Conversion between Renin and High-Molecular-Weight Renin in the Dog

By SUSUMU FUNAKAWA, YOSHIHIKO FUNAE and KENJIRO YAMAMOTO
Department of Pharmacology, Osaka City University Medical School, Osaka, Japan

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Two forms of renin, one of mol.wt. 43 000 and the other 60 000, were found in the dog kidney. Conversion between the two forms of renin was reversible at neutral pH. Though the molecular weight of renin in kidney-cortex homogenate was 43 000, it was completely converted into high-molecular-weight renin in the presence of substances that react with thiol groups. On the contrary, stored renin in the granules was the form of normal size (mol.wt. 43 000) regardless of the absence or presence of such substances. The present experiments indicated that renin is stored in the granules as the form of normal size and might be converted into high-molecular-weight renin when it is released from the granules and attached to some substance in the soluble fraction of renal-cortical tissue.

Renin (EC 3.4.99.19) is generally defined as an enzyme of renal origin, of mol.wt. ranging from 37 000 to 43 000, which produces proangiotensin (angiotensin I) from renin substrate in circulating plasma. A number of studies on high-molecular-weight forms of proteinases (zymogens) and polypeptide hormones (prohormones) have been reported. The existence of high-molecular-weight renin of mol.wt. greater than 55 000 has also been demonstrated in several species (Boyd, 1972, 1974; Leckie & McConnell, 1975; Day *et al.*, 1975; Levine *et al.*, 1976; Inagami & Murakami, 1977; Inagami *et al.*, 1977; Barrett *et al.*, 1977) and it has been variously postulated as a reversibly regulated inhibitor–renin complex, a pathological form of renin, or a biosynthetic precursor of renin. However, the origin and physiological role of high-molecular-weight renin are not yet known, and the relationship to the renin of normal size seems to be discussed by inference. Though renin is stored in specific granules in the juxtaglomerular cells (Cook, 1971), whole homogenate of the kidney has been used mostly as experimental material. Stored renin in the granules must be examined to clarify the above problems. The present study describes the storage form of renin in the granules and reversible interconversion between renin and high-molecular-weight renin in the dog kidney cortex.

Materials and Methods

Crude tissue extract

For this 1 g of frozen or fresh dog kidney cortex, medulla or liver was minced and homogenized for 5 min in 7 ml of 10 mM-KH₂PO₄/40 mM-Na₂HPO₄, pH 7.2, containing 0.1 M-NaCl (buffer I), by using a Waring blender at 10 000 rev./min. The resulting

homogenate was centrifuged at 10 000g for 20 min to remove insoluble material, and the supernatant was used as a crude extract. Protein contents, measured by the method of Lowry *et al.* (1951), in these preparation of kidney cortex and medulla and liver were approx. 10.5, 5.3 and 20.5 mg/ml respectively. Renin activity in the extract of kidney cortex was 4.0–10.0 μ g of proangiotensin/h per ml. The extract of medulla and liver contained no renin activity. A portion of the extract of kidney cortex was dialysed against 50 mM-glycine/HCl buffer, pH 3.0, containing 0.1 M-NaCl, for 20 h at 4°C (acidification) and subsequently dialysed against buffer I for 20 h at 4°C. Inhibitors of proteinases, thiol-group blockers and a metal chelator were used for suppressing the destruction of renin. The inhibitors used were 5 mM-EDTA, 2 mM-phenylmethanesulphonyl fluoride, 0.2 mM-diisopropyl phosphorofluoridate, 5 mM-sodium tetrathionate, 10 mM-N-ethylmaleimide and 5 mM-o-iodosobenzoic acid. Phenylmethanesulphonyl fluoride and diisopropyl phosphorofluoridate were used in solution in 0.5% (v/v) methoxyethanol. These inhibitors were added separately or in combination to the extraction buffer before homogenization. Cysteine (50 mM) and dithiothreitol (50 mM) were used as reductants.

Extraction of renin from the isolated renin granules

Renin granules were prepared by the method of Morimoto *et al.* (1972): 1 g of fresh dog kidney cortex was sliced and gently homogenized for 40 s in 7 ml of buffer I by using a Potter–Elvehjem homogenizer with a loose pestle at 800 rev./min. The homogenate obtained was centrifuged at 500g for 10 min to remove cell debris and nuclei and then the supernatant was placed on a discontinuous sucrose-density-gradient solution (1.2 to 1.7 M with 0.1 M intervals, 7 ml of each

sucrose solution in water) and centrifuged at 60000g for 90min in a Hitachi 65P centrifuge. Renin granules were mainly equilibrated at 1.6M-sucrose: 1ml of this renin-granule fraction was suspended in 1.0ml of water to lower the sucrose concentration and centrifuged at 75000g for 20min. The sediment was resuspended in 1.0ml of buffer I and homogenized again for 2min by using a tight pestle at 2000 rev./min. The resulting homogenate was centrifuged at 75000g for 20min and the supernatant (protein, 0.45 mg/ml; renin activity, 0.2–0.6 μ g of proangiotensin/h per ml) was retained.

Determination of molecular weight of renin

The molecular weight of renin was estimated by gel filtration. Sample (1ml) was applied to a Sephadex G-100 column (1.6cm $\times$ 90cm; Pharmacia column), previously equilibrated with buffer I, and eluted with the same buffer at a flow rate of 8 ml/h, and 1ml fractions of each eluate were collected at 4°C. Void volume (V_0) of the column was estimated by using Blue Dextran. Bovine serum albumin (mol.wt. 67000), ovalbumin (mol.wt. 45000), α -chymotrypsinogen A (mol.wt. 25000) and cytochrome *c* (mol.wt. 12900) were used as molecular-weight standards.

Renin assay

Renin activity was measured by radioimmunoassay of the proangiotensin generated (Haber *et al.*, 1969) by using the CEA-IRE-SOLIN kit (Solin Biomedica, 13040 Saluggia, Italy) after incubation with partially purified dog renin substrate prepared by the method of Morimoto *et al.* (1970). The incubation mixture consisted of 0.1ml of the sample containing renin, 0.1ml of dog renin substrate (1500pmol of proangiotensin equivalent/ml), 0.8ml of 0.03M-KH₂PO₄/0.07M-Na₂HPO₄, pH 7.0, 10mM-EDTA, 3.2mM-2,3-dimercaptopropan-1-ol and 1.6mM-8-hydroxyquinoline sulphate. Incubation was carried out at 37°C for 1–3h and terminated by cooling.

Chemicals

Phenylmethanesulphonyl fluoride, di-isopropyl phosphorofluoridate, *o*-iodosobenzoic acid, Blue Dextran and the molecular-weight-standard proteins were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., and sodium tetrathionate was from Nakarai Chemicals, Kyoto, Japan. Other chemicals used were obtained from Wako Pure Chemical Industries, Osaka, Japan.

Results

Molecular weight of renin in the crude extract

The elution patterns of extract of dog kidney cortex from Sephadex G-100 are shown in Fig. 1.

Fig. 1. Gel filtration of the crude extract of dog kidney cortex prepared without inhibitors of proteinases (a), with inhibitors (b), and of the extract acidified to pH3.0 before gel filtration (c)

The supernatant (10000g) of the homogenate was applied on a Sephadex G-100 column (1.6cm $\times$ 90cm), eluted with 0.05M-phosphate buffer, pH 7.2, containing 0.1M-NaCl, and 1ml fractions of each eluate were collected. Inhibitors used were: 5mM-EDTA, 0.2mM-di-isopropyl phosphorofluoridate, 2mM-phenylmethanesulphonyl fluoride and 5mM-sodium tetrathionate. Renin was assayed before (●) and after (○) acidification of each fraction. Renin activity was expressed as pmol of proangiotensin/h per ml. ---, A_{280} .

Renin activity in the extract of dog kidney cortex prepared without proteinase inhibitors showed a single peak at 89ml of eluate (Fig. 1a). When a mixture of proteinase inhibitors, consisting of EDTA (5mM), phenylmethanesulphonyl fluoride (2mM), di-isopropyl phosphorofluoridate (0.2mM) and sodium tetrathionate (5mM), was present in the homogenizing buffer, the elution volume of renin activity moved to an apparent higher molecular weight (Fig. 1b), similar to the result with hog renin (Inagami *et al.*, 1977). The relative elution volume of the renin in the former experiment (1.62) corresponds to an apparent mol.wt. of 43000 and in the latter (1.43) to 60000, which were calibrated by authentic proteins (bovine serum albumin, 1.37; ovalbumin, 1.57; α -chymotrypsinogen A, 1.89; cytochrome *c*,

2.21). Thus two forms of renin were found in the extract of dog kidney. These results indicate the following two possibilities for the nature of high-molecular-weight renin. (1) It is a protein-renin complex that is converted into the renin of normal size by proteinase(s). (2) The native form of renin stored in the kidney is exclusively high-molecular-weight renin and the renin of normal size is an artifact

produced by an attack by proteinase(s) during the extraction procedure.

When sodium tetrathionate was omitted from the mixture of inhibitors, high-molecular-weight renin was not detectable. On the contrary, in the presence of sodium tetrathionate alone without other inhibitors, still only high-molecular-weight renin was detected. Addition of sodium tetrathionate to the extract that had been prepared without inhibitors resulted in complete conversion of the renin of normal size into high-molecular-weight renin. When other blockers of thiol groups, e.g. *o*-iodosobenzoic acid and *N*-ethylmaleimide, were substituted for sodium tetrathionate, high-molecular-weight renin was also detected (Fig. 2). These results suggested that the conversion between high-molecular-weight renin and renin of normal size was reversible and that thiol groups were required for this conversion.

Dithiothreitol (50mM) was added to the extract prepared with sodium tetrathionate, incubated for 15min at 37°C, and then applied on the Sephadex column. Renin activity was eluted from the column at the same volume of eluate as for the extract prepared without inhibitors. A similar result was observed in the presence of cysteine (50mM) instead of dithiothreitol. These results confirm the requirement for thiol groups for the conversion of high-molecular-weight renin into the renin of normal size. Detectable forms of renin in the extract of dog kidney in various experimental conditions are summarized in Table 1.

In this experiment we found two forms of renin in dog kidney cortex that could be interconverted. However, from these data we cannot determine which form of renin is the true storage form. In the next experiment, renin granules were isolated and the molecular weight of renin therein was determined.

Fig. 2. Gel filtration of the crude extract of dog kidney cortex prepared with 5mM-*o*-iodosobenzoic acid (a) and 10mM-*N*-ethylmaleimide (b)

The supernatant (10000g) of the homogenate was applied on a Sephadex G-100 column (1.6cm×90cm), eluted with 0.05M-phosphate buffer, pH7.2, containing 0.1M-NaCl, and 1ml fractions of each eluate were collected. Renin activity was expressed as pmol of proangiotensin/h per ml. ---, *A*₂₈₀.

Table 1. Detectable forms of renin in various experimental conditions

For full experimental details see the text. * Prepared without inhibitors; ** prepared with inhibitors; *** supernatant (75000g, 20min) of homogenate was used; +, detected; −, not detected.

Preparation	Addition	Renin	High-molecular-weight renin
Crude extract*	—	+	—
**	—	—	+
*	Sodium tetrathionate (5mM)	—	+
** (acid-treated)	Sodium tetrathionate (5mM)	+	—
**	Dithiothreitol	+	—
**	Cysteine	+	—
Renin granule*	—	+	—
**	—	+	—
**	Renal cortex,***	—	+
	sodium tetrathionate (5mM)		
**	Renal medulla,***	+	—
	sodium tetrathionate (5mM)		
**	Liver,***	+	—
	sodium tetrathionate (5mM)		

Molecular weight of renin in renin granules

The molecular weight of renin stored in the granules was revealed to be 43 000, as shown in Fig. 3, in either the absence or the presence of blockers of thiol groups during preparation procedures. If the stored renin in granules is considered to be the native form, renin of normal size is evidently the native form of renin, and high-molecular-weight renin is seemingly a complex of renin and renin-binding substance. When the extract (0.5 ml) of granules and the supernatant fraction (0.5 ml) of renal-cortex homogenate (which was prepared as gently as possible to avoid destruction of renin granules) were mixed, incubated for 30 min at 4°C with sodium tetrathionate (5 mM) and then applied on the Sephadex column, renin of normal size in the extract of granules was completely converted into high-molecular-weight renin. This conversion was not observed when the extract of granules was incubated with the supernatant (0.5 ml) of renal medulla or liver homogenate (Table 1). These results indicate that a specific renin-binding substance must be present only in the soluble fraction of renal-cortical tissue.

Fig. 3. Gel filtration (Sephadex G-100 column, 1.6 cm $\times$ 90 cm) of the extract of renin granules isolated from dog kidney cortex in the absence of sodium tetrathionate (a) and in the presence of 5 mM-sodium tetrathionate (b).

For experimental details of the preparation of the extract of renin granules see the text. Renin was assayed before (●) and after (○) acidification of each fraction. In (b) no significant change in renin activity was found after acidification. Renin activity was expressed as pmol of proangiotensin/h per ml.

Acidification

Renin activity in the extract of the kidney did not change significantly after dialysis against buffer I for 40 h at 4°C. The pH of the dialysis residue gradually reached about 3 within 10 h of dialysis against 50 mM-glycine/HCl buffer, pH 3.0. As shown in Fig. 1(b), renin activity in each fraction eluted from the Sephadex column corresponding to high-molecular-weight renin increased by about 50% by acidification, whereas little change was observed in the eluate corresponding to renin of normal size (Figs. 1a and 3a). Acidification of the extract prepared with sodium tetrathionate resulted in an increase in renin activity by 40–70%, and a concomitant decrease of the molecular weight of renin to that of the form of normal size (Fig. 1c). The conversion of renin of normal size into high-molecular-weight renin could not be demonstrated in the renal extract that was acidified before addition of sodium tetrathionate.

Discussion

Although numerous studies on high-molecular-weight renin have been reported in recent years, there are wide variations in the molecular-weight profile, and the presumed biochemical nature frequently differs among investigators. Differences in methodology and species have made it difficult to assess the physiological significance of high-molecular-weight renin, and the relationship between high-molecular-weight renin and that of normal size is yet to be clarified. It seems inadequate to examine the biochemical nature of renin only in the extract of whole tissue, because renin is stored in secretory granules. For example, Inagami *et al.* (1977) reported the existence of high-molecular-weight renin (mol.wt. 60 000) in rat kidney, whereas Morris & Johnston (1976) showed that the molecular weight of inactive renin (prorenin) stored in the juxtaglomerular granules was 44 000. In the present study, gel filtration of the extract of renin granules isolated from dog kidney showed a single peak of renin activity at a relative elution volume corresponding to the molecular weight of the renin of normal size. Only renin of normal size was found in the renin-granule fraction prepared by sieving with a membrane filter (M. Kawamura & K. Yamamoto, unpublished work). It is noteworthy that this renin of normal size was completely converted into high-molecular-weight renin when it was incubated with sodium tetrathionate and the supernatant fraction of the homogenate of renal cortex, but not with that of renal medulla or the liver.

Day *et al.* (1975) reported the existence of high-molecular-weight renin associated with certain diseases, so it was considered to be a pathological form. In the crude renal extract of normal dogs, high-

molecular-weight renin could be found without exception when thiol groups were blocked. The reversible conversion between high-molecular-weight renin and the renin of normal size at physiological pH indicates that high-molecular-weight renin is a renin-protein complex. Therefore it is unlikely that high-molecular-weight renin is a pathological form of renin.

Boyd (1974) reported that high-molecular-weight renin (renin B) of pig kidney was converted into the form of normal size (renin A) with a simultaneous increase in pressor activity by acidification to pH 2.5 at 4°C. Leckie & McConnell (1975) observed similar results with rabbit kidney renin. In the present experiment, acidification of the extract of dog renal cortex resulted in an irreversible conversion of high-molecular-weight renin into the form of normal size and a concomitant increase in renin activity. As a significant change in activity of the renin of normal size was not observed by acidification, renin-binding ability seemed to be irreversibly lost on acidification.

Activation of inactive renin in human plasma and amniotic fluid by treatment with trypsin (Day & Luetscher, 1975; Cooper *et al.*, 1977) and cathepsin D (Morris, 1978) has been shown. These data strongly suggested that endogenous proteinases must be involved in the conversion of high-molecular-weight renin into the form of normal size under physiological conditions. A confirmation of the requirement for thiol groups in the conversion of high-molecular-weight renin was provided in the present experiment. The renin of normal size was converted into high-molecular-weight renin in the presence of sodium tetrathionate, and high-molecular-weight renin was reconverted into the form of normal size by dithiothreitol or cysteine. We conclude that renin is stored in the granules as the form of normal size and can be converted into high-molecular-weight renin when it is released from the granules and attached to

some substance in the soluble fraction of renal-cortical tissue, probably in the juxtaglomerular cells. Further study on the localization and biochemical characteristics of the substance and enzymes involved in the conversion between the two forms of renin is required.

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