Chemical Characteristics of a High Molecular Weight Renin from the Renal Cortex of the Dog

D. Mark Potter, Walter J. McDonald, Patrick M. Dunn, and James Metcalfe

SUMMARY We found an acid extract of normal dog kidneys to contain two distinct molecular weight forms of renin-like activity. Gel filtration chromatography showed peaks of activity at estimated molecular weights of 65,000 and 41,000. The high molecular weight fraction (HMW) comprised only 1% of the total activity of the extract. Both HMW and low molecular weight (LMW) fractions were inhibited by anti-human renin antibody and had similar broad pH-dependent activity optima between pH 6.0 and 7.5 in homologous substrate. The Michaelis constant (Km) of HMW was 3.6 times the Km of LMW. Both renins bound reversibly to concanavalin A-Sepharose with comparable affinities. HMW and LMW eluted from DEAE-Sephadex at similar salt concentrations without conversion of HMW to LMW. Transient acidification effected partial conversion of HMW to LMW without changing the total activity. Preincubation of HMW with trypsin increased the activity 40% and effected complete conversion of HMW to LMW. The apparent molecular weight difference between HMW and LMW is probably due to a covalently bound fragment(s) and not to a noncovalently bound moiety such as has been described in the rabbit and the hog. Both HMW and LMW are glycoproteins whose terminal sugar constituents possibly are similar. HMW dog renin is a new molecular form of renin that is convertible to a more active lower molecular weight renin with trypsin proteolysis.

SINCE Lumbers' report in 1971 that renin in human amniotic fluid exhibited high specific activity following transient acidification and the finding by Morris and Lumbers in 1972 that the same effect could be attained by pretreatment of amniotic fluid renin with proteolytic enzymes, large quantities of data have been acquired concerning multiple molecular forms of renin. These have been the subject of several recent reviews. Hog and rabbit renal renins have been described which are high in molecular weight and are reversibly convertible to lower molecular weight forms with ion exchange chromatography or transient acidification. Human renal and plasma renins characterized by high molecular weight and low specific activity have also been described. These human renin forms are susceptible to conversion to high specific activity by acidification or enzymatic proteolysis but, in contrast to hog and rabbit forms, do not convert to lower molecular weight forms as they are activated. Similarly, high molecular weight renin has been described in the rat kidney and mouse plasma.

We report finding a true high molecular weight (HMW) renin in normal dog kidneys. We have described its biochemical characteristics, using in vitro experiments which allow its comparison to HMW renins described for other species.

Methods

Renin Preparation

Renal cortical tissue, dissected from mongrel dog kidneys that had been frozen and thawed, was extracted by the method of Deodhar et al. and Haas et al., 602 g of tissue were homogenized on ice in distilled water, and H2SO4 was added to pH 2.5. Ten minutes later, the pH was restored to pH 6.5 with KOH. After centrifugation, the supernatant extract was titrated to pH 4.5 with H2SO4 and enough (NH4)2SO4 was added to make the solution 2.4 M. After another centrifugation, the precipitate was suspended in distilled water, dialyzed against distilled water for 14 hours at 4°C, and again centrifuged. The supernatant extract was then titrated to pH 2.8 with H2SO4, and a quantity of −20°C ethanol was added sufficient for a final concentration of 10% ethanol. After 24 hours at 4°C, the precipitate was discarded and Na2-EDTA, 1 mg/ml, was added to the supernatant fluid, followed by dialysis against 0.05 M phosphate buffer, pH 7.4, at 4°C. Eighty-one milliliters of recovered extract were stored at −70°C in 3.5 mM diisopropyfluorophosphate (DFP).

Protein concentration of the extract was estimated by absorbance at 280 nm against a human serum albumin reference standard (Miles Laboratories). Total protein recovery was 1.077 g. The specific activity of the extract was determined using...
a human renin standard (Medical Research Council Research Standard A 68/356) and partially purified sheep renin substrate dissolved in 0.05 M phosphate and 0.15 M NaCl, pH 7.5 (see below). Total activity recovered was 75.4 Goldblatt units (GU), and the specific activity of the crude extract was 0.07 GU/mg. No angiotensinase activity was detectable in the extract in the maximum concentration used experimentally, and the activity of the extract was linear with serial dilution in buffer containing egg lysozyme, 1 mg/ml (Sigma Chemical Co.).

Substrate Preparation

For assay of renin activity of eluates from the chromatographic experiments to be described, a pool of renin-free substrate was prepared. Three dogs were nephrectomized 2 days prior to exsanguination under pentobarbital anesthesia. Blood was collected in EDTA to a final concentration of 1 mg/ml, and the plasma was separated by centrifugation at 4°C. Following storage of the plasma at −20°C, DFP was added to a final concentration of 3.5 mM and the plasma was titrated to pH 6.2 with 0.38 M citric acid. Renin substrate concentration, defined by exhaustive incubation with homologous renin, was approximately 1500 pmol/ml. Endogenous renin activity was undetectable after 10 hours of incubation at 37°C. Angiotensinase activity was undetectable for up to 20 hours at 37°C, defined by recovery of added angiotensin I (A I).

For standardization of renin activity of the extract against human standard renin, a sheep was nephrectomized under pentobarbital anesthesia and exsanguinated into heparin-containing flasks 2 days afterward. Using the method of Skinner et al.,18 we desalted the plasma on a 5- × 100-cm column of Sephadex G-50 (Pharmacia Fine Chemicals) with 0.02 M phosphate buffer, pH 7.5. After application of the desalted plasma to a column of DEAE-Sephadex A-50, the renin substrate was eluted with a NaCl gradient, dialyzed against distilled water in the cold, and lyophilized. The renin substrate concentration used for standardization of the dog renin preparation was 3000 pmol/ml defined by exhaustive incubation with human renin.

Renin Assay

Assay of renin activity of chromatographic fractions was performed in a standard fashion except as described in individual experiments. Ten microliters of eluate were added to 200 μl of ice-cold renin substrate and incubated for 60 minutes at 37°C in a Dubnoff shaking incubator. The reaction was stopped by plunging the samples into an ice-water bath; subsequently they were stored frozen at −20°C until assay of generated A I. The rate of generation of angiotensin was linear with time if less than 200 ng/ml were generated. Samples whose renin activity initially was found to exceed that limit were appropriately diluted prior to assay and the activity corrected for dilution.

A I was measured by radioimmunoassay by the method of Cohen et al.,19 using rabbit anti-A I antibody,125I-labeled A I from New England Nuclear, and A I standard from Schwarz/Mann calibrated against Medical Research Council Research Standard A, code 71/328. The lower limit of quantitative detection of A I was 0.3 ng/ml of the original sample. Each experimental sample was assayed in triplicate in the same assay with all other samples of that particular experiment. Intra-assay variability was ±3%.

Gel Filtration

Gel filtration chromatography of the crude renin extract was performed on a 2.6 × 92-cm column of Ultrogel AcA-54 (LKB Instruments) at 4°C with a buffer of 0.15 M NaCl, 0.05 M sodium phosphate, and 0.01% sodium azide. Void volume was estimated with blue dextran. Total volume was estimated with 125I. Calibration was completed with bovine serum albumin, ovalbumin, chymotrypsinogen A, and ribonuclease A. Protein in the eluate was monitored continuously at 280 nm. Calibration and experimental elutions were performed with 1- to 1.2-ml samples at a flow rate of 13 ml/hr. The eluate was collected in 2-ml fractions, and every other fraction was assayed for renin activity.

pH Dependence of Renin Activity

To determine whether both of the two peaks of renin activity obtained from gel filtration demonstrated the characteristic broad peak of pH-dependent activity around pH 6 that is characteristic of dog renin,20 HMW and LMW fractions were separated and then incubated simultaneously for 1 hour at 37°C and assayed for A I. In the pH range examined, recovery of added A I was complete.

Antibody Inhibition of Renin Activity

As a further check on the specificity of HMW and LMW peaks as renin, inhibition of activity by anti-human renin antibody (kindly supplied by Dr. C. P. Lucas, Detroit, Mich.) was determined. To 100 μl of dog substrate were added identical volumes of appropriately diluted human renin21 (also supplied by Dr. Lucas), HMW or LMW fraction, and either 5 μl of undiluted renin antiserum, normal rabbit serum, or egg lysozyme (12.5 mg/ml) in phosphate buffer. Samples were preincubated for 24 hours at 4°C and then incubated 1 hour at 37°C and assayed for generated A I.

Kinetic Comparison of HMW and LMW Renins.

Estimates of Michaelis constants (K_m) of HMW and LMW renin fractions were performed as follows. Egg lysozyme (12.5 mg/ml) was added to eluates from gel filtration after addition of DFP to 3.5 mM and titration to pH 6.2 with 0.38 M citric acid.
acid. Dog substrate was diluted with a solution of egg lysozyme, 12.5 mg/ml, in 0.15 M NaCl, 0.05 M sodium phosphate, 3.5 mM DFP, and Na2-EDTA, 1 mg/ml, pH 6.2. Actual substrate concentrations of the various dilutions were determined by exhaustive incubation with excess dog renin. Samples of comparable activity of HMW or LMW fractions were added to duplicate samples of substrate at each substrate concentration, and all samples were incubated simultaneously for 2 hours at 37°C. Rates of A I generation were linear with time at all substrate concentrations. K_m was determined by Lineaweaver-Burk analysis.

Effect of Acid Dialysis on Renin Activity and Molecular Weight

Samples of HMW and LMW fractions from gel filtration were divided into three parts for determination of the effect of transient acidification on renin activity. The first sample was kept at 4°C for 48 hours but was not dialyzed. The second was dialyzed for 24 hours at 4°C against 0.05 M glycine-HCl buffer, pH 3.3, then for an additional 24 hours against 0.05 M phosphate buffer, pH 7.5. The third sample was dialyzed for 48 hours against 0.05 M phosphate buffer, pH 7.5. Renin activity of the samples was subsequently determined as previously described. The three samples were then chromatographed on the AcA-54 gel column to determine whether the apparent molecular weight of the renin had changed with acidification.

Effect of Trypsin on Activity and Molecular Weight

To determine whether incubation of either HMW or LMW renin with trypsin altered renin activity, pools of each renin were divided into three aliquots. To the first, trypsin (Bovine Type III, Sigma Chemical Co.) was added to a final concentration of 50 μg/ml at 4°C and pH 7.5, a sample was taken to determine baseline renin activity, and the mixture was then incubated up to 90 minutes at 37°C. Additional samples were taken at various times to determine the rate of change in renin activity. At each sampling, the reaction of trypsin with the renin was quenched with lima bean trypsin inhibitor (Sigma) at a final concentration of 50 μg/ml and placed in an ice-water bath. The second aliquot was incubated and sampled identically, but without the addition of trypsin, to serve as a control for the effect of incubation; the third was kept at 4°C without any treatment, to serve as an additional control. After determination of the time required for maximal change of activity, additional HMW fractions were incubated 30 minutes at concentrations of trypsin varying between 50 and 400 μg/ml to confirm the maximal change in activity (see Results). It was also determined that neither trypsin nor trypsin inhibitor had any intrinsic ability to generate angiotensin with dog substrate.

Following the experiments described above, matched samples incubated 30 minutes with trypsin, 50 μg/ml, incubated without trypsin, or not incubated at all were concentrated by pressure ultrafiltration under nitrogen using an Amicon UM-10 membrane and chromatographed to determine changes in apparent molecular weight of the renin activity.

Ion Exchange Chromatography

Fractions of chromatographed extract containing samples from peaks of HMW and LMW renin activity which had been approximately matched for velocity of generation of A I were dialyzed for 24 hours at 4°C against 0.02 m phosphate buffer, pH 7.5, and applied to a 0.75- × 3-cm column of DEAE-Sephadex A-50. Renin was eluted with a linear gradient of NaCl from 0 to 0.3 m in starting buffer after being washed with three volumes of starting buffer. One-milliliter fractions were collected at a rate of 8 ml/hour. Renin activity of each fraction was determined as previously described. Salt concentrations of the fractions were measured by electrical conductance (Beckman Instruments), and peaks of renin activity were plotted as a function of salt concentration. Following ion exchange chromatography, fractions containing peaks of renin activity were concentrated by pressure ultrafiltration and reapplied to the AcA-54 column to determine possible conversion of HMW to LMW renin.

Concanavalin A Binding Studies

Binding of HMW and LMW renin to concanavalin A-Sepharose (Pharmacia Fine Chemicals) was determined by dialyzing HMW and LMW fractions of the crude extract against a buffer consisting of 1 M NaCl, 0.01 m sodium acetate, and 0.001 m MgCl2, CaCl2, and MnCl2, pH 6, at 4°C. Diafiltered eluate (2.5 ml) was incubated 30 minutes at 4°C with 0.5 ml of concanavalin A-Sepharose. The samples were centrifuged at 40 g for 10 minutes and the supernatant fluids were assayed for unbound renin activity. After determination that both renins bound almost completely to concanavalin A, identical columns of concanavalin A-Sepharose were constructed with a bed volume of 1.5 ml. The columns were washed with 30 volumes of the buffer described above, and diafiltered eluates containing HMW, LMW, or a mixture of the two renins were applied. The columns were eluted with an additional 10 ml of starting buffer and a linear gradient of α-methyl-d-mannopyranoside was begun. Final sugar concentration in the elution gradient was 0.2 M. Eluates were collected in 1-ml fractions. Every other fraction was assayed for renin activity as previously described. Neither the buffer constituents nor the α-methyl-d-mannopyranoside had significant effect on renin activity in dog substrate at the final concentrations employed.
Results

Figure 1 shows a representative elution profile of crude extract which had been concentrated from 6 ml to 1 ml prior to application to the AcA-54 column. Clear resolution of a HMW peak of activity is seen. The estimated molecular weight of HMW renin is 65,000 ± 3,000 (SD), and the weight of LMW renin, 41,000 ± 3,500 (n = 6). The renin activity of the HMW fraction comprises 1% of the total renin activity of the crude extract.

Because enzymes other than renin are capable of generating angiotensin under varying conditions, it is important, in impure preparations such as ours, to attempt to define the identity of the enzymatic activity as being due to either renin or another enzyme. Figure 2 shows pH-dependent activity curves of HMW and LMW peaks of activity. Their similarity to one another and dissimilarity to pseudorenin provides reassurance that both are renin. The inhibition of human renin and both HMW and LMW dog renins by anti-human rabbit antiserum was comparable at 62%, 49%, and 49%, respectively, whether compared to normal rabbit serum or lysozyme controls. This specific active-site antibody inhibition strongly suggests that the HMW activity is renin.

The estimated $K_m$ of HMW renin was 5700 pmol/ml and of LMW renin, 1600 pmol/ml (correlation coefficient of both linear regressions = 0.99). Whether conversion of HMW to LMW renin with trypsin (see below) involved a change in $K_m$ or $V_{\text{max}}$ or both was not determined.

Acid dialysis neither increased nor decreased the activity of either HMW or LMW renin. Dialysis at pH 3.3 for 24 hours did, however, result in the partial conversion of HMW to LMW renin activity (Fig. 3). These results compare with the recent report of Levine et al. that a high molecular weight...
hog renin converted to a LMW form during acid dialysis without an increase in activity. Our results contrast with several reports of acid activation or complete conversion of HMW renin in that the effect of acid dialysis is far from complete at 24 hours.

Figure 4 shows the rate of activation of HMW renin at a trypsin concentration of 50 μg/ml at 37°C and pH 7.5. The maximum activation of approximately 40% was also seen when the concentration of trypsin was increased up to 400 μg/ml and incubation carried out for 30 minutes. LMW renin was neither activated nor inactivated during a 90-minute incubation with trypsin. Our findings are in contrast with those of Day et al. who demonstrated an approximate 20-fold increase in activity of HMW human renin following trypsin digestion and also reported that activation was followed by a rapid decrease in activity within minutes of the reaction reaching maximum activation. Similarly, Morris and Lumbers found that trypsin digestion of human amniotic fluid renin resulted in at least a 3-fold increase in subsequent renin activity.

Gel filtration of HMW dog renin which had been completely activated during incubation with trypsin showed that the renin activity in the HMW fraction had disappeared entirely. All the remaining activity appeared in the elution volume identical to that of LMW renin, while both the incubated and unincubated controls showed no change in the elution volume of the original HMW renin (Fig. 5). Assay for renin activity in elution volumes other than those corresponding to the original HMW and LMW peaks of activity found with gel filtration of the original crude extract failed to locate additional peaks of renin activity. There were no discrete peaks of activity intermediate between the HMW and LMW positions following trypsin digestion, even when chromatography of incompletely activated HMW fractions was performed (Fig. 6).

The results of DEAE-Sephadex ion exchange chromatography are shown in Figure 7. Boyd and Leckie and McConnell have shown for the hog and rabbit that conversion of HMW to LMW renin can
occur on ion exchange chromatography. Levine et al. have suggested that this conversion may occur with sufficient rapidity to appear that both HMW and LMW forms elute at identical salt concentrations in the elution buffer. That dog HMW renin does not undergo such a conversion is shown in Figure 8. This figure illustrates the results of gel filtration chromatography of the pooled eluates from DEAE-Sephadex chromatography shown in Figure 7. The proportions of HMW and LMW renin activity illustrated are in direct proportion to the amounts of each renin fraction originally applied to the DEAE-Sephadex column.

The results of concanavalin A-Sepharose affinity chromatography are shown in Figure 9. The appearance of small amounts of renin activity in eluates not containing sugar is a curious but quite reproducible phenomenon for which we have no explanation. The elution of renin bound to free concanavalin A which has dissociated from the gel or the existence of renin which is chemically different from the bound activity are two possible explanations for this phenomenon.

Discussion

This constitutes the first report of a high molecular weight renin in the dog. The antibody inhibition studies, pH-dependence curves for enzymatic activity, and the ability to generate A I in plasma are evidence that the high molecular weight renin activity is true renin (in contrast to other known A I-generating enzymes).

Dog HMW renin is similar in some respects to HMW renins previously reported in other species. It differs from all these other forms, however, in at least one property. Table 1 presents a summary of similarities and dissimilarities between our dog HMW renin preparation and other selected HMW renins. The molecular weight is comparable in all...
except the rat preparation, ranging from 55,000 to 70,000. Dog HMW renin is different from all others except that reported by Levine et al., with respect to the lack of acid activation of dog and hog HMW renins. Dog HMW renin is so far the only renin described which is converted to LMW renin following limited proteolysis with trypsin.

These data imply that there are multiple forms of HMW renin. Alternatively, the ascribed differences may involve experimental artifacts, as most studies of renin chemistry, including our own, are conducted with very impure preparations of renin. The problem is compounded in that many of these studies are carried out in very complex biological fluids such as plasma or amniotic fluid. Thus, questions which are appropriately applied to interpretation of any study of HMW renin must include (1) is the enzymatic activity truly renin or another angiotensin generating proteolytic enzyme? (2) are the experimental manipulations applied directly affecting the renin molecule itself or indirectly affecting it by coincident changes in other enzymes in the preparation which subsequently result in modification of the renin molecule? and (3) are moieties affecting apparent molecular weight differences among renins covalently bound to the molecule, or are they loosely bound incidental "satellites"?

In spite of the difficulties of interpretation of the many reports of HMW renins now available, it seems likely that HMW renins really exist. The resistance to conversion of HMW human renin to a LMW form reported by Day et al. strongly suggests that the moiety accounting for the molecular weight difference is tightly, and probably covalently, bound to the LMW form. Similarly, under the conditions of the experiments we have described, the proteolytic activity of trypsin is essential to effect the conversion of HMW dog renin to the LMW form. Two features of this tryptic conversion suggest the possibility that the cleavage site may be one that is important in the hypothetical in vivo conversion of HMW to LMW dog renin. One is that there are no apparent intermediate forms present during tryptic digestion, although this may simply reflect inactivity of such potential forms or lack of resolution of our gel filtration system. The other is that, after the inhibitory moiety is removed by trypsin, no additional degradation of the LMW product appears to occur.

The finding that HMW dog renin is only partially converted to LMW renin during 24 hours of dialysis at pH 3.3 is at variance with reports of the conversion of other renins during acid dialysis. Preliminary experiments in our laboratory (unpublished observations) have shown no further change in HMW activity or apparent molecular weight when acid dialysis is extended past 24 hours. The explanation(s) for this limited conversion remains speculative at this time but may include the possibility of more than one form of HMW dog renin, the absence of potential activating cofactors, or an equilibrium between interconvertible HMW and LMW forms.

The elution of HMW and LMW dog renin from DEAE-Sephadex at identical salt concentrations and the failure to convert HMW to LMW renin during ion exchange chromatography are at variance with the findings of Leckie and McConnell in the rabbit and Boyd in the hog. The fact that they used a cellulose-based ion exchanger and we used a Sephadex-based exchanger is a possible but highly unlikely explanation for the variance. It is tempting to assume that similarity of binding of HMW and LMW dog renin to concanavalin A establishes the fact that they share identical sugar moieties, but the binding of glycoproteins to concanavalin A is too complex to allow such an assumption. Inagami and Murakami have demonstrated by direct analysis that LMW hog renin is a glycoprotein and binds to concanavalin A. This is the first report to suggest that HMW renin, as well as LMW renin, is a glycoprotein. If HMW renin is indeed a precursor to LMW renin in vivo, it would seem highly possible that they would share at least one common glycosylated moiety.

In conclusion, we have found a HMW renin in normal dog kidneys which is convertible to a lower molecular weight renin with trypsin digestion and partial conversion by transient acidification. The portion of the molecule which accounts for the weight difference between HMW ad LMW appears, especially in view of the results of tryptic digestion, to be a covalently bound fragment(s). While HMW dog renin shares properties with other high molecular weight renins which have been described previously, it appears to be a unique form. It has recently been pointed out by Levine et al. that it is careless to equate "activation" of renin with con-
version of HMW forms to LMW forms, as one may occur without the other.\textsuperscript{7,23} The increase in activity of HMW dog renin coincident with trypsic digestion may not necessarily be a result of conversion of HMW to LMW renin, and could be the result of destruction of renin inhibitors coincidently present in the HMW chromatographic fraction. Nevertheless, the conversion of HMW to LMW dog renin under the conditions of the experiments we performed is incontrovertibly dependent upon the presence of trypsin and strongly suggests that HMW renin may be a true LMW precursor in the dog. Whether this phenomenon occurs in vivo and whether it is of biological significance of course remain to be seen.

Acknowledgments

We wish to thank Linda Niemi and Mark Bellis for their expert secretarial assistance and Dorothy Macfarlane for her patient technical work.

References

Chemical characteristics of a high molecular weight renin from the renal cortex of the dog.
D M Potter, W J McDonald, P M Dunn and J Metcalfe

Circ Res. 1978;43:832-839
doi: 10.1161/01.RES.43.6.832

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1978 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/43/6/832