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Cloning, Characterization and Site-Directed Mutagenesis of Canine Renin

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Inhibition of renin has been shown to be successful in managing hypertension and maintaining cardiac health. Canine models have played a key role in preclinical assessment of renin inhibitors. Here we report the cloning of canine prorenin gene. The amino acid sequence of mature canine renin was ~70% identical to that of human renin. The full-length prorenin was expressed in HEK 293 cells, purified and converted to its active form by trypsin-mediated cleavage of the 43 residue propeptide. The mature enzyme was characterized by steady-state kinetics using a peptide corresponding to the canine angiotensinogen sequence, Ac–Asp–Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu–Leu–Val–Tyr–Ser–OH (cleavage between Leu^{10}–Leu^{11}). The reaction followed Michaelis–Menten kinetics with a K_M of 120 μM and a second-order rate constant (k_{cat}/K_M) of $1.7 \times 10^5\ M^{-1}s^{-1}$. The enzyme was inhibited by various human renin inhibitors, but at reduced potency compared to the human renin.
The basis of the species specificity was investigated by mutagenesis. Based on primary sequence and structural alignments, three mutants were prepared (G149S-S150T, V286L, G149S-S150T-V286L). Each mutant yielded catalytically active enzymes with lower specific activities than native canine renin. V286L had the greatest effect on substrate specificity, while G149S, S150T mutations produced enzymes with inhibitor profiles similar to human renin.

Key words: hypertension, proteases, recombinant-canine-renin, renin–angiotensin–aldosterone system, renin-inhibition, species-specificity.

Abbreviations: AI, angiotensin I; AII, angiotensin II; BES, *N,N-bis*(2-hydroxyethyl)-2-aminoethanesulfonic acid; RACE, Rapid amplification of cDNA ends; RAAS, renin–angiotensin–aldosterone system; TFA, trifluoroacetic acid.

Renin is the rate-limiting enzyme in the renin–angiotensin–aldosterone system (RAAS), playing a critical role in blood pressure control (*1*). Renin is produced primarily in the juxtaglomerular cells of the kidneys. It is synthesized as a pro-enzyme and processed to the active form by a cleavage of the N-terminal 43 amino acid propeptide. Renin cleaves the N-terminus of angiotensinogen yielding the 10 amino acid peptide, angiotensin I (AI). Angiotensin I is further processed by the angiotensin converting enzyme (ACE) to produce angiotensin II (AII), the final effector of the RAAS system. AII binds to the AII receptor, triggering a chain of events leading to elevation of blood pressure and volume increase. The binding of AII to the receptor also causes deleterious effects, such as promoting tissue growth leading to cardiac and renal organ damage (*2*).

The RAAS cascade is a major target for the clinical management of hypertension. Current clinical treatments include angiotensin converting enzyme inhibitors (ACEi) and AII receptor blockers (ARBs). Renin inhibitors have been pursued as a novel class of antihypertensive drugs within the RAAS cascade due to renin's rate-limiting role in AII production. Clinical studies conducted with renin inhibitors proved them to be as effective as ACE inhibitors (*3*). Most importantly, inhibitors of renin may have a number of potential advantages over ACEi and ARBs. Renin is specific for angiotensinogen and will not cause the kinin-mediated side effects of ACE inhibitors (*2*). Additionally, inhibition of renin would greatly suppress the accumulation of AII, which builds up as a result of the ARB blockade, leading to other AII-derived peptides and uncertain ancillary pharmacology. Currently, only one renin inhibitor is making its way to the market, aliskiren (*4*). The development of renin inhibitors has been hindered by poor bioavailability and complex synthesis. Additionally, strict species specificity has been observed for a range of substrates and inhibitors.

The only known naturally occurring substrate for renin is angiotensinogen (*5*). At least eight residues in positions P_4–P_4' of angiotensinogen contribute to

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recognition by renin (*6*, *7*). The N-terminus of angiotensinogen comprising AI residues is identical for most species, therefore changes in this portion of the molecule are unlikely to explain species specificity. The species specificity of renin for native angiotensinogen likely resides on the P_1'–P_4' segment of the substrate. For example, angiotensinogen from non-primate species have the Leu–Leu scissile bond, while human angiotensinogen is cleaved on the Leu–Val bond. Human renin is capable of cleaving the Leu–Leu bond in angiotensinogens from other species (*8*). However, it has significantly lower activity versus murine angiotensinogen having Tyr in the P2′ position. Non-primate renins do not cleave human angiotensinogen to any appreciable extent (*9*), but are capable of cleaving human Leu–Val substrates when Ile at the P_2' position is replaced with Val (*8*, *10*).

This species substrate specificity translates into poor cross-species affinity for inhibitors of human renin. Most of these compounds are significantly weaker inhibitors of mouse, rat and dog enzymes. This drop in affinity limits the assessment of pharmacodynamics and efficacy in standard canine and rat models. With the continued interest in the development of renin inhibitors to treat hypertension (*11–14*), *in vitro* characterization of canine renin would be valuable for evaluating human–canine cross-inhibition of renin inhibitors and evaluation of these compounds in canine models. Here we report cloning, expression and characterization of canine renin. In addition, we describe the mutation of several amino acids in the substrate binding site of canine renin replacing them with their human orthologs. The mutated canine enzymes were characterized with respect to substrate specificity and cross-inhibition with human renin inhibitors.

MATERIALS AND METHODS

Cloning of Canine Renin—Frozen canine kidney (U.S. Biological, Swampscott, MA) was used as a source for total RNA isolation. The tissue was disrupted and homogenized using the mortar and pestle method outlined in the RNeasy Mini kit (Qiagen, Valencia, CA). Samples were quantified and inspected for purity by spectrophotometry at OD_{260} and OD_{280}. Synthesis of cDNA was performed with SuperScript II reverse transcriptase according to the protocol outlined for use with Oligo(dT) primer (Invitrogen, Carlsbad, CA). The putative canine renin gene (lacking 5′ and 3′ ends) was amplified using primers designed for the ovine renin sequence[1] (FWD 5′CACCGATGCCTCTCTGGGGACT CC3′; REV 5′AAACTCCGTGTAGAACTTGCGGAT3). The polymerase chain reaction conditions were optimized using Elongase polymerase mixture (Invitrogen, Carlsbad, CA). Sequencing was done by BigDye terminator reaction chemistry for sequence analysis on the ABI Prism 377 (Kimmel Center Nucleic Acid Facility, Thomas Jefferson University, Philadelphia, PA) using canine-renin-specific primers based on the published 179 bp sequence[2] (FWD 5′ CACCCTTGGTCTGTG AGGAGGGCTG-3′; REV 5′GAGGTGGAAGGAGATGT CGGGGAGT 3′) and the ovine primers. Utilizing the incomplete canine prorenin sequence, gene-specific primers were designed for use in the 5′ and 3′ Rapid Amplification of cDNA Ends (RACE) procedures. (5′ RACE: GSP1 5′GACTATGGGAAGTACG3′ GSP2 5′TGTAAGTGGTAGGCGATGCC 3′; GSP3 5′CATGATG CCGCTCTAACCGT3′; 3′ RACE: GSP1 5′GACCACTCC ATGCAGCCCT3′; GSP2 5′GGGAGTGTAGGCACCTGG TT3′). 5′ and 3′ RACE procedures were performed independently and according to manufacturer protocols (Invitrogen, Carlsbad, CA).

Following sequence confirmation of RACE fragments, the full-length canine prorenin sequence was constructed using Vector NTI (Invitrogen). Using this information, amplification of the full-length canine prorenin (~1.2 kb) was performed by polymerase chain reaction using Elongase DNA polymerase and primers based on the sequence upstream and downstream of the coding region (FWD 5′CGTCTAAGCTTCACCCAGGCCAAGCACC3′; REV 5′CGGTCAATTCTAGACTGCGGGCCAAGGCAA AG3′). The 5′-sense primer introduced a consensus Kozak translation initiation sequence and a *HindIII* restriction site. The 3′ antisense primer introduced an *XbaI* restriction site. The PCR product was digested to completion with *HindIII* and *XbaI* (NEB, Beverly, MA) and cloned into the mammalian expression vector pcDNA3.1B (Invitrogen, Carlsbad, CA) in-frame and upstream of the V5 epitope and 6× histidine tags. The complete canine renin sequence was confirmed by DNA sequencing as described.

Site-Directed Mutagenesis—Amino acid residues in the canine renin amino acid sequence were modified by site-directed mutagenesis utilizing the Quikchange II mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer's protocols. The following primers were designed to introduce amino acid changes at position 84, 85 and 224: Canine Renin G149S/S150T Fwd: cattcaccatccgctacagcacagggaaggtcaaagg. Canine Renin G149S/S150T Rev: cctttgaccttccctgtgctgtagcggatggtgaatg. Canine Renin V286L Fwd: gagggctgcatggtactagtcga taccggtgca. Canine Renin V286L Rev: tgcaccggtatcgac tagtaccatgcagccctc. Confirmation of the changes in the nucleotide sequence was confirmed by sequencing as described earlier.

Expression of Secreted Canine Prorenin—Human embryonic kidney cells, HEK 293 (ATCC, Manassas, VA) were cultured in Eagle's Minimum Essential Medium, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 μg/ml penicillin/streptomycin (Mediatech Inc., Herndon, VA). Transfection experiments were conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's protocols. Transient expression of soluble canine renin in culture supernatants was determined 72 h post-transfection by Western blot analysis using antibodies to the V5 tag (Invitrogen, Carlsbad, CA). Stable transfectants were obtained by selection in media supplemented with Geneticin (800 μg/ml), and single clones were obtained by limiting dilution. A single clone was scaled up and switched to serum-free media C293 (Invitrogen, Carlsbad, CA) for purification.

[1] GenBank Accession Number L43524.
[2] Genbank Accession Number U67198.

Purification and Activation of Secreted Canine Prorenin—Serum-free supernatant containing the recombinant protein was cleared by centrifugation at 6000 rpm and filtered using PES (Polyethersulfone) filters (Corning Inc., Acton, MA). Tris–HCl was added to the filtered medium to a final concentration of 20 mM and a final pH of 7.5 prior to initiating nickel-chelating chromatography. The buffered medium was loaded by gravity flow onto a 20 ml Ni-NTA column (Qiagen, Valencia, CA) pre-equilibrated with 20 mM Tris–HCl, pH 7.5 and 200 mM NaCl. The column was washed with 20 mM imidazole in the same buffer, and the protein was eluted by increasing the concentration of imidazole to 250 mM. The purified canine prorenin was dialysed against 20 mM Tris–HCl, pH 7.5 and 200 mM NaCl overnight at 4°C. The prorenin was converted to active renin by incubation with trypsin (trypsin/prorenin 1:100, w/w) for 60 min on ice. Western blot analysis using antibodies to the V5 tag was performed to ensure proteolytic removal of the N-terminal propeptide. Trypsin was removed with trypsin inhibitor agarose beads (Sigma, St Louis, MO), and the activated renin was loaded onto a 3 ml Concanavalin A column (Amersham Biosciences, Piscataway, NJ) pre-equilibrated with 20 mM Tris–HCl, pH 7.5, 200 mM NaCl, 0.1 mM $CaCl_2$ and 0.1 mM $MnCl_2$. Renin was eluted with 250 mM methyl-α-D-mannopyranoside in 20 mM Tris–HCl, pH 7.5 and 200 mM NaCl. The protein was further purified on a gel-filtration column Superdex 75 (Amersham Biosciences, Piscataway, NJ). Protein samples (~10 pmol/μl) were sent to the Wistar Institute (University of Pennsylvania, Philadelphia, PA) for matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) and to Macromolecular Resources (Colorado State University, Fort Collins, CO) for Edman's N-terminal sequencing.

SDS–PAGE and Western Blotting—Protein analysis was performed by electrophoresis on NUPAGE Novex 4–12% gradient Bis–Tris gels (Invitrogen, Carlsbad, CA). Gels were stained with Coomassie blue dye for purity determination. Western blotting was performed by transfer to nitrocellulose membranes using the Xcell II Blot Module (Invitrogen, Carlsbad, CA). Protein was probed using the α-V5-HRP antibody (Invitrogen, Carlsbad, CA). Blot development was performed using the enhanced chemiluminescence (ECL) system (Amersham Biosciences, Piscataway, NJ).

Enzyme Assays—Initial assays were performed using acetylated canine angiotensinogen 1–14 fragment Ac–Asp–Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu–Leu–Val–Tyr–Ser–OH (Bachem Biosciences, King of Prussia, PA). All reactions were carried out at room temperature in 50 mM Tris–HCl, pH 8, 100 mM NaCl, 1 mM EDTA and 0.2–3% dimethylsulfoxide (DMSO). Each reaction mixture contained 5–10 μM substrate and 0.2–2 nM canine renin. Reactions were initiated by addition of enzyme and incubated for 30 min (<20% product conversion). The reactions were quenched by addition of 1.2% TFA and analysed by high-performance liquid chromatography (HPLC) on a Zorbax SB-C8 column $4.6 \times 50\,mm^2$ (Agilent, Palo Alto, CA) using a gradient from 15 to 40% of acetonitrile in 0.1%TFA in 6 min at 1 ml/min and monitoring absorbance at 280 nm on Agilent 1100 series system equipped with either a multi-wavelength or diode-array detector (Agilent, Palo Alto, CA). Retention times were 6.0 min for the substrate, 5.4 min for the 1–10 product (Ac–AngI) and 1.8 min for the 11–14 product (tetrapeptide LVYS).

Fluorimetric enzyme assays were performed in 50 mM BES buffer, pH 7.0, containing 0.25 mg/ml of BSA, 100 mM NaCl, 2% DMSO, 4–8 μM of fluorogenic peptide substrate and 2–5 nM of the activated renin in a total volume of 100–200 μl. The following fluorogenic peptides were used in the assay: canine renin substrate CR–MZ Mca–Ile–His–Pro–Phe–His–Leu–Leu–Val–Tyr–Ser–Lys(Dnp)–NH_2 (AnaSpec, San Jose, CA); and human renin substrate AnasSpec-24479 DABCYL-γ-Abu–Ile–His–Pro–Phe–His–Leu–Val–Ile–His–Thr–EDANS (AnaSpec, San Jose, CA). Incubation was performed at room temperature in white opaque 96-well plates (Optiplates™, PerkinElmer, Wellesley, MA). Fluorescence ($\lambda_{ex} = 335$ nm, $\lambda_{em} = 460$ nm for CR–MZ; $\lambda_{ex} = 336$ nm, $\lambda_{em} = 495$ nm for AnaSpec-24479) was measured repeatedly over extended periods of time (2–6 h) on either Victor V or Fusion plate reader (PerkinElmer, Wellesley, MA) and the intensity of fluorescence was regressed against reaction time to derive velocities. The reaction rates were used for calculating percent inhibition values using uninhibited enzyme as a positive control and either no enzyme or enzyme inactivated with 2 μM SR 42128 (Bachem, King of Prussia, PA) as a negative control. IC_{50} values were calculated by fitting the percent inhibition values versus inhibitor concentration into a four-parametric model using XLFit software (IDBS, Guildford, UK).

RESULTS AND DISCUSSION

Cloning of Canine Renin—Much of the early work on the development of renin inhibitors was performed in canine models of hypertension. These early studies found that the potency of renin inhibitors differed between human and canine enzymes (Table 1). As part of our effort to develop renin inhibitors, the potency of compounds against canine enzyme needed to be determined. Due to the lack of a commercial source of the enzyme, we cloned, expressed and purified the enzyme from cultured mammalian cells.

The cloning strategy relied on a previously published partial sequence (179 bp) of canine renin to assist in elucidating the full-length gene. Analysis of the available partial sequence of canine renin sequence by BLAST (ncbi.nlm.nih.gov/BLAST/) revealed that the species ortholog with highest homology to the partial canine sequence was ovine renin. Primers were designed from 5′ and 3′ extremes of the ovine prorenin open reading frame. The cDNA was generated by oligo (dT) second-strand synthesis from total RNA isolated from canine kidney. A larger portion of the canine prorenin cDNA was then amplified. Following PCR, a single band of ~1.1 kb was generated (data not shown). Alignment of the sequence data from the PCR product was found to contain regions homologous with the available 179 bp sequence of canine renin and the full-length ovine and human prorenins. In order to obtain the 5′ and 3′

Table 1. **Cross-inhibition of canine and human renin with investigational drugs aimed at renin inactivation.**

Compound name	Human renin IC_{50}, nM	Canine renin IC_{50}, nM	Literature
A-74273	3	43	(*38*, *39*)
A-72517 (Zankiren)	1	110	(*40*)
CGP 44099A	0.3	0.007	(*41*)
CP-71,362	20	0.003	(*42*, *43*)
SC-56525	1.16	4.58	(*44*)
KRI-1314	4.7	79	(*45*, *46*)

extremes of the canine renin cDNA and remove the potential contamination from ovine sequence, we designed gene-specific primers for use in 5′ and 3′ RACE procedures. Sequence data were from the original PCR and the sequences from the 5′ and 3′ RACE procedures were used to construct a full-length sequence of canine prorenin. Primers were designed to amplify this sequence directly from canine kidney cDNA. The PCR product was sequenced.[3] Figure 1 shows the identity between human and canine renin proteins (71.1% for the full-length pre–pro-proteins, 70.8% for pro-proteins and 70.5% for mature enzymes) with conservation of the trypsin cleavage site and active site aspartic acid residues Asp 103 (Asp 32 by porcine pepsin numbering) and Asp 288 (Asp 215 by porcine pepsin numbering).

Purification, Characterization and Activation of Secreted Canine Prorenin—Recombinant canine renin was expressed in HEK 293 cells as an in-frame fusion with a C-terminal V5-His_6 tag. The secreted prorenin was purified by Ni-NTA chromatography. The prorenin was converted to the active form by digestion with trypsin. Active renin was purified by affinity chromatography on Concanavalin column followed by size exclusion chromatography with a yield of ~250 μg/l of conditioned media. Purified enzyme is shown by SDS–PAGE in Fig. 2. The 42 kDa molecular weight observed by SDS–PAGE is similar to that of canine renin purified from dog kidneys (*15*). MALDI analysis showed MW range 41–43 kDa implying glycosylation. The material bound quantitatively to Concanavalin A resin further indicating glycosylation. In addition, N-terminal sequence analysis revealed that the first seven amino acid residues in renin processed by trypsin were LSSGXST, where X is most likely glycosylated asparagine, as seen with human renin.

Steady-State Kinetic Characterization—Steady-state kinetic parameters of the activated canine renin were measured using the canine angiotensinogen 1–14 substrate (Ac–Asp–Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu–Leu–Val–Tyr–Ser–OH, cleavage at the Leu^{10}–Leu^{11}site). The rate of formation of acetyl-Angiotensin I and tetrapeptide Leu–Val–Tyr–Ser as a function of substrate concentration followed hyperbolic pattern consistent with Michaelis–Menten kinetics, and can be fit into Equation (1).

$$\text{Velocity} = \frac{k_{cat} \times [E] \times [S]}{K_M + [S]} \quad (1)$$

[3] Genbank Accession Number AY630442.

From the fit, the Michaelis constant K_M was determined to be 120 μM. This value is significantly higher than the K_M previously reported for the canine angiotensinogen tetradecapeptide with renin purified from dog kidneys [$K_M = 7$ μM (*16*)]. However, the literature data were for unacetylated tetradecapeptide bearing a free amino-terminal residue. In our hands, this substrate exhibited strong substrate inhibition at concentrations above 50 μM (Fig. 3). The single micromolar K_M value for this substrate reported in the literature may be the artifact of the substrate inhibition and limited dynamic range of the RIA analysis used for quantitation of the reaction product (*16*).

The rate of proteolysis was linear with the concentration of canine renin between 0.2 and 2.2 nM. The linear relationship indicates that canine renin is rate limiting for the reaction. Second-order rate constants were determined using full progress curve analysis at 10 μM substrate ($[S] \ll K_M$) and 1.8 nM canine renin. Under these conditions, the reaction progress follows first order kinetics (Fig. 4). The data were fit into the first-order rate equations [Equation 2(a) and (b)]. The k_{obs} was determined to be $3.1 \times 10^{-4}\,s^{-1}$ and the second-order rate constant [Equation (3)] or k_{cat}/K_M was calculated as $1.7 \times 10^5\,M^{-1}s^{-1}$.

$$P_t = S_0 \times \left(1 - e^{-k_{obs} \times t}\right) \quad (2a)$$

$$S_t = S_0 \times e^{-k_{obs} \times t} \quad (2b)$$

$$\frac{k_{cat}}{K_M} = \frac{k_{obs}}{[E]} \quad (3)$$

This value is similar to the k_{cat}/K_M values of $1.4 \times 10^5\,M^{-1}s^{-1}$ derived from substrate saturation kinetics [Equation (1)] and to the value of $2.7 \times 10^5\,M^{-1}s^{-1}$ reported in the literature for renin purified from dog kidneys (*16*). Thus, the catalytic efficiency of canine renin appears to be same order of magnitude as the second-order rate constant of the hydrolysis of human angiotensinogen tetradecapeptide by human renin previously reported as $3 \times 10^5\,M^{-1}s^{-1}$ (*17*) and $6.9 \times 10^5\,M^{-1}s^{-1}$ (*18*).

Determination of Optimal pH—Enzymatic activity was measured between pH 4 and 9 in full pH units. The maximum activity is observed at pH 6, similar to purified human renin (*19*), with the activity decreasing at the lower and higher pH ranges with the unity slopes indicating that a single ionized group is responsible for the activity at the pH extremes. The velocity for hydrolysis of the canine substrate as a function of pH was fit into Equation (4) to determine pK_a and pK_b values. Since our experiments were performed at $[S] \ll K_M$, the ionization constants are indicative of free enzyme and free substrate.

$$\text{Velocity} = \frac{k}{\left(10^{-pK_b}/10^{-x}\right) + \left(10^{-x}/10^{-pK_a}\right) + 1} \quad (4)$$

The experimentally determined values of pK_a and pK_b of 4.8 and 8.1 are different from the two pairs of pK values of 5.9 and 6.2 (acidic maximum) and 6.9 and

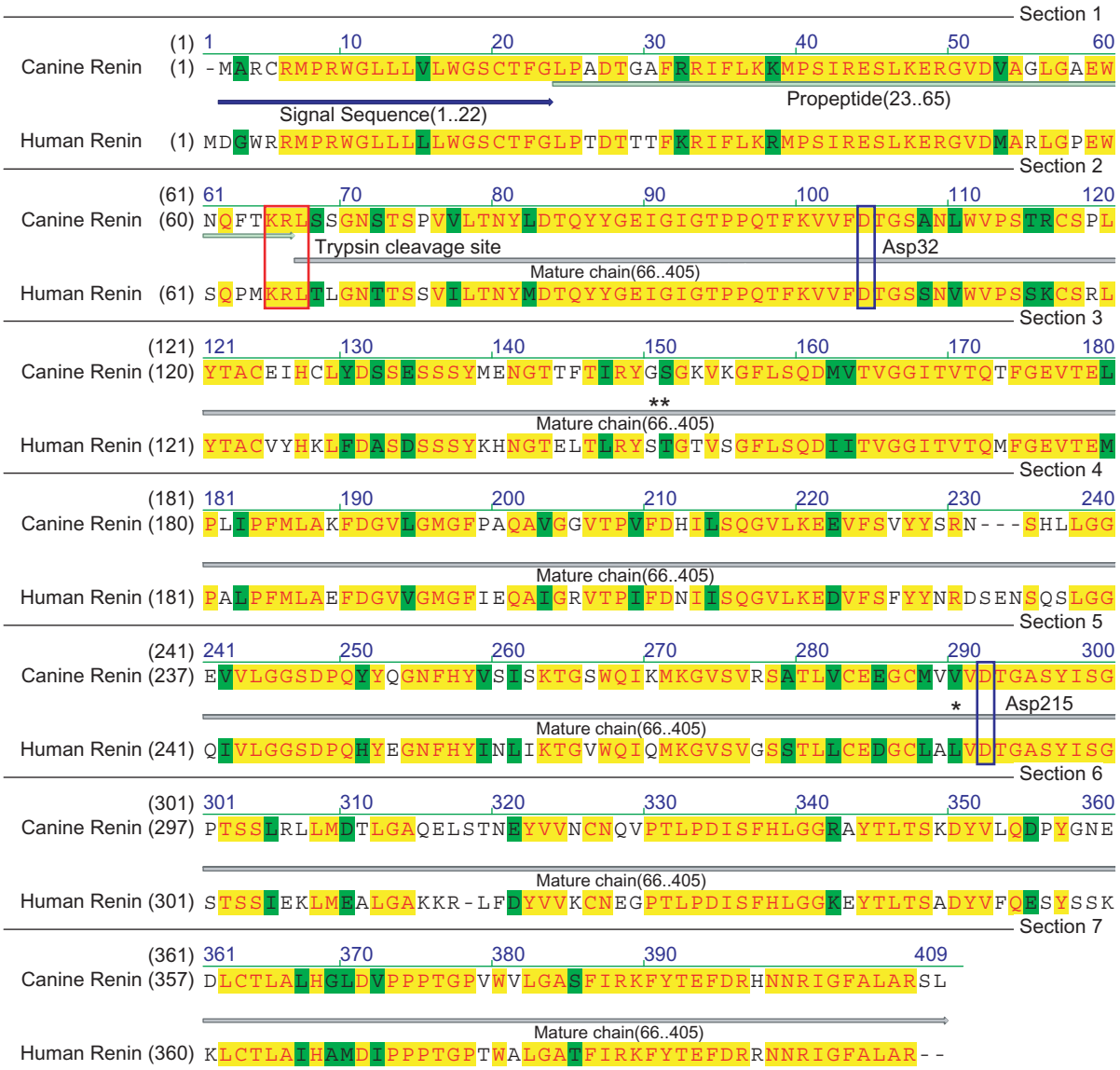

Fig. 1. **Alignment of the canine prorenin amino acid sequence with the human pro-enzyme.** Conserved motifs include the two aspartic acid residues Asp 103 (Asp 32 by porcine pepsin numbering) and Asp 288 (Asp 215 by porcine pepsin numbering) forming the enzyme active site and the trypsin cleavage site necessary for activation of prorenin. The residues Gly-149 (Gly-76 by pepsin numbering), Ser-150 (Ser-77 by pepsin numbering)) and Val-286 (Val-213 by pepsin numbering) selected for site-directed mutagenesis are labeled with asterisks.

8.5 (basic maximum) observed for hydrolysis of recombinant sheep angiotensinogen by human renin (*20*, *21*). The differences between canine and human pH profiles may reflect differences in conformation of the two proteins. Alternatively, the full-length angiotensinogen may exhibit different pH profile of hydrolysis when compared to its N-terminal tetradecapeptide, since the secondary structure of the full-length protein substrate may play a role in substrate recognition. For example, the pH dependence of human renin proteolysis of the full-length angiotensinogens from different species varied in shape of the pH optima (*21*).

Site-Directed Mutagenesis Studies—Multiple sequence alignment of renin sequences from different species along with the crystal structures of human (*22*) and mouse (*23*) renin reveal high degree of homology between the substrate binding sites. The amino acid residues participating in binding of the AI portion of the substrate are identical as are the AI sequences in all mammalian species. Therefore, the species specificity of renin proteins may depend on several amino acid alterations found in the binding sites of the C-terminal portion of the substrate, specifically within the S_1'–S_2' subsites. The difference between the scissile bonds in human (Leu–Val) versus non-primate (Leu–Leu) angiotensinogens may be a direct result of the divergence between S_1'–S_2' binding sites in human and non-primate renin. In particular, replacements of Ser-150, Thr-151 and Leu-290 (Fig. 1) neighbouring the P_1' substrate residue in human renin with Gly, Ser and Val, respectively, are highly conserved

in dog, mouse and rat. This observation prompted us to revert these residues in canine renin to their human orthologs and investigate functional consequences of these mutations. Three mutant enzymes were prepared MUT1 (G149S, S150T), MUT2 (V286L) and MUT3 (G149S, S150T, V286L). All mutants retained catalytic activity, albeit at a lower rate compared to the wild-type canine renin (Fig. 5).

All mutants were purified and the concentration of active enzyme was determined by active-site titration. These concentrations of active renin were used to calculate specific activities observed with the wild-type and mutated canine renin while cleaving either canine or human renin substrates (Table 2). The rates of proteolysis of the human substrate with canine renin mutants were significantly lower compared to those exhibited with the canine fluorogenic peptide. Therefore, these mutations did not result in complete reversal of substrate specificity from canine to human renin. Mutation in the active site 'flap' (G149S, S150T) led to a ~50% loss in specific activity with both human and canine substrates.

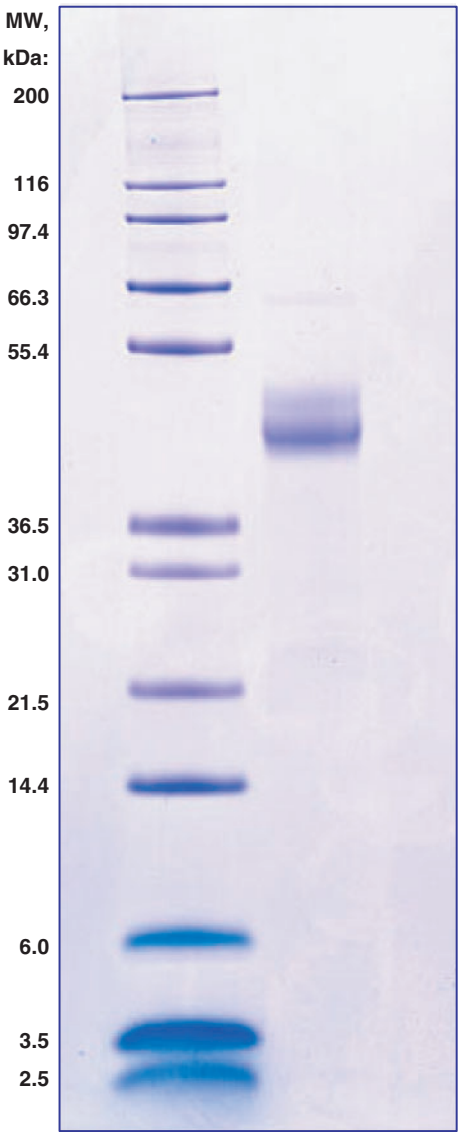

Fig. 2. **SDS–PAGE of canine renin after the final step of purification. Renin is heterogeneous due to its glycosylation.**

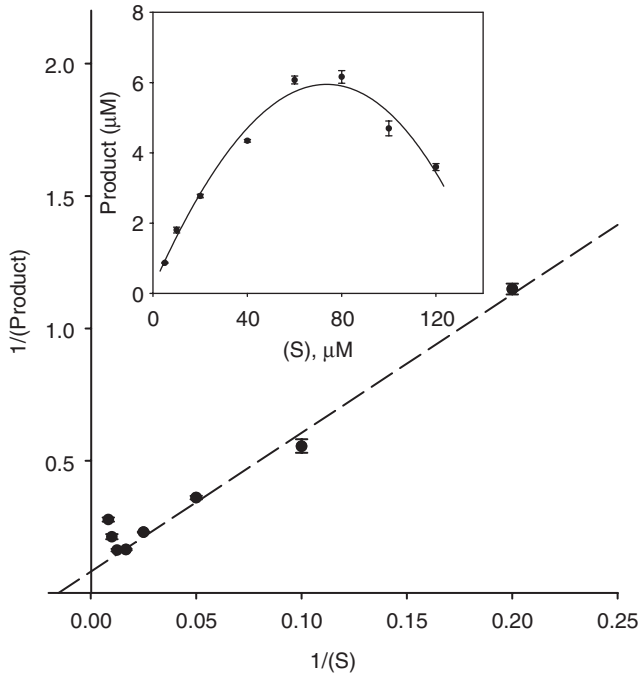

Fig. 3. **Lineweaver–Burk plot for hydrolysis of unacetylated tetradecapeptide by canine renin.** The product concentration was determined by monitoring the peak of the tetrapeptide LVYS using HPLC. The concave shape of the curve reflects deviation from the standard Michaelis–Menten kinetics and shows substrate inhibition for concentrations of tetradecapeptide above 50 μM. The insert shows the v versus s plot for the same reaction. The data were analysed as described in (*37*), and the following values were obtained: $K_M = 65\,\mu M$, $K_{is} = 87\,\mu M$.

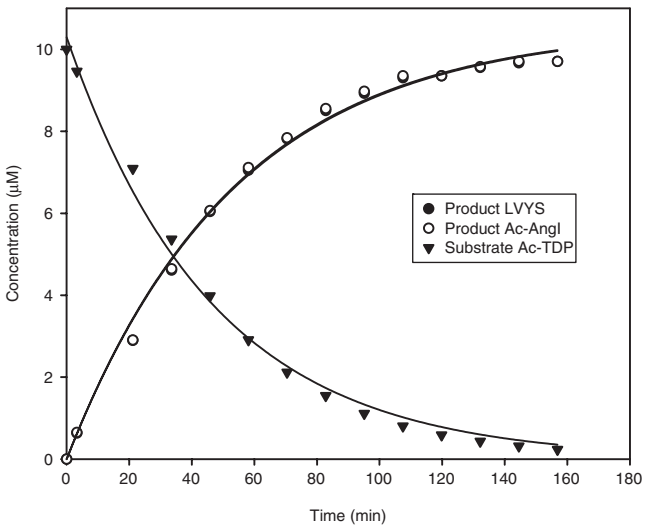

Fig. 4. **Full progress curves of canine renin observed with 10 μM Ac-TDP (substrate concentration $\ll K_M$).** Data were fit to Equations 2(a) and 2(b) to determine the first-order rate constant.

In contrast, both mutants carrying the V286L mutation exhibited the highest specific activity for cleaving the Leu–Val bond of the human substrate while being least active with the canine substrate. Thus, the V286L mutation resulted in the largest drop in substrate selectivity from ~26-fold to 4.4-fold, measured as a ratio of specific enzymatic activities observed with the canine versus human fluorogenic substrates. The G149S, S150T mutation had no effect on substrate specificity of canine renin.

All mutations in the S_1' site aimed at replacing canine S_1'-amino acids with their human orthologs had a deleterious effect on the specific activity of the mutants to cleave canine substrate. Even though the V286L mutation did decrease species specificity of canine renin ~6-fold, the effect was somewhat limited, since this mutation led to more than a 78% drop in its specific activity for native substrate, while activity towards human substrate rose by only 28%. This limited effect of the S_1' mutations on substrate specificity of dog renin is not entirely unexpected, since there is mounting evidence in the literature implicating the S_2' subsite as a key element of substrate recognition. For example, while human renin is capable of cleaving the Leu–Leu bond in angiotensinogens from other species, it cleaves rat angiotensinogen very slowly (*9*) due to the substitution of Tyr for Ile at the P_2' site. Also, non-primate renins do not cleave human angiotensinogen or its analogs having Ile at the P_2' position, but they do cleave substrate analogs with Val at this position (*8*, *10*). Therefore, further alterations in the S_2' site of canine renin may be necessary for efficient cleavage of human substrate with engineered canine renin.

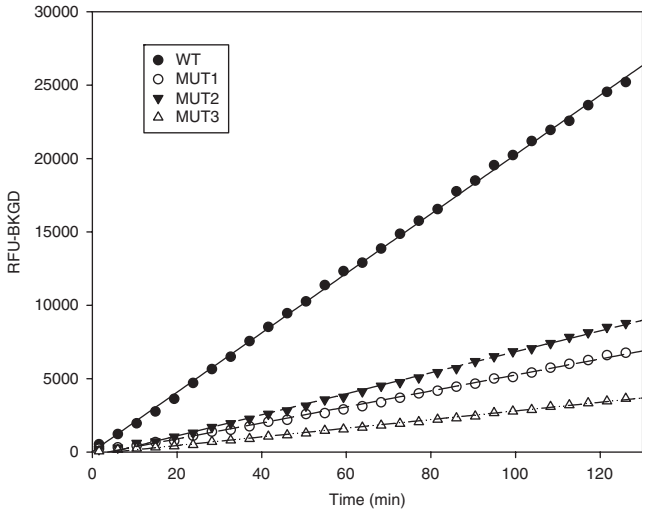

Fig. 5. **The time-course of hydrolysis of the fluorogenic canine substrate with the wild-type canine renin (WT) and the enzyme carrying mutations G149S, S150T (MUT1), V286L (MUT2) and G149S, S150T, V286L (MUT3).**

Inhibition of Canine Renin with Human Renin Inhibitors—The activity of a collection of inhibitors was determined for human and canine renin along with the mutant enzymes. These inhibitors included commercially available statines, the small molecule inhibitor aliskiren and related analogues.

Pepstatin A is a potent inhibitor of several aspartic proteases including cathepsin E [IC_{50} of 0.2 nM (*24*)], cathepsin D [IC_{50} of 0.4 nM (*25*)] and HIV protease [IC_{50} of 250 μM (*26*)]. Human renin is poorly inhibited by pepstatin A [IC_{50} of 15 μM (*27*) and 36 μM (*28*)], while porcine renin exhibits sub-micromolar inhibition constants with this compound [IC_{50} of 0.58 μM (*29*) and 0.32 μM (*27*)]. We found that canine renin resembles porcine renin in its inhibition by pepstatin A with the IC_{50} value of 0.8 μM (Fig. 6).

SR 42128 is a pepstatin analog that inhibits human and dog plasma renin activities with the IC_{50} values of 28 nM and 48 nM, correspondingly (*30*). The compound is more potent against purified recombinant human renin with an IC_{50} of 2.3 nM (data not shown) and inhibited purified canine renin with similar IC_{50} of 2.0 nM (Fig. 6).

Canine renin is also inhibited by aliskiren, a potent human renin inhibitor currently marketed for hypertension (*31*). Aliskiren is selective for human renin with the IC_{50} of 0.6 nM (*32*). The potency of aliskiren for canine renin is 3 nM (Fig. 6) in line with the previously reported value of 7 nM (*32*).

Aliskiren acts as a transition state mimetic, inhibiting renin via hydrogen bonding of both the central hydroxyl and amino groups to the catalytic Asp32 and Asp215 residues (*33*). In the X-ray structure of aliskiren co-crystallized with human renin, the P_3–P_1 portion of aliskiren is accommodated by a large hydrophobic S_3–S_1 pocket formed in part by the flap region in its closed conformation. The S_3 sub-pocket accommodates the aromatic alkoxy side chain (*32*).

Table 2. **Specific activities of the wild-type canine renin (WT) and its mutants G149S,S150T (MUT1), V286L (MUT2) and G149S, S150T, V286L (MUT3) measured with either canine renin substrate CR-MZ or human renin substrate AnaSpec-24479.**

	WT	MUT1	MUT2	MUT3
Enzyme Conc. (nM):	22	11	35	21
Activities with 4uM CR-MZ (canine renin substrate):				
Rate (RFU/min):	186 ± 24	46 ± 12	63 ± 12	25 ± 7
Specific Activity (RFU/min/nM):	8.4 ± 1.1	4.4 ± 1.1	1.8 ± 0.3	1.2 ± 0.3
Activities with 4uM AnaSpec-24479 (human renin substrate):				
Rate (RFU/min):	7.1	1.8	14.4	7.5
Specific Activity (RFU/min/nM):	0.32	0.17	0.41	0.36
Ratio Canine/Human	26.1	25.9	4.4	3.3

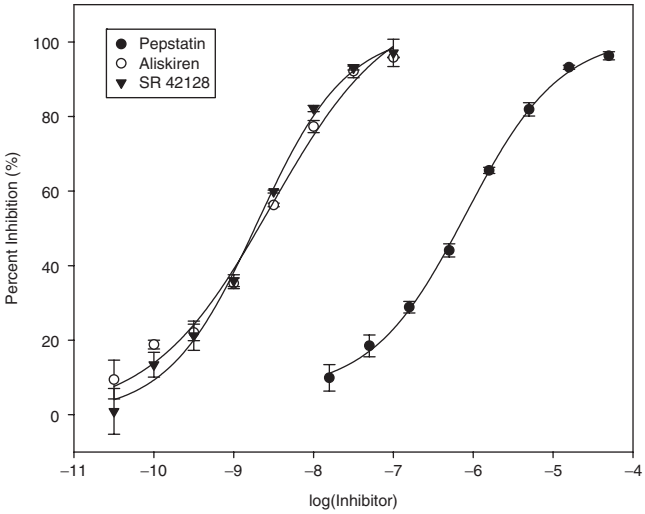

Fig. 6. **Inhibition of canine renin by various inhibitors.** The percent inhibition is plotted as a function of inhibitor concentration for aliskiren (open circle), SR 42128 (filled inverted triangle) and pepstatin (filled circle). The line through the data is the least squares fit to a four-parameter equation describing inhibition, and the IC_{50} values determined are 3 nM (aliskiren), 2 nM (SR 42128) and 800 nM (pepstatin).

Table 3. **Effect of mutations in the S1′ of the substrate binding site of canine renin on cross-inhibition with human renin inhibitors.**

Canine renin construct	The slope of the linear fit of IC_{50} canine versus IC_{50} human	The ratio IC_{50} canine IC_{50} human
Native canine renin	8.72 ± 0.91	14.8 ± 10.2
MUT1, G149S,S150T	1.25 ± 0.16	2.1 ± 1.5
MUT2, V286L	3.97 ± 0.42	6.8 ± 4.3
MUT3, G149S,S150T, V286L	0.67 ± 0.07	1.15 ± 0.7

The P_3–P_1 pharmacophore along with the hydroxyethylamine (HEA) warhead of aliskiren provide strong interaction with the enzyme. The portion of aliskiren lacking the prime side exhibits low micromolar IC_{50} against human renin (data not shown). A series of inhibitors of human renin were synthesized where the HEA transition state isostere was replaced with diaminopropanol group. The latter is an attractive low molecular weight replacement for statine and homostatine extensively used for design of inhibitors of renin (*34*) and other aspartic proteases, such as HIV protease (*35*) and beta-secretase (*36*).

We analysed inhibition of the mutated enzyme with a series of compounds that differed only by the moieties interacting with the S_1' and perhaps S_2' substrate binding pockets (Table 3). When the library of compounds was tested against the wild-type canine and human renin, the inhibitors were fairly specific for the human enzyme. Substitution V286L (MUT2) in canine renin led to a stronger cross-inhibition of the mutated canine renin. More interestingly, the alteration in the 'flap' region (MUT1, G149S, S150T) resulted in nearly equipotency of human renin inhibitors against the engineered canine renin. Further mutation of the canine enzyme (MUT3: G149S, S150T, V286L) led to stronger inhibitor binding.

In conclusion, the full-length open reading frame of canine pre-prorenin has been cloned from total RNA isolated from dog kidney. This is the first report of the primary amino acid sequence of the canine pre-proenzyme. Canine protein shared 70.4% identity with human pre-prorenin. The protein was expressed in human cell line HEK 293. The native canine signal peptide directed processing and secretion of prorenin into culture medium. Active renin was obtained by the cleavage of the 43 amino acid N-terminal propeptide with trypsin and purified to homogeneity. Steady-state kinetic characterization of canine renin revealed that its efficiency to cleave the native tetradecapeptide substrate was practically indistinguishable from the catalytic efficiency of previously described renin isolated from dog kidneys. The activity is the same order of magnitude as the k_{cat}/K_M values reported previously for human renin.

Analysis of the primary sequences of canine and human renin allowed identification of several substitutions in the active sites of the enzymes that may be critical for species specificity of renin. We mutated three residues in the S_1' subsite of canine renin, G149, S150 and V286 to corresponding amino acids in human renin, S150, T151 and L290 (Fig. 1). We studied the effect of the mutations on substrate specificity and cross-inhibition of canine renin with human renin inhibitors. Mutation V286L had the greatest impact on both specific activity (4-fold loss) and substrate selectivity (6-fold loss) of the canine renin mutant. A panel of 38 human renin inhibitors sharing the same P_3–P_1 moiety and differing only by their prime-site chemistries was tested versus human renin and mutated canine enzymes. Changes in the 'flap' region of canine renin (G149S, S150T) resulted in almost complete loss of discrimination between inhibition of human renin and the mutated canine renin. This dramatic effect of limited point mutations on catalytic activity and inhibitor affinities agrees well with the structural models. Our results indicate that point-substitutions in the S_1' pocket complementary with the substrate structure have a great effect on species specificity of renin.

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