the data indicates that for both hK6 and rat MSP autolytic cleavage in the region 74-76 results in inactivation of the enzyme. Thus, with regard to enzymatic activity towards myelin-related and extracellular proteins, and autolytic properties, hK6 and rMSP appear to be true functional homologues. The ability of both rat MSP and hK6 to degrade myelin-associated proteins, coupled with data showing that this enzyme is abundantly expressed within inflammatory cells at sites of demyelination in murine models of multiple sclerosis and associated lesions, supports the idea that this enzyme may play a pivotal role in demyelinating disease. and its robust expression in the brain and central nervous system, suggests that it may play a role in demyelinating diseases (18,26,27,50).

**Autolytic activity of hK6.** Determination of the x-ray structure of hK6 provides an opportunity to further characterize the autolytic regulation of MSP/hK6. Unlike the mouse kallikreins, and similar to trypsin, autolysis of hK6 leads to inactivation. Thus, autolysis represents a potential
regulatory mechanism in controlling the activity of hK6. The locations of the autolytic sites of hK6, in juxtaposition to the active site region, are shown in Fig. 5. Not surprisingly, Arg 76 (a site of autolysis in hK6) is the most solvent accessible arginine residue in the structure. Although the sites of autolysis in hK6 and trypsin are not identical, both proteases autolyze within the amino terminal domain (Fig. 5). The two canonical sites of autolysis in the mouse kallikreins, which are not associated with inactivation (51,52), are located within the extended kallikrein loop at position 95 and within the carboxyl terminal domain at position 148 (Fig. 5). Cleavages at the locations within the amino terminal domain in trypsin and hK6 may result in destabilization of the structure, and inactivation by autolysis may represent a stability-based mechanism of inactivation. The autolytic properties of the regulatory protease thrombin provide another contrast to hK6. Thrombin contains Arg residues at positions 73 and 75. Autolysis at these positions in thrombin does not result in inactivation, rather, it affects substrate specificity (abolishing the specificity for fibrinogen) (53). Since autolysis in hK6 abolishes catalytic activity, the autolytic properties of hK6 are more similar to the digestive enzyme trypsin than to thrombin.

The natural pro-peptide sequence of hK6 is Glu-Glu-Gln-Asn-Lys (19), and cleavage after the Lys residue produces mature active hK6. Rat MSP has a similar activation pro-peptide sequence of Glu-Asp-Gln-Asp-Lys (48) and is not activated by autolytic digestion (27). This inability of rat MSP to self-activate has led to the proposal that a distinct, Lys-specific protease is responsible for activation of rat MSP in vivo (27). Similarly, the preference for cleavage after Arg versus Lys residues in the P1 position suggests that a distinct Lys-specific protease is hypothesized to activate pro-hK6 in vivo.
Overall structural relationship of hK6 with other serine proteases. The secondary structure of
hK6 is composed of thirteen β-strands, two α-helices, two 3_10-helices, and eight identifiable loop
regions. These loop regions have varying functions that, based upon the structures of related
serine proteases, include defining substrate specificity (54-57) and autolytic regulation
(27,58,59). In addition, these loops can provide sites for N-glycosylation that may serve to
regulate activity in this class of enzyme (60).

The overall structure of hK6 is more similar to that of bovine trypsin than the mouse
kallikrein mK13 (pro-renin converting enzyme, one of the few available mouse kallikrein
structures) and the superimposed structures have rms deviations of 0.79 Å and 1.06 Å,
respectively. When comparing the x-ray structure of hK6 with either bovine trypsin or mK13,
there are three immediately identifiable loop regions adjacent to the active site that exhibit
structural heterogeneity. These include residue positions 91-103 (the "kallikrein loop"), 141-152
and 172-178 (Fig. 5). The “kallikrein loop” is a sequence of up to eleven amino acids inserted
between the sixth and seventh β-sheets (after residue 94) in the kallikrein family of enzymes.
hK6 has no inserted residues in this region and thus lacks the classical kallikrein loop. This loop
in hK6 is indistinguishable in length in comparison to the degradative proteases trypsin and
chymotrypsin, and shorter than that seen in mouse kallikreins or other regulatory type proteases
(Fig. 6). Although the amino acid sequences within this region differ between hK6 and trypsin,
the structures are essentially identical (Fig. 5 and 6).

The short surface loop comprising residue positions 172-178 is identical in length for the
different proteases compared in Fig. 6. The amino acid sequence for hK6 within this region is
identical to that of bovine trypsin with the exception of position 178 (Fig. 6), and adopts an
essentially identical structure as bovine trypsin (Fig. 5). This short loop is oriented away from
the active site, and contrasts with the homologous region in mK13, which is oriented towards the active site (Fig. 5).

The loop region 141-152 in hK6 is shorter than that in trypsin (Fig. 6), and leads to a conformation that orients this loop away from the active site in comparison to trypsin (Fig. 5). In the comparison with other proteases (Fig. 6) the broad-specificity degradative proteases generally have a shorter length loop in this region, whereas the regulatory proteases have longer loops that afford more extensive structural determinants of the substrate binding site.

The structural data for the variable surface loop regions that border the active site of hK6 describe loops that are both short and generally oriented away from the substrate binding site. Thus, their contribution to formation of the S2 and S3 sites within the protease appears limited. This is a characteristic feature of the degradative type proteases, exemplified by the digestive enzymes trypsin and chymotrypsin (61). Thus, the original hypothesis (18) that rat MSP is a trypsin-like digestive enzyme expressed in the CNS, is supported by both the enzymatic properties of MSP (27) and the biochemical and structural data reported here for the human homologue hK6. The activity of hK6 towards small peptide substrates indicates relatively large values for $K_m$ (table III). This apparently weak binding affinity may reflect limited interactions within the S2 and S3 sites, as is suggested from the general structural data of the active site. Thus, hK6 may function effectively only with larger peptide substrates with the potential for extended contact interactions beyond the S2 and S3 sites. The rapid digestion of myelin basic protein is consistent with this hypothesis.

$S1$ site structural features. Residues 189-195, 214-220, and 224-228 in addition to the catalytic triad define the S1 binding pocket. The presence of a bound benzamidine inhibitor in the x-ray
structure of hK6 permits an evaluation of how the guanidino group of a substrate P1 Arg side chain might fit within the active site. In trypsin, each of the nitrogen groups of the bound benzamidine inhibitor hydrogen bonds to an oxygen moiety of the Asp189 in the "bottom" of the S1 binding pocket (Fig. 7). In porcine kallikrein (an available kallikrein structure with a bound benzamidine inhibitor) the Oγ moiety of the Ser side chain at position 226 displaces one of the benzamidine amide groups and forces a rotation of the benzamidine ring of approximately 60° away from the Ser sidechain (61). Similar to trypsin, hK6 has a Gly residue at position 226 and the interaction of benzamidine with the Asp189 side chain is virtually indistinguishable from that of trypsin (1CE5), and distinctly different from the orientation in porcine kallikrein (2PKA) (Fig. 7).

Further structural similarity of the S1 site between hK6 and trypsin is achieved due to structural changes within the local region 215-220. This region in trypsin adopts a conformation that results in a hydrogen bonding interaction between the main chain carbonyl of residue Gly 218 with a benzamidine nitrogen group (Fig. 7). Although region 215-220 in hK6 has an amino acid insertion in comparison to the same region in trypsin, it adopts a conformation that positions the main chain carbonyl of residue Asn 217 in an almost identical location as that of Gly 218 in trypsin (Fig. 7). Although region 215-220 in porcine kallikrein has the same length as in hK6, there are slight conformational changes, presumably in response to the Ser226 residue. These conformational changes position the main chain carbonyl of residue 217 further away from the bound benzamidine and permit a hydrogen bonding interaction with the alternatively oriented benzamidine nitrogen (Fig. 7). These structural features in hK6 suggest a generally optimized fit for a P1 guanidino group within the active site that translates into a much higher catalytic efficiency towards substrates with an Arg versus Lys residue in this position.
Site of glycosylation. It has been reported that N-linked oligosaccharides within the "kallikrein loop" of neuropsin (the apparent mouse homologue of KLK8) affect the size of the S2 pocket and that mutations in this region result in a significant decrease in both $k_{\text{cat}}$ and $K_m$ (while maintaining the overall $k_{\text{cat}}/K_m$) (60). As previously mentioned, hK6 lacks the equivalent "kallikrein loop" characteristic of the regulatory proteases, including the N-linked Asn residue at position 95 (Fig. 6). However mass spectrometry data suggests there is a potential N-linked glycosylation site (sequence Asn-Xxx-Thr) at position Asn132 that is not present in any of the other known kallikrein structures. In contrast to the N-glycosylation site found on the kallikrein loop in other kallikreins, residue 132 is quite distant from the active site and lies at the “rear” of the enzyme. There is electron density present in this region that is indicative of possible sugar residues, but the density is not sufficient for accurate modeling. The function of this site of glycosylation has yet to be determined, but due to its distal location from the active site it is hypothesized not to significantly affect enzyme specificity or function.

In conclusion, the present study provides biochemical and phylogenetic data to support the identification of hK6 as the homologue of rat MSP. The biochemical and structural data also support the original hypothesis by Isackson and coworkers (18) that the MSP/KLK6 gene codes for a trypsin-like degradative protease that is expressed in the brain. Since our recent studies implicate excess MSP/hK6 activity in the development of immune-mediated demyelination in both animal models of MS and in human MS lesions (27,50) the availability of an atomic model of mature hK6, reported herein, may prove useful in the design of specific, and potentially therapeutic, inhibitors of this unique enzyme.
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Footnotes

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Figure Legends

Figure 1. Majority rule consensus tree (modified from TreeView (62) output) of three phylogenetic inference estimates from protein sequence. Horizontal branch length is proportional to evolutionary divergence in units of substitutions per site according to the scale bar at the bottom, whereas nothing is implied by vertical order. Node support values greater than 50% are printed at appropriate nodes and are an average of Tree-Puzzle and bootstrapped neighbor-joining support values. Human sequences are only labeled with gene/protein identifiers; rodent sequences are labeled mouse or rat along with the gene identifier.

Figure 2. Left panel: MALDI-TOF mass spectrum of purified, active recombinant hK6, with an indicated mass of 25.866 kDa (also shown is a peak at 12.932 kDa that represents intact hK6 at one-half the m/z ratio). Right panel: Enlarged view of the peak at 25.866 kDa showing the presence of six forms. The average difference in molecular mass between each peak is 184 Da, or one hexose unit, indicating that the mass heterogeneity is related to heterogeneity of glycosylation.

Figure 3. Left panel: degradation of myelin basic protein (MBP) by hK6 (see experimental procedures for details). Lanes: 1, control with no added hK6; 2, 10 minute incubation; 3, 30 min; 4, 60 min; 5, 2 hr; 6, 4hr; 7, molecular mass markers. Middle panel: 7.5% SDS-PAGE (reducing) showing digestion of fibronectin by hK6 at 310.15 K in 2.7 mM KCl, 340 mM NaCl, 8 mM sodium phosphate, 1.5 mM potassium phosphate, and 10% glycerol, pH 7.3. Lanes: 1, molecular mass markers; 2, fibronectin + hK6, 0 hr; 3, fibronectin + hK6, 1 hr; 4, fibronectin + hK6, 24 hr; 5, fibronectin control, 24 hr. Right panel: 7.5% SDS-PAGE (reducing) showing digestion of
laminin by hK6. Lanes: 1, molecular mass markers; 2, laminin + hK6, 0 hr; 3, laminin + hK6, 1 hr; 4, laminin + hK6, 24 hr; 5, laminin control, 24 hr.

Figure 4. Autolysis of hK6 (see experimental procedures for details). Lanes: 1, molecular mass markers; 2, purified active hK6 at 0 time point; 3, 1 hr; 4, 2 hr; 5, 4 hr; 6, 6 hr; 7, 8 hr; 9, 10 hr; 10, 24 hr.

Figure 5. Relaxed stereo ribbon diagrams of hK6 (top panel), bovine trypsin (1CE5; middle panel) and mouse glandular kallikrein 13 (mK13) (1AO5; lower panel). Orientation is intended to show the active site cleft with locations of catalytic triad (His57, Asp102 and Ser195), S1 site (Asp192) and bound benzamidine inhibitor (if present). Also indicated are the locations of the autolysis sites in hK6 and bovine trypsin. The two canonical autolysis sites in the mouse kallikreins are indicated using the structure of mK13. Also shown are the locations of the loop regions 92-102 (blue), 141-152 (magenta) and 172-178 (green) that border the active site.

Figure 6. X-ray structure-based alignment of the primary sequence of the loop regions 92-102, 141-152, 172-178 and 214-230 for hK6, MSP, trypsin, chymotrypsin, mouse kallikrein 13, neuropsin and porcine kallikrein (PDB accession codes are given).

Figure 7. Relaxed stereo diagram showing details of the S1 binding pocket in hK6 (upper panel), bovine trypsin (1CE5, middle panel) and porcine kallikrein (2PKA, lower panel). The hydrogen bonding interactions of the bound benzamidine inhibitor are shown using broken lines (residue positions 191-193 are omitted for clarity).
Table I. Sequences in phylogenetic analysis. Gene symbols without organism identifiers are all from human and all sequences are in same order as in Fig. 1.

| Gene     | other names                             | NCBI GI designation                  | GenBank accession                  |
|----------|-----------------------------------------|--------------------------------------|------------------------------------|
| KLK10    | breast normal epithelial, PRSSL1, NES1 | 10799395, 124476, 3065711             | AC011473, AF243527, AF055481, AF024605 |
| KLK12    | KLKL5                                   | 6249632, 6166249, 10799397, 11244770 | AF135025, AC011473, AF243527       |
| KLK2     | glandular kallikrein I                  | 386842, 7527776, 6425046, 4261522, 11244761 | M18157, AC037199, AF188746, S9329, AF243527 |
| KLK13    | KLKL4                                   | 6063386, 10799398                     | AF135024, AC011473                |
| KLK14    | KLKL6                                   | 6715552, 10799399                     | AF161221, AC011473                |
| KLK15    | ACO protease                            | 9957760, 11244759                     | AF242195, AF243527                |
| KLK5     | KLKL2, SCTE, stratum corneum tryptic-like | 4589283, 6063033, 11244763           | AF135028, AF168768, AF243527      |
| KLK4     | KLKL1, PRSS17, enamel matrix serine protease, prostatase | 8896096, 11244762, 7920367, 6136038, 4589272, 5020095, 5020096, 9296995 | AF259969, AF243527, AF228497, AF135023, AF113140, AF113141, AF148532, AAD21580, AAD21581, AAD38019 |
| KLK7     | PRSS6, SCCE, stratum corneum chymotrypsin | 2297543, 532504, 5733684               | L33404, A42048, AF166330, AF243527 |
| KLK8     | PRSS19, NRPP, BISP1, TADG14, neutpsin, ovasin | 3176837, 4768607, 5672473, 5624797, 5918518, 11244766 | AF135026, AC011473, AF243527     |
| KLK9     | KLKL3                                   | 5919239, 10156093, 10799394, 11244767 | AF135026, AC011473, AF243527      |
| KLK11    | PRSS20, TLSP, hippostasin, prostate-type hippostasin, keratinocyte trypsin-like protease | 5713131, 6681454, 9269698, 8574439, 3649791, 10799396, 11244769 | AF164623, AB013730, AB041036, AB012917, AC011473, AF243527 |
| KLK6     | PRSS9, protease M, zyme, neurosin, myelencephalon specific protease | 1518788, 1505849, 2318115, 5791636, 11244764 | U62801, D78203, E13209, I95869, AF013988, AF149289, AF243527 |

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Table II. Crystal, data collection, and refinement statistics

| **A. Crystal data** |  |
|---------------------|------------------|
| Space group         | P2₁2₁2₁          |
| Cell dimensions (Å) | a = 39.1 b = 62.1 c = 85.8 |
| Molecules/asymmetric unit | 1               |
| Matthews’ constant (Vₘ) (Å³/Da) | 1.80            |
| Maximum resolution (Å) | 1.75            |

| **B. Data Collection and Processing** |  |
|-------------------------------------|------------------|
| Total/unique reflections            | 495,027/21,777    |
| Completion (43-1.75 Å)/(1.79 – 1.75 Å) (%) | 96.0/82.7      |
| I/σ (43 – 1.75 Å)/(1.79 – 1.75 Å) | 43.0/4.9         |
| R_merge (43 –1.75Å)/( (1.79 – 1.75 Å) (%) | 5.7/38.2        |
| Wilson temperature factor (Å²)     | 26.6             |

| **C. Refinement** |  |
|--------------------|------------------|
| R_cryst (43-1.75 Å) (%) | 20.9              |
| R_free (43-1.75 Å) (%)  | 24.1              |
| rms bond length deviation (Å) | 0.005            |
| rms bond angle deviation (°) | 1.35             |
| rms B-factor deviation (σ)  | 2.83              |
| Ramachandran plot (%)      |                   |
| Most favored region       | 87.6              |
| Additional allowed region | 12.4              |
| Generously allowed region | 0                 |
| Disallowed region         | 0                 |
| Number of atoms/molecule  |                   |
| Non-H protein             | 1,685             |
| Water/ion                  | 139/1             |
| Average thermal factors (Å²) |               |
| All atoms                  | 30.9              |
| Protein atoms              | 30.3              |
| Solvent molecules          | 27.7              |
| Benzamidine                | 32.0              |
Table III. Kinetic constants for hydrolysis of Tos-GlyProArg-AMC and Tos-GlyProLys-AMC substrates by mature hK6 (50 mM Tris, 0.1 mM EDTA, 4% DMSO, pH 8.5, 37 °C)

| Substrate               | $k_{cat}(s^{-1})$ | $K_m(\mu M)$ | $k_{cat}/K_m$ (M$^{-1}$s$^{-1}$) |
|-------------------------|-------------------|--------------|----------------------------------|
| **hK6**                 |                   |              |                                  |
| Tos-Gly-Pro-Arg-AMC     | 6.84 ± 0.79       | 1562 ± 266   | 4.40 ± 0.30 x 10$^3$             |
| Tos-Gly-Pro-Lys-AMC     | 0.026 ± 0.004     | 777 ± 181    | 3.30 ± 0.20 x 10$^1$             |
| **rMSP**$^a$            |                   |              |                                  |
| Tos-Gly-Pro-Arg-AMC     | 14.4 ± 0.40       | 408 ± 19     | 3.53 ± 0.08 x 10$^4$             |
| Tos-Gly-Pro-Lys-AMC     | 0.13 ± 0.01       | 269 ± 10     | 4.80 ± 0.10 x 10$^2$             |
| **bovine Trypsin I$^b$**|                   |              |                                  |
| Tos-Gly-Pro-Arg-AMC     | 29.0 ± 1.0        | 3.5 ± 0.5    | 9.00 ± 1.0 x 10$^6$              |
| Tos-Gly-Pro-Lys-AMC     | 16.9 ± 0.08       | 12.3 ± 1.2   | 1.36 ± 0.05 x 10$^6$             |

Values from $^a$(27) and $^b$(63)
Figure 1.
Figure 2.
Figure 4.
Figure 6.
Crystal structure and biochemical characterization of human kallikrein 6 reveals a trypsin-like kallikrein is expressed in the central nervous system
Matthew J. Bernett, Sachiko I. Blaber, Isobel A. Scarisbrick, Pushparani Dhanarajan, Steven M. Thompson and Michael Blaber

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