Complete Purification of Dog Renal Renin†

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ABSTRACT: Canine renal renin was purified to homogeneity by using an eight-step procedure. Proteolytic enzyme inhibitors in all buffers utilized during the procedure improved the stability of renin. Separation of renin from other proteolytic enzymes by carboxymethylcellulose and pepstatin affinity chromatography was essential for optimal yield and stability of the product. A 600 000-fold purification was obtained with a 16% overall recovery. The specific activity of the pure enzyme was 4200 Goldblatt units/mg of protein. The criteria for homogeneity were the following: (1) a symmetrical peak of renin activity eluted from the final gel filtration chromatography with uniform activity across the peak; (2) a single stained band of protein by polyacrylamide disc gel electrophoresis, pH 8.9 and 7.8, assay of the slices from the pH 7.8 gel revealed that renin activity eluted as a symmetrical peak and corresponded to the stained band; (3) sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis yielded a single protein band; and (4) isoelectric focusing yielded a single band. The molecular size of purified renin was 36 000 daltons by $NaDodSO_4$ gel electrophoresis and 42 000 daltons by gel filtration chromatography; the isoelectric point was pH 5.7. The amino acid composition was similar to that reported for hog renal renin and mouse submaxillary gland renin. Pure canine renin was stable at 4 or −20 °C to 8 weeks. The availability of purified renin permits specific antibody production for application in immunoassay and physiologic studies.

The enzyme renin (EC 3.4.99.19) is a protease that acts at the amino-terminal end of renin substrate to release the decapeptide angiotensin I which is in turn cleaved by other enzymes to angiotensin II, a potent pressor agent and a primary stimulus for adrenocortical release of aldosterone. The role of this enzyme and its products in blood pressure homeostasis and the pathogenesis of hypertension remains only partially understood and highly controversial. The unavailability of purified enzyme has retarded progress in this field. Many prior purification efforts have failed because of the exceedingly low concentration of the enzyme in renal tissue (Haas et al., 1954) as well as its apparent instability.

We undertook the purification of renin from canine kidneys with four goals in mind: first, a biochemical characterization of the enzyme as well as an interspecies comparison; second, the development of antibody specific for the enzyme; third, the application of the antibody in the direct measurement of enzyme concentration as well as the identification of potential zymogens; and fourth, the application of antibody as a highly specific inhibitor of renin activity in physiologic experiments in the intact dog.

In this communication we detail a 600 000-fold purification of the enzyme by utilizing conventional methods as well as an affinity chromatography step which separated renin from other proteases and rendered it stable. In subsequent papers we shall report the production of renin-specific antibody and the application of the antibody in immunoassay and in physiologic studies.

Materials and Methods

Renin Assay. Renin enzymatic activity was determined by the rate of generation of angiotensin I generally according to the method of Haber et al. (1969). Nephrectomized dog plasma with no renin activity was used as substrate and the reaction mixture incubated in the presence of angiotensinase inhibitors (ethylenediaminetetraacetic acid, 8-hydroxyquinolone, and dimercaptopropanol) at pH 7.4 for 1 h. Angiotensin I released was measured by a radioimmunoassay utilizing [^{125}I]angiotensin I (Schwarz/Mann). Activity was expressed in Goldblatt units (GU) by comparison with a standardized canine renin preparation that had been bioassayed in the dog (kindly provided by Dr. Erwin Haas). One Goldblatt unit generated 7 μg of angiotensin I per h in the assay described above.

After partial purification (step 6, subsequently), renin was free from other proteolytic enzymes and the fluorometric assay of Reinharz & Roth (1969) as modified by Corvol et al. (1977) for rapid determination of peak activity could be used. N-Acetylated tetradecapeptide substrate synthesized in our laboratory (Dr. G. Matsueda) was incubated with renin for 1 h at 37 °C in 0.1 M sodium phosphate, pH 6.6. The reaction was terminated by immersion in boiling water. The new amino terminus generated by cleavage of the peptide was quantified by reaction with fluorescamine in 0.5 M borate buffer, pH 8.0. Fluorescence was recorded on an Aminoco-Bowman spectrofluorometer with an excitation wavelength of 390 nm and an emission wavelength of 475 nm.

Protein Determination. Protein concentrations were determined by the Folin–phenol method of Lowry et al. (1951). The Folin–phenol reagent was purchased from Fisher Scientific Co., NJ. The purified enzyme's protein concentration was also determined by the fluorescamine method (Udenfried et al., 1972) using bovine serum albumin (Sigma) as standard and by amino acid analysis.

Nonspecific Proteolytic Activity Assay. The method of Anson (1937) was used at pH 3.5, utilizing hemoglobin (Sigma) as substrate.

Preparation of the Affinity Column. Pepstatin (Peptide Institute, Osaka) was coupled to aminohexyl-Sepharose (Pharmacia) by the method of Murakami & Inagami (1975); 4.5 μmol/mL of wet gel was incorporated. Before coupling, an aliquot of pepstatin solubilized in methanol–NaOH–acetic acid was tested for its capacity to inhibit plasma renin activity by the method of Corvol et al. (1977). The activity of pepstatin–aminohexyl-Sepharose was evaluated by pepsin binding at pH 2.2 by the method of Marciniszyn et al. (1976).

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was run in duplicate on 5.5-cm gels containing 10% polyacrylamide with 2% cross-linking in a multiphasic

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Table I: Dog Renal Renin Purification

	total protein (mg)	renin act. (ng of A_I/h)[a]	sp act. ng/mg	sp act. GU/mg	x-fold purifn	yield (%)
kidney powder	7.5×10^6	3.5×10^8	47	0.007	1.0	100
crude extract	1.0×10^6	3.0×10^8	100	0.014	2.0	86
DEAE-cellulose batch	3.1×10^5	2.1×10^8	677	0.08	11.0	60
acid-NaCl ppt	9.0×10^4	1.0×10^8	1100	0.16	23.0	30
ammonium sulfate ppt	3.0×10^4	9.0×10^7	3000	0.43	61.0	26
CM-cellulose chromatography	5.0×10^3	7.3×10^7	1.4×10^4	2.0	285.0	21
pepstatin affinity chromatography	17	6.1×10^7	3.6×10^6	514.0	73 000.0	18
Sephadex G-100	2	5.9×10^7	3.0×10^7	4200.0	600 000.0	16

[a] A_I = angiotensin I.

buffer system at pH 7.8, 4 °C, with a constant current of 1 mA/gel for 2 h according to the method of Rodbard & Chramback (1971). One gel from each pair was stained by Coomassie Blue overnight at room temperature. The other was sliced into 1-mm sections; each slice was eluted in 0.5 mL of 100 mM Tris-HCl buffer, pH 7.5, for 24 h at 4 °C for subsequent renin activity determination.

Polyacrylamide gel electrophoresis was also performed by the method of Davis (1964) at pH 8.9.

$NaDodSO_4$ gel electrophoresis was performed according to the method of Weber & Osborn (1969). Renin was prepared by overnight dialysis against 1% $NaDodSO_4$ and 1% mercaptoethanol in phosphate-buffered saline. Protein was stained by Coomassie Blue. Proteins used for molecular size calibration included bovine serum albumin (Sigma) (M_r 67 000), rabbit immunoglobulin heavy chain (M_r 50 000) and light chain (M_r 25 000), myoglobin (M_r 17 000), and lysozyme (Worthington) (M_r 13 000).

Stability. Stability was determined by storage of renin aliquoted after purification steps 5–8 at 4 and −20 °C. Renin aliquoted after step 8 was subjected to multiple freezing and thawing and to lyophilization.

Isoelectrofocusing. Isoelectrofocusing was performed between pH 3 and 8 according to the original method of Vesterberg & Svensson (1966).

Amino Acid Analysis. One hundred micrograms of purified renin was dialyzed against 5 mM sodium acetate, pH 5.5, and dried, and amino acid analysis was performed on a Durrum D-500 amino acid analyzer after timed hydrolysis in 6 N HCl for 24 h at 110 °C. Tryptophan was determined after alkaline hydrolysis, and cysteine was determined as cysteic acid after performic acid oxidation.

Purification Procedure. Twenty kilograms of dog kidneys was collected from anesthesized animals prior to sacrifice and was immediately frozen and stored at −20 °C. Prior to use, the cortex was dissected and then subjected to one freeze–thaw cycle before it was minced by an electric meat grinder. The tissue was then frozen, immediately arranged in 2-cm thick layers in trays, and lyophilized. The dry tissue was pulverized in a blender and defatted by stirring with 6 L of diethyl ether for 1 h. After filtration, the powder was air-dried overnight in a fume hood.

The dry powder was extracted with 20 L of a 30% acetone–water mixture containing 5 mM ethylenediaminetetraacetic acid (EDTA), 2 mM phenylmethanesulfonyl fluoride (PMSF), and 5 mM sodium tetrathionate. After being stirred for 1 h at 4 °C, the mixture was centrifuged in a Sharples (Warminster, PA) model continuous flow centrifuge at 13 500 rpm (1500*g*) for 20 min and the supernatant collected.

DEAE-cellulose (Whatman DE-52) was washed with deionized water. Wet resin (1500 g) was added to the supernatant, and the mixture was stirred for 30 min and then collected in a Büchner funnel. The cellulose was then washed with 4 L of 5 mM sodium acetate, pH 5.0, and eluted with 8 L of 100 mM sodium acetate and 200 mM sodium chloride, pH 5.0, containing the proteolytic enzyme inhibitors, 0.25 mM EDTA, 0.25 mM sodium tetrathionate, and 0.1 mM PMSF. These inhibitors were used in all solutions at each subsequent step through step 7.

The eluate was acidified to pH 3.0 with 5 N HCl; 5 g/100 mL sodium chloride was then added, and the resultant precipitate was removed by centrifugation. Ammonium sulfate (2.3 kg) was added to the supernatant to achieve 70% saturation. The precipitate obtained for centrifugation was dissolved in 500 mL of 20 mM sodium acetate, pH 5.0, and dialyzed overnight against 40 L of 20 mM sodium acetate, pH 5.0.

The dialysate was then applied to a carboxymethylcellulose column (190 × 4 cm) equilibrated with 20 mM sodium acetate, pH 5.0, at a flow rate of 60 mL/h. After elution with 4 L of the same buffer, absorbance at 280 nm was negligible. Subsequent elution was carried out with a linear concentration gradient between 1500 mL each of 20 mM sodium acetate, pH 5.0, and the same buffer containing 100 mM potassium chloride (Figure 1). The fractions containing renin activity were pooled and dialyzed overnight in 40 L of 5 mM sodium acetate, pH 5.5.

Fifty milliliters of pepstatin–aminohexyl-Sepharose (4.5 μmol/mL of wet gel) was mixed with the renin activity pool for 2.5 h at 4 °C. The mixture was poured into a 25 × 2.5 cm column, and the column was washed with 2 L of 20 mM sodium acetate and 1 M sodium chloride, pH 5.0. The column was eluted with a linear concentration gradient between 200 mL each of 100 mM Tris-HCl buffer, pH 7.5, and the same buffer containing 2 M lithium bromide at a flow rate of 90 mL/h (Figure 2).

Fractions containing renin activity were pooled and subjected to gel filtration on a Sephadex G-100 column (190 × 4 cm) developed with 5 mM sodium phosphate, pH 7.5, 100 mM sodium chloride, and 1% EDTA at 100 mL/h.

Results

The result of each purification step is summarized in Table I. A 600 000-fold purification was obtained with 16% recovery. Twenty kilograms of kidney yielded 2 mg of renin. The specific activity was 4200 GU/mg of protein.

The initial extraction procedure, modified from Rubin (1972), resulted in a 40-fold higher renin activity yield than simple homogenization of kidney cortical tissue in aqueous buffer. The addition of protease inactivators diminished losses of renin throughout the experiment.

Pepstatin affinity chromatography proved to be the most effective purification step, providing a 350-fold purification if utilized after ammonium sulfate precipitation or a 200-fold increase when preceded by CM-cellulose chromatography. Renin was optimally bound when equilibrated with 5 mM

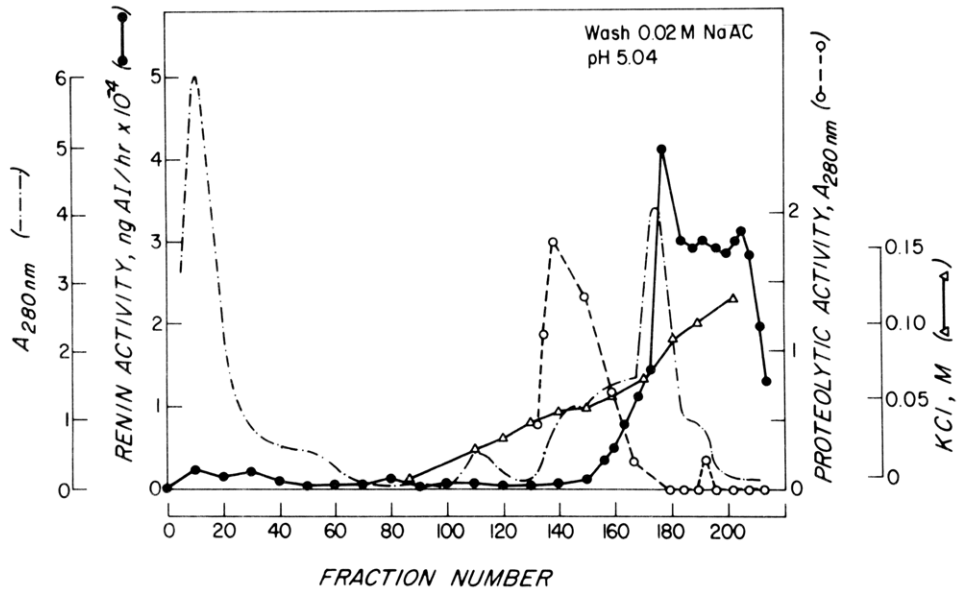

FIGURE 1: Elution pattern of dog renal renin on a 190 × 4 cm carboxymethylcellose column. Elution was carried out by using a linear concentration gradient of potassium chloride (Δ) in 20 mM sodium acetate, pH 5.0. This resulted in the separation of nonrenin protease (O) from renin activity (●). Each fraction volume was 15 mL.

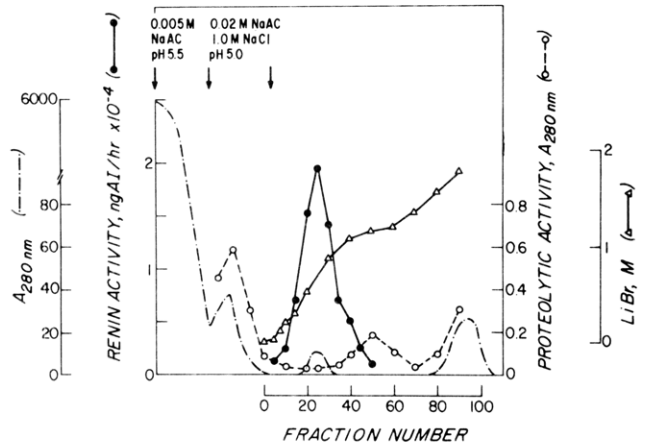

FIGURE 2: Affinity chromatography using a pepstatin–aminohexyl-Sepharose column (1.5 × 2.5 cm) equilibrated with 5 mM sodium acetate, pH 5.5. Elution using a linear concentration gradient of lithium bromide (Δ) in 100 mM Tris, pH 7.5, yielded a symmetrical peak of renin activity (●), separating it from nonrenin protease (O). Each fraction was 10 mL in volume.

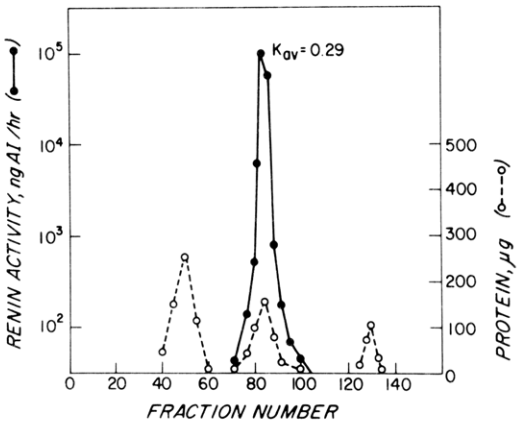

FIGURE 3: Gel filtration of semipurified renin on a Sephadex G-100 column (190 × 4 cm). Fractions of renin eluted from the pepstatin column (Figure 2) were pooled, concentrated two- to threefold with an Amicon PM-10 membrane, and applied to the G-100 column at 100 mL/h. A symmetrical peak of renin activity (●) with a specific activity of 4200 GU/mg was obtained. K_{av} of renin = 0.29.

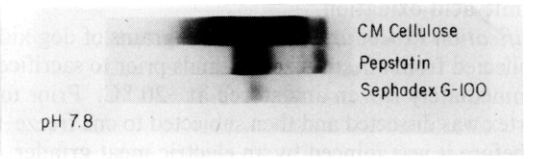

FIGURE 4: Polyacrylamide disc gel electrophoresis of dog renal renin obtained from Sephadex G-100, pepstatin, and CM-cellulose chromatography steps. Gels were 5.5 cm in length and contained 10% polyacrylamide with 2% cross-linking. Electrophoresis was performed at pH 7.8, 4 °C, with a constant current of 1 mA/gel for 2 h. Protein staining was performed with Coomassie Blue. Similar results were obtained at pH 8.9.

sodium acetate, pH 5.5. The lithium bromide elution program described resulted in maximal yields (85%). In contrast, elution with 100 mM acetic acid, pH 3.2, yielded less than 30% of the renin activity applied to the column.

When pepstatin affinity chromatography was employed immediately after ammonium sulfate precipitation, a small but significant amount of nonrenin protease coeluted with renin which resulted in instability of renin on storage. Prior CM-cellulose chromatography separated the proteases from renin (Figure 1).

Gel filtration indicated a symmetrical absorbance peak at 280 nm corresponding to the renin activity (Figure 3). Specific activity was uniform across the peak. Following final gel filtration, polyacrylamide disc gel electrophoresis with both the pH 8.9 and 7.8 systems showed a single protein band (Figure 4). Elution of slices from the pH 7.8 gel revealed that renin activity eluted as a symmetrical peak and corresponded to the stained band (Figure 5).

$NaDodSO_4$–polyacrylamide gel electrophoresis with or without mercaptoethanol yielded a single protein band with an R_f of 0.39, corresponding to a molecular size of 36 000 daltons (Figure 6). Molecular size by gel filtration was 42 000 daltons. The isoelectric point was pH 5.7. The amino acid composition (Table II) of dog renal renin is similar to that reported for hog renin (Inagami & Murakami, 1977; Corvol et al., 1977).

During the course of purification, renin was unstable through pepstatin affinity chromatography. After the removal of nonrenin proteases at this step, the enzyme was relatively stable to storage at 4 and −20 °C in pH 5.0 buffers. A 10%

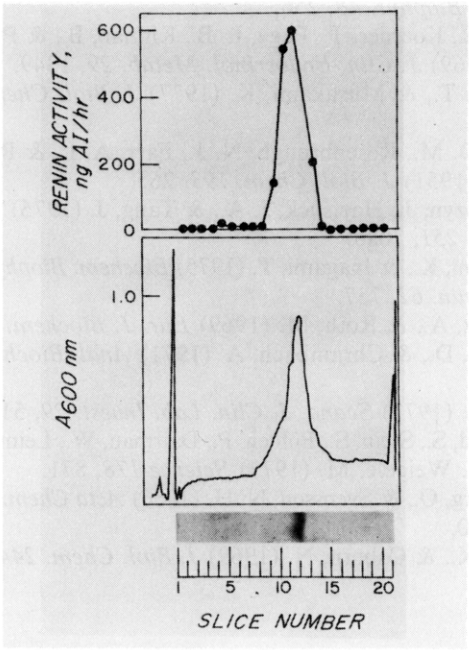

FIGURE 5: Profile of renin activity and protein migration on polyacrylamide disc gel electrophoresis at pH 7.8 (details are outlined in Figure 4). Protein was profiled by scanning the absorbance of the stained gel at A_{600nm} with the Gilford spectrophotometer. One-millimeter slices of a simultaneously run unstained gel were assayed for renin activity by the method of Haber et al. (1969). Renin activity corresponded precisely to the protein band.

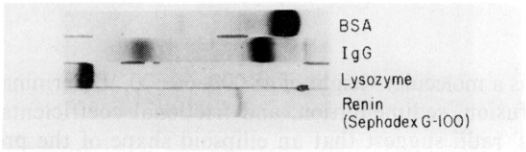

FIGURE 6: $NaDodSO_4$ (1%)–polyacrylamide gel electrophoresis in the presence of 1% mercaptoethanol of pure dog renin obtained from the Sephadex G-100 column (R_f 0.39). Gels were 5.5 cm in length and ran with a constant current of 1 mA/gel for 2 h at pH 7.5. Protein staining was performed with Coomassie Blue.

loss of renin activity was observed after 3 weeks of storage. After final purification, renin did not lose measurable activity either at 4 °C or at −20 °C when followed up to 8 weeks. Repeated freezing and thawing, however, resulted in a loss of renin activity associated with the appearance of new bands on $NaDodSO_4$ gel electrophoresis, suggesting aggregation and fragmentation. Lyophilization led to a loss of 10–25% of the renin activity.

Discussion

The purification of canine renal renin was accomplished by an eight-step procedure. The final product gave a single homogeneous protein band on polyacrylamide discontinuous gel electrophoresis at pH 7.8 and 8.9, $NaDodSO_4$ gel electrophoresis with or without mercaptoethanol, and isoelectric focusing. Enzymatic activity corresponded to the position of the protein band on disc gel electrophoresis. After a 600 000-fold purification, a specific activity of 4200 GU/mg of protein with 16% recovery was achieved. Thus, 2 mg of pure enzyme can be obtained from 20 kg of dog kidney. The specific activity reported is considerably higher than that of purified renin from other species but may simply be related to the standard employed in the assay. The Goldblatt unit is defined in terms of a pressor effect in the dog. Canine renin may simply be a more effective pressor agent in the homologous species than heterologous renins.

Table II: Amino Acid Composition of Dog Renin

	dog renin		hog renin	
amino acid	wt (g)	residues/ protein molecule[a]	residues/ protein molecule[b]	residues/ protein molecule[c]
Asp	7.70	24.3	25.9	40.4
Thr	5.00	17.8	26.2	20.7
Ser	10.10	41.8	31.9	36.4
Glu	12.70	35.5	30.8	36.0
Pro	4.50	16.7	17.6	18.2
Gly	6.75	42.6	33.6	43.6
Ala	4.14	21.0	15.7	21.8
Cys	0.58	2.1	2.5	
Trp	2.40	4.8	5.0	
Val	6.52	23.7	34.2	17.1
Met	0.76	2.1	3.7	2.4
Ile	2.54	8.1	12.2	8.0
Leu	7.75	24.7	30.3	25.8
Tyr	2.50	5.6	16.4	6.4
Phe	6.29	15.4	16.9	13.0
His	2.32	6.1	5.0	8.2
Lys	3.77	10.6	10.6	5.0
Arg	3.12	7.2	9.4	9.5

[a] Calculations based on a renin molecular weight of 36 000. [b] Calculations based on a renin molecular weight of 36 400 (Inagami & Murakami, 1977). [c] Calculations based on a renin molecular weight of 36 800 (Corvol et al., 1977).

As has been the experience in prior renin purifications, difficulty was encountered on account of the marked instability of the enzyme throughout purification. Despite the use of protease inhibitors (EDTA, PMSF, and sodium tetrathionate), renin was unstable after ammonium sulfate fractionation. Pepstatin affinity chromatography immediately after this step failed to separate renin from proteases. Carboxymethylcellulose, utilized prior to affinity chromatography, resulted in sufficient separation of renin from proteases so that subsequent affinity chromatography resulted in a product that was fairly stable to storage at 4 or −20 °C for up to 3 weeks.

Affinity chromatography, although not entirely specific, was the most effective purification step, resulting in a 200-fold purification, similar to that reported by Murakami & Inagami (1975) and Corvol et al. (1977). Canine renin, however, behaved differently from hog renin in several respects: (a) binding was favored by conditions of very low ionic strength; (b) acid elution resulted in a recovery of less than 30% of the applied activity, whereas lithium bromide elution effected 85% recovery; and (c) pepstatin also bound contaminating proteases, requiring prior carboxymethylcellulose chromatography.

Canine renal renin has many properties similar to those of hog and human renin. The molecular size estimated by $NaDodSO_4$ gel electrophoresis was 36 000 daltons and that by gel filtration 42 000 daltons. These values compare favorably with 36 800 (Corvol et al., 1977) and 36 400 (Inagami & Murakami, 1977) daltons for hog renin. Mouse submaxillary gland renin has a molecular weight of 37 200 (Cohen et al., 1972). The isoelectric point of dog renin was 5.7 as compared to 5.15 (Corvol et al., 1977) or 5.2 (Inagami & Murakami, 1977) for hog renin and 5.3–5.6 for the mouse enzyme (Cohen et al., 1972).

Pure dog renin is stable at 4 and −20 °C when tested up to 8 weeks. Repeated freezing and thawing, however, resulted in fragmentation and aggregation and loss in renin activity. The enzyme can also be stored in the lyophilized form. About 10–25% of the renin activity is lost with lyophilization and subsequent redissolving.

The availability of purified canine renin provides a significant opportunity for studying this enzyme in a species par-

ticularly suited to cardiovascular investigation. Antibodies to the purified protein may not only contribute insight to renin biosynthesis and the nature of renin precursors but also may provide a highly specific renin antagonist in physiologic experiments.

Acknowledgments

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Purification and Characterization of High-Affinity Cyclic Adenosine Monophosphate Phosphodiesterase from Dog Kidney†

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ABSTRACT: High-affinity cyclic adenosine monophosphate (cAMP) phosphodiesterase was purified from dog kidney homogenate to apparent homogeneity. The high-affinity form of the enzyme system was separated from the bulk of low-affinity cAMP–cGMP phosphodiesterase by preparative DEAE-cellulose chromatography and further purified by hydroxylapatite chromatography, DEAE-cellulose rechromatography, and Sephacryl S-200 gel filtration in a stabilizing buffer system. Criteria of purity were sodium dodecyl sulfate–acrylamide gel electrophoresis, sedimentation velocity, sedimentation equilibrium, and gel filtration. The enzyme shows no evidence of subunit structure, is an acidic protein as determined by amino acid composition and isoelectric focusing (pI = 4.8), and has a molecular weight of 48 000–60 600. Determinations of diffusion, sedimentation, and frictional coefficients and Stokes' radii suggest that an ellipsoid shape of the protein apparent in a low-polarity medium is more stable than a more spheroidal shape apparent in a high-polarity medium. The purified enzyme displays Michaelis–Menten kinetic behavior for cAMP and cGMP hydrolysis with relative affinities of 2.2 and 312 μM, respectively. cAMP hydrolysis is not affected by known activator or inhibitor proteins. We conclude from these studies that this purified high-affinity enzyme form is the basic catalytic subunit of mammalian cyclic nucleotide phosphodiesterases.

Cyclic nucleotide phosphodiesterase (EC 3.1.4.17) catalyzes the hydrolysis of cAMP and cGMP, the only known catabolic mechanism for these important regulatory nucleotides. Both kinetic and physical criteria have supported the existence of multiple molecular forms of this enzyme system in a wide variety of tissues. High- and low-affinity enzyme forms have been identified that differ in size, substrate specificity, and modulation by effectors such as cAMP or cGMP, a calcium-dependent regulator (calmodulin), modulator binding proteins (inhibitor), cell–cell interactions, proteases, hormones, drugs, and genetic influences [for reviews, see Appleman & Terasaki (1975), Wells & Hardman (1977), Strada & Thompson (1978), and Thompson & Strada (1978)].

Purification of mammalian high- and low-affinity cyclic nucleotide phosphodiesterases has not previously been achieved, and thus the biochemical elements of this enzyme system were not known. This stands in contrast to effectors of one of the lower affinity forms of the enzyme, calcium-dependent regulator or calmodulin and inhibitor proteins, which have been purified to homogeneity (Lin et al., 1974; Watterson et al., 1976; Vandermeers et al., 1977; Klee & Krinks, 1978; Wang & Desai, 1977; Jarrett & Penniston, 1978; Sharma et al., 1978). Some nonmammalian sources of the enzyme have been extensively purified, but these appear to differ markedly in their kinetic and physical properties (Rosen, 1970; Morishima, 1974; Fujimoto et al., 1974; Miki et al., 1975) from mammalian enzyme forms.

Partial purification of the low-affinity form of the enzyme has been reported from heart (Butcher & Sutherland, 1962;

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