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DEMONSTRATION OF THE FORMATION OF RENIN AND RENIN-BINDING PROTEIN COMPLEX USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatograph equipped with the newly developed gel, TSK G3000SW, was used to study the interaction between renin and renin-binding protein (RBP). Previously, the interaction could only be demonstrated after overnight gel chromatography in the presence of a non-physiological sulfhydryl reagent. However, this new high-speed gel chromatography provided a clear separation of renin and renin—RBP complex within 40 min. It also demonstrated that the renin—RBP complex was formed at 37°C in the absence of sulfhydryl reagent. These results indicate that the binding protein may play an important role in blood pressure regulation.

INTRODUCTION

Since the report of Boyd in 1974 [1] that a high molecular weight renin in hog kidney extract was a combination of renin and a carrier protein in a complex which is reversible under some conditions, and because of similar findings by Leckie and McConnell [2] for rabbit kidney, it has been hypothesized that kidneys contain renin-binding protein (RBP) which can be separated from and then recombined with renin. More recently, higher molecular weight forms of renin have been described in blood samples taken from patients suffering from Wilms' tumor of the kidney [3] or from stroke-prone spontaneously hypertensive rats in a malignant phase [4]. Since these observations suggest that the plasma level of higher molecular weight forms of renin would be a useful diagnostic aid, RBPs in the kidney and in the circulation have been extensively studied.

However, concerning the molecular weight of this renin-binding protein and the mechanism of the association, there have been large discrepancies among

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the results reported from different laboratories: Boyd [1] and Yamamoto et al. [5] have suggested molecular weights of over 50,000, while Leckie and McConnell reported a value of only 13,000 for their RBP preparation; Boyd [1] and Leckie and McConnell [2] have shown that warming a mixture of renin and RBP solution to 37°C led to the complex formation, while Kawamura et al. [6], using dog kidney, have reported that warming did not stimulate the slow association they observed at a low temperature.

On the other hand, Funakawa et al. [7] and Murakami et al. [8] have found that modification of sulfhydryl group(s) in RBP or renin seemed necessary to produce rapid association. Differences in kidney sources used by the above-mentioned investigators might account for some of these discrepancies, but, at least concerning the effect of temperature, we rather suspected that the low temperatures to which samples were exposed overnight during the analysis by gel filtration might affect the equilibrium between renin and renin—RBP complex.

To minimize the analysis time we employed in the present study high-performance liquid chromatography (HPLC) with a recently developed rigid and porous gel which provided rapid separation of proteins at high flow-rates [9]. This procedure gave good resolution and high reproducibility in separating the protein-bound forms of renin and demonstrated that the interaction between renin and RBP was in fact temperature-dependent and that at 37°C it proceeded within minutes.

EXPERIMENTAL

Preparation of hog kidney extracts

The hog kidneys obtained from a slaughterhouse were stripped of the capsule and bisected. The cortices were separated from the medulla and homogenized in 4 volumes of ice-cold 0.01 *M* sodium pyrophosphate buffer (pH 6.5) containing 0.1 *M* NaCl. The homogenate (20 ml) was centrifuged at 100,000 *g* for 1 h using a Beckman preparative ultracentrifuge L5 and the resulting supernatant was used as hog kidney extract.

Detection of renin—RBP complex

High-performance liquid chromatographic (HPLC) gel filtration was used to detect renin—RBP complex on the basis of molecular weight difference between renin and the renin—RBP complex. Analysis was conducted on a Toyo Soda Model HLC-803 instrument fitted with two coupled G3000SW columns (60 × 0.75 cm) using 0.01 *M* sodium pyrophosphate (pH 6.5) containing 0.1 *M* sodium chloride and 0.02% sodium azide as solvent. The G3000SW columns are packed with a rigid, hydrophilic, porous, spherical silica gel with a particle size of 10 ± 2 μm. The system was run at 100 kg/cm², 1 ml/min flow-rate, at room temperature. The column effluent was monitored with an UV detector at 280 nm. Samples (50 μl) containing 0.1 m*M* bovine serum albumin (BSA, M_r = 68,000) and 0.1 m*M* egg albumin (EA, M_r = 45,000) as internal molecular weight standards were injected through a loop injector. Fractions (0.25 ml) were collected every 15 sec for 45 min and aliquots were then assayed for renin activity.

Preparation of samples for HPLC

The effect of temperature on the interaction between renin and RBP was examined using hog kidney extracts incubated for fixed periods at 37°C.

When studying the effect of sulfhydryl reagents and sodium chloride on the interaction, hog kidney extracts were treated with 10 m*M* sodium tetrathionate, 10 m*M* dithiothreitol or 4 *M* sodium chloride at 37°C for 45 min. The precipitate formed during the incubation was removed by centrifugation before injecting on to the HPLC column.

To examine the pH-dependency of the renin—RBP complex formation, 2 g of hog kidney cortices were homogenized in 8 ml of 0.5 *M* Na_2HPO_4—citric acid buffer (pH 3—8) of constant ionic strength and centrifuged as described above; the supernatant was used as hog kidney extract.

RESULTS

Effect of temperature on the formation of renin—RBP complex

In an effort to solve the controversial problem whether incubation at 37°C stimulates the binding of RBP to renin or inactivates RBP, we incubated hog kidney extracts at 37°C for fixed periods of time and subjected them to the high-speed gel chromatography. As shown in Fig. 1, a single run on the column was completed in 40 min with a clear separation of the various forms of renin. This result indicated that the procedure could provide a better analysis of the reversible interaction between renin and RBP than conventional gel filtration which is considered not suitable for the analysis of systems in temperature-dependent equilibrium, since it usually requires overnight elution and therefore is carried out at low temperatures to preserve the activity of these biologically active materials. Fig. 1A and B illustrate the stimulatory effect of heat on the renin—RBP complex formation. Incubation of the extract for 1 min at 37°C produced an appreciable amount of higher molecular weight forms of renin (Fig. 1A). After 45 min incubation, most of the renin originally present in the extract was converted to higher molecular weight forms of renin (Fig. 1B). Their molecular weights were 70,000* ± 3,000 and 52,000 ± 2,000 (Fig. 1B), which correspond, respectively, to the high molecular weight and intermediate molecular weight renin, as reported in our previous paper [8]. This conversion was hardly affected by the presence of dithiothreitol, a protective reagent for sulfhydryl groups, eliminating the possibility that during the incubation at 37°C thiol groups in renin and/or RBP were oxidized. Fig. 1C shows that the high molecular weight renin formed by the warming was unstable at 4°C, and was dissociated into its components, intermediate molecular weight renin and RBP, by the cold treatment. Further dissociation of the renin did not occur, indicating that the intermediate form is relatively stable at low temperatures. In control experiments, we observed that the incubation of pure hog renin at 37°C, in the absence of RBP, did not produce higher molecular weight renins.

*This value was higher than that (60,000 ± 2,500) estimated using Ultrogel AcA 44 or Sephadex G-100 [8]. This discrepancy is probably due to the difference in the supporting medium or the elution time on gel filtration.

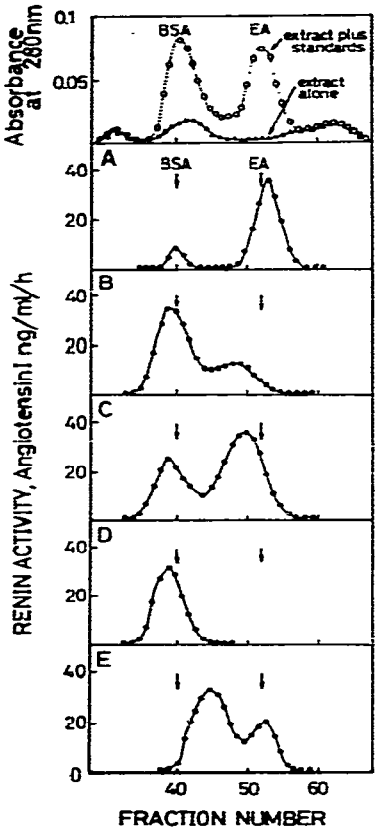

Fig. 1. HPLC separation of renin and renin—RBP complex formed by various treatments of hog kidney extracts. Incubation conditions in hog kidney extract: (A) 37°C for 1 min; (B) 37°C for 45 min; (C) 37°C for 45 min followed by incubation at 4°C for 16 h; (D) 37°C for 15 min with 10 m*M* sodium tetrathionate; (E) 37°C for 45 min with 4 *M* sodium chloride. Typical elution profiles, monitored by measuring absorbance at 280 nm, of the hog kidney extract with and without internal molecular weight standards are shown on the top panel; since nearly the same profile was obtained for the samples analyzed, only renin activity profiles are shown in the other panels. Renin activity was determined by the rate of formation of angiotensin I from hog renin substrate as described in our previous paper [8]. BSA = bovine serum albumin; EA = egg albumin.

Stimulation of the renin—RBP complex formation by the modification of thiol groups

It is well established that at low temperatures the interaction between renin and RBP occurs very slowly; however, the rate of the interaction is markedly enhanced by the addition of thiol blocker. To see if this is the case even at high temperatures, we incubated the extract in the presence of 10 m*M* tetrathionate at 37°C. As shown in Fig. 1D, the addition of tetrathionate to the incubation mixture did indeed facilitate the complex formation; in less than 15 min the complete conversion from renin to high molecular weight renin was attained.

Effect of pH and sodium chloride on the interaction

Boyd [1] has shown that almost complete dissociation of renin—RBP com-

plex occurs in the presence of 3 *M* sodium chloride. To confirm by gel chromatography this dissociative effect of sodium chloride and to determine which process in the two conversions (renin to intermediate molecular weight renin, or the intermediate form to high molecular weight renin) is most affected by the presence of sodium chloride, the hog kidney extract was incubated in 4 *M* sodium chloride at 37°C for 30 min and analyzed by HPLC. Most of the renin after the incubation was converted to intermediate molecular weight renin but not to the high molecular weight form (Fig. 1E). Similarly, high molecular weight renin was dissociated to the intermediate molecular weight form in the presence of 4 *M* sodium chloride at 37°C but no further dissociation occurred.

Fig. 2 shows the effect of pH on the stability of the renin—RBP complex. At pH values below 4, both high molecular weight and intermediate molecular weight renins were quite unstable and converted to renin. About 20% of these higher molecular weight forms of renin escaped the conversion during 2-min acidification at pH 3; however, 15-min acidification of the high molecular weight renin resulted in its complete conversion to normal renin.

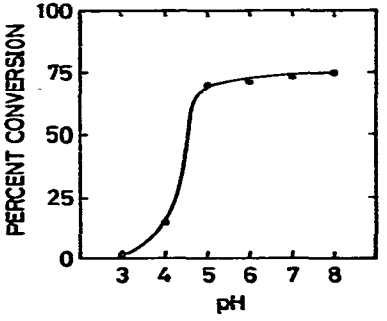

Fig. 2. Effect of pH on the interaction between renin and RBP. The percentage values indicate the proportion of the renin—RBP complex (high molecular weight renin) in the total renin activity.

DISCUSSION

Introduction of the gel TSK G3000SW in HPLC has made it possible to separate proteins according to their size and shape within 40 min [9]. We examined possible applications of this gel to studies on interconversions among various forms of renin. As a result of the interaction with renin-binding protein, active renin has been shown to exist in three forms: renin, and intermediate molecular weight and high molecular weight renins [8]. Although these three forms were distinguished by conventional gel filtration on Sephadex G-100 or Ultrogel AcA 44, the separation was unsatisfactory. The newly developed SW-type gel, however, gave satisfactory resolution. In addition, it drastically reduced elution time, opening up exciting possibilities for elucidating, in more detail, the mechanism and factors which contribute to the formation of the renin—RBP complex. We have shown that at 37°C the interaction can occur spontaneously at a reasonable speed even in the absence of sulfhydryl reagents. This fact, together with our previous observation [10, 11] that the binding capacity of RBP is specific for renin, strongly suggests that RBP may play an important role in

blood pressure regulation. These conclusions are in substantial agreement with those reached by Boyd [1] who used different approaches to study the protein-bound form of renin. Also, our previous finding [8] of the presence of an intermediate molecular weight form of renin, was confirmed using the newly developed gel, TSK-Gel G3000SW.

The present series of experiments has further established the experimental conditions under which the intermediate molecular weight renin is a major form among other interconvertible forms of renin; an intermediate molecular form becomes dominant at low temperatures or at high sodium chloride concentrations, suggesting that the conversion from the intermediate molecular weight renin to the high molecular weight form is a temperature- and ionic strength-sensitive process.

Attempts to demonstrate the presence of the three forms of renin by a single run have so far been unsuccessful. This is mainly due to the nature of the equilibria among them. At 37°C or in the presence of sulfhydryl reagents, the equilibria are shifted entirely to the higher molecular weight forms of renin, making the detection of low molecular weight renin difficult; in the kidney extracts freshly prepared at 4°C or in the samples acidified or treated with sodium chloride, the presence of low molecular weight renin can be demonstrated, but one or both of the higher molecular weight forms of renin are unstable under these conditions. Further studies are required to clarify the nature of equilibria and the mechanism of the interconversions among the various forms of renin.

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