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PHYSICOCHEMICAL PROPERTIES OF NON-ACTIVATED AND ACTIVATED RENIN FROM HUMAN AMNIOTIC FLUID

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Summary

The main physicochemical and enzymic properties of non-activated and activated human amniotic renin (EC 3.4.99.19) were studied in order to clarify the relationships between the two enzymes. Human amniotic renin was activated by dialysis against acidic buffer (pH 3.3), direct acidification or trypsin treatment. All procedures produced similar activation. The physicochemical characteristics of non-activated and activated renin were compared to those of human renal renin. Non-activated renin had a molecular weight of 45 500. A similar molecular weight was obtained by gel eluate activation and by acid treatment of renin prior to gel filtration. Similar isoelectric points were also found for non-activated and activated renin. One major renin peak focused at pH 6.6, whereas no similar renin peak was detected in extracts from normal human kidney. In addition, non-activated and activated renin forms were found to have the same optimal pH, the same K_m and the same inhibiting pepstatin concentrations.

Introduction

Several recent studies report the presence of an activatable form of renin (EC 3.4.99.19) in biological fluids such as amniotic fluid [1—4], in plasma [3—15] and in kidneys [17—21]. The most common finding is that this inactive renin (called elsewhere non-activated renin, prorenin or reninogen) is transformed into active renin by exposure to cold, acidic pH or treatment with proteolytic enzymes such as trypsin, pepsin or cathepsin D [16]. However, the biochemical

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characteristics of inactive renin differ according to the authors or material studied. In several reports inactive renin has a larger molecular weight than renin itself and for this reason has been called 'big' renin [5,18]. However, other studies reported a similar or identical molecular size for both inactive and active renin [4,14].

Lumbers [1] and Morris and Lumbers [2] were the first to demonstrate that acidification of human amniotic fluid greatly increases its renin concentration. Since in this fluid the percentage of activatable renin was extremely high and the amount of angiotensinogen very low, it seemed an appropriate model for the study of the main physicochemical and enzymic characteristics of inactive and active renin. Another advantage of this source of material in comparison to the kidney was that no renin extraction was needed, since in some cases extraction procedures activate renin [22]. Moreover it was of interest to investigate the biochemical mechanism involved in converting inactive into active renin.

Materials and Methods

Amniotic fluid was collected from normal pregnant women undergoing voluntary abortion at the beginning of the second trimester and by amniocentesis at the third trimester. Blood contamination was avoided and the amniotic fluids were immediately centrifuged at 4° C for 20 min at 1000 × *g* and frozen at −30° C. All amniotic fluid samples were pooled. For determination of molecular weight and isoelectric points, the amniotic pool was concentrated by 3 M $(NH_4)_2SO_4$ precipitation: the precipitate was dissolved in 0.1 M, phosphate buffer pH 7.0 and dialyzed against the same buffer. This procedure did not alter the renin concentration. Human renal renin was partially purified by acidification, $(NH_4)_2SO_4$ precipitation and DEAE-cellulose chromatography according to Peart et al. [23]. Standardization of the renin assay was obtained by standard human renal renin kindly supplied by the Medical Research Council (Holly Hill, London). Hog substrate was partially purified from the plasma of binephrectomized hogs according to Skeggs et al. [24]. This pool was devoid of any detectable renin activity and contained 40 to 60 pmol angiotensinogen per mg protein.

Bovine serum albumin, α-chymotrypsinogen-A, trypsin and soybean trypsin inhibitor were obtained from Sigma. Ovalbumin was purchased from Miles. Protease inhibitors were purchased from the following: ethylene-diaminetetraacetic acid, phenylmethane-sulfonyl-fluoride, sodium tetra-thionate and diisopropylfluoro phosphate (Merck); D-tryptophan methylester, 2,3-dimercaptopropanol and 8-hydroxyquinoleine (Sigma); benzamidine (ICN). Pepstatin was a generous gift from Dr. Umezawa and Dr. Aoyagi.

Renin activation

Samples were activated by three different procedures: (1) dialysis against 0.2 M glycine/HCl buffer, pH 3.3, for 20 h at 4° C according to Morris and Lumbers [2]. Samples were then brought back to pH 7.5 by further dialysis against 0.2 M phosphate buffer for 20 h. (2) Direct acidification by adjusting the amniotic fluid to pH 3.0 with 0.5 N HCl. After 20 h activation at 4° C, 5 μl of the acidified amniotic fluid was added to 0.5 ml incubation mixture for

renin concentration measurement. We verified that the pH of the incubation mixture (pH 7.5) did not change after addition of acidified amniotic fluid. (3) Trypsin treatment according to the method of Day and Luetscher [7], slightly modified as follows: 0.5 ml samples were incubated with 100 μg trypsin at pH 7.5 for 5 min at 37° C. The reaction was stopped by adding 200 μg soybean trypsin inhibitor. In the absence of renin, trypsin and soybean trypsin inhibitor did not exhibit any detectable renin-like activity on hog substrate. In each case, the renin concentration was measured by radioimmunoassay of angiotensin I as described below.

Protease inhibitors ($2 \cdot 10^{-3}$ M) were added to the amniotic fluid pool before activation by direct acidification for 20 h at 4° C. Each experiment was run in triplicate. We verified that none of these inhibitors, when added singly to the renin mixture, modified the renin reaction.

Renin assay. Renin samples (10—25 μGoldblatt Units) were incubated with an excess of hog renin substrate (250 pmol/incubation) for 30 min, pH 7.5 at 37° C. The angiotensin generated was measured by radioimmunoassay as already described [25]. The standard curve ranged from 5 to 80 pg of angiotensin I. The results were converted into milliGoldblatt Units (mG.U.), using human renin standard. 1 mG.U. of renin generated 9.9 ± 0.8 ng (mean ± S.E.M.) of angiotensin I under the conditions of the assay.

Gel filtration. Concentrated amniotic fluid corresponding to 50 mG.U. was applied to a 2.5 × 92 cm column of Sepharose-acrylamide gel (Ultrogel AcA 44) at 4° C. Upward elution was developed in 0.1 M phosphate buffer, pH 7.0 flow rate, 0.3 ml/min. In each experiment, 20 mg bovine serum albumin, 30 mg ovalbumin and 15 mg chymotrypsinogen-A were chromatographed as standard proteins, for detection of minor changes in molecular weight. 2.15 ml fractions were collected for absorbance measurement at 280 nm and for determination of non-activated and activated renin. Activated renin was also applied on the column.

Isoelectric focusing. 50 mG.U. concentrated amniotic fluid was submitted to isoelectric focusing in a pH gradient of 4-6 on an LKB column, model 8101. 700 V were applied for 48 h at 4° C and 2.0 ml fractions were collected for pH, activated and non-activated renin determinations. In another experiment, renin was activated by acidification before the isoelectric focusing.

Optimal pH. Incubations of non-activated and acid-activated renin were performed between pH 4 and 9.5, using 0.2 M acetate buffer for pH below 5.5, 0.2 M phosphate buffer for pH 5.5—9.0 and 0.2 M Tris-HCl when pH exceeded 9.0. No activation of non-activated renin or denaturation of hog renin substrate occurred during the reaction.

Michaelis constant determination. The Michaelis constant of non-activated and acid-activated renin was studied at pH 7.5 by incubating renin with different amounts of hog substrate (62.5—500 pmol).

Results were plotted according to Lineweaver-Burk. The least square method was used to calculate the regression coefficient and regression line slope.

Inhibition by pepstatin. 10 μG.U. of non-activated and acid-activated renin were incubated with 10^{-7} to 10^{-4} M pepstatin and the amount of angiotensin I generated was determined.

Results

Renin activation

In preliminary experiments time-course studies of renin activation by acid dialysis or direct acidification were performed on the amniotic fluid. Maximum activation was obtained at 20 h for both procedures, and the renin concentration remained constant up to 72 h. In subsequent experiments, 20 h acidification was therefore selected.

Direct acidification of amniotic fluid at pH 3.0 was as effective in activating renin as dialysis against acidic buffers. Moreover the two acidic methods and the trypsin activation procedure produced similar results: direct acidification, 11.4 ± 1.2 mG.U./ml; dialysis acidification, 11.5 ± 1.6; trypsin treatment, 11.6 ± 0.4; before treatment, 3.8 ± 0.8 mG.U./ml. Since an enzymic process might be involved in renin activation, several protease inhibitors were tested for their ability to prevent this activation. Ethylenediaminetetraacetic acid, phenylmethanylsulfonylfluoride, sodium tetrathionate, diisopropylfluorophosphate, D-tryptophan methylester, 2,3-dimercaptopropanol and 8-hydroxyquinoleine. All these protease inhibitors failed to prevent renin activation in our preliminary results: identical renin activation was obtained in absence or in presence of all the inhibitors tested.

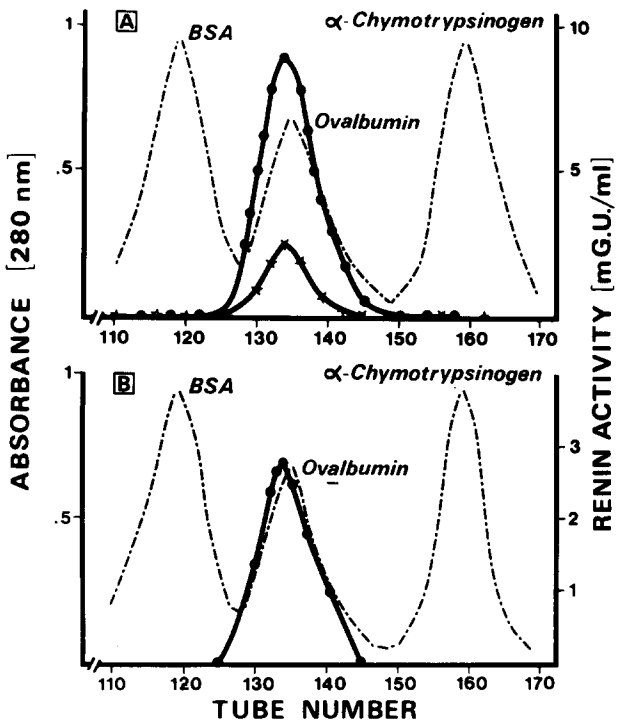

Fig. 1. Agarose-acrylamide gel filtration of renin. (A) 1 ml concentrated amniotic fluid was chromatographed on a 2.5 X 92 cm column. Renin concentration was detected directly in the eluates (★———★) and in eluates activated by dialysis against pH 3.3 buffer (●———●). The same pattern (not shown) was found when trypsin instead of acidic dialysis was used for activation. (B) acidification before application. BSA, bovine serum albumin.

Physicochemical characterization of non-activated and activated renin

Chromatography on Sepharose-acrylamide gel of non-acidified amniotic renin showed a single peak. From 3 different experiments, an apparent molecular weight of 45 000 ± 500 was found (Fig. 1a). Gel eluates were activated either by acid-dialysis acidification or trypsin. Both procedures clearly increased the renin concentration. The size and the height of the activated renin peak was similar in the two methods. The maximum renin activity after activation of the eluates was always found in the same fraction as before activation, and renin was never detected in fractions devoid of renin activity before activation. In another series of experiments, amniotic renin was activated by acidification prior to its application to the gel. A molecular weight of 43 000 ± 900 (n = 3) was found (Fig. 1b). The molecular weight of semi-purified human renin was 40 200 ± 200 (n = 3). Fig. 2a shows the amniotic renin pattern on isoelectric focusing. Non-acidified renin focused in 5 different peaks, between pH 5.1 and 6.6. Activation of each fraction by dialysis acidification showed an increase in renin concentration in every peak except in the one focusing at pH 5.4. The same pattern was found in another individual experiment. In a different study, (Fig. 2b) amniotic renin was activated before isoelectric focusing

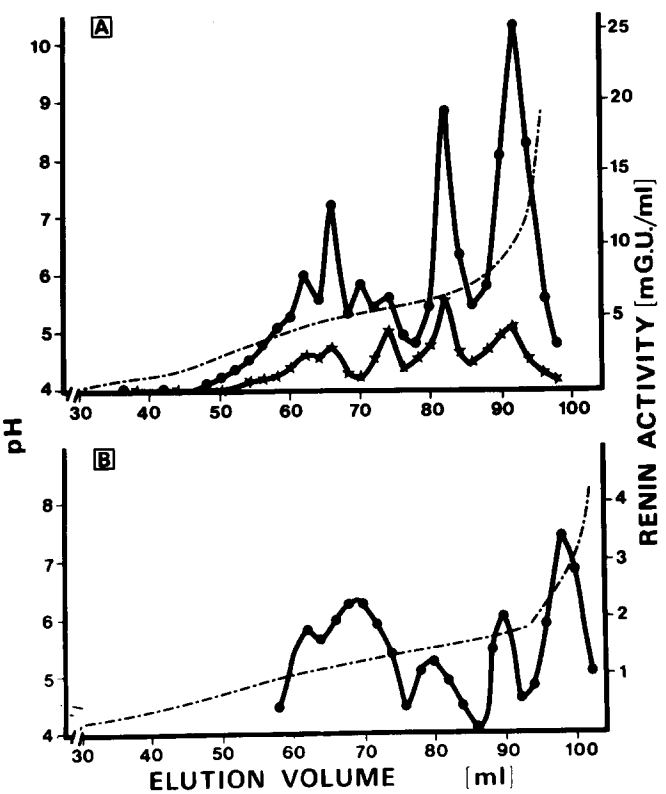

Fig. 2. Isoelectric focusing of renin. 1 ml concentrated amniotic fluid was submitted to isoelectric focusing in a pH gradient between 4 to 6 (· — · — ·). (A) Renin concentration was detected in the eluates before activation (★———★) and after activation by dialysis acidification (●———●). (B) Isoelectric pattern for renin activated before focusing.

TABLE I

PHYSICOCHEMICAL PARAMETERS OF AMNIOTIC AND RENAL RENIN

Renin source		Physicochemical parameters	
		Molecular weight	Isoelectric point
Amniotic fluid	Non-activated renin	45 500 ± 500	5.1 5.2 5.4 5.6 6.6
	Activation of gel eluates	45 500 ± 500	5.1 5.2 5.3 5.4 5.6 6.6
	Activated renin	43 800 ± 200	5.1 5.25 5.5 5.7 6.5
Kidney	Acid-treated renin	40 200 ± 200	4.9 5.2 5.3 5.5

and focused at the same pH values as the non-activated renin. Isoelectric focusing of human renal renin showed two main peaks with isoelectric points (p*I*) of 5.15 and 5.35 and two minor peaks (p*I*: 4.95, 5.50). Contrary to amniotic renin, kidney renin did not exhibit any peak at values more alkaline than pH 5.50. Table I gives the main physicochemical characteristics of non-activated and activated amniotic renin, and of renal renin.

Enzymic studies

Both non-activated and acid-activated renin exhibited similar optimum pH curves (Fig. 3). Lineweaver-Burk representations for non-activated and activated renin are given in Fig. 4. From 3 separate experiments using 3 different renin concentrations, the Michaelis constants calculated $3.1 \pm 0.3 \cdot 10^{-7}$ M

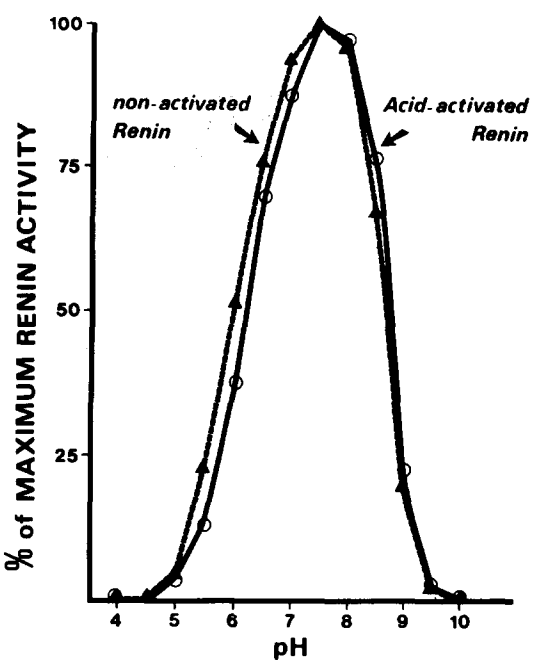

Fig. 3. Optimum pH renin curve on hog angiotensinogen. Optimum pH was determined in non-activated (▲———▲) and in acid-activated renin (○———○).

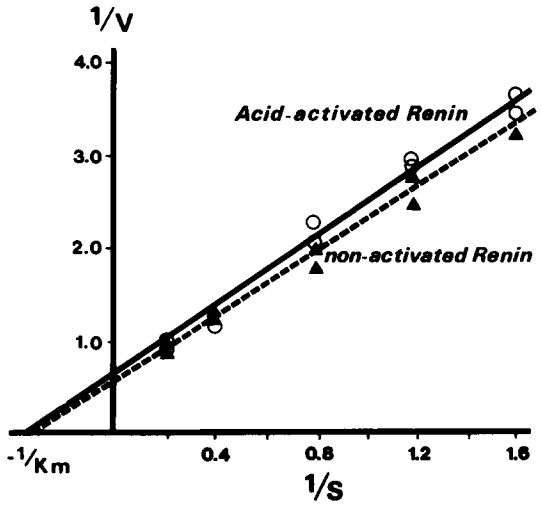

Fig. 4. Determination of Michaelis constant for renin with hog angiotensinogen. The same amount (0.18 μG.U./incubation) of non-activated renin (▲———▲) and acid-activated renin (○———○) was incubated at pH 7.5 with different amounts of hog substrate. Ordinate: reciprocal plot of angiotensinogen I generated during incubation. Abscissa: reciprocal plot of angiotensinogen concentration.

and $2.9 \pm 0.3 \cdot 10^{-7}$ M for non-activated and acid-activated renin respectively. Finally, the same pepstatin inhibition curve was obtained for the two different renins. 50% inhibition at pH 7.5 was recorded at pepstatin concentrations of 3.0 and $3.1 \cdot 10^{-5}$ M for non-activated and acid-activated renin, respectively.

Discussion

The main physicochemical properties of human amniotic renin were studied in an attempt to elucidate the relations between active and inactive renin and possibly the nature of renin activation. When human amniotic renin was activated by dialysis against acidic buffer, direct acidification or trypsin, the increase in renin concentration was identical suggesting that the same mechanism was operating and that conversion might be maximal. This agrees with the results of Shulkes et al. [4] who showed that pepsin caused no further activation of amniotic fluid pretreated with acid. The direct acidification procedure allowed experiments on small amounts of material and had the advantage of simplicity over dialysis against acidic buffer. The present study shows the important similarities between the physicochemical properties of activated and non-activated renin. Molecular gel sieving using internal standards in three separate experiments showed a slight difference of almost 2000 daltons between non-activated and activated renin, but this difference was too small to be significant. Moreover, molecular weight determinations by gel filtration must be cautiously interpreted since both hog [26,27] and human renin [28] are known to be glycoproteins which can behave abnormally on molecular gel sieving. No 'big' renin with a molecular weight of 60 000 [29—30] or 'big big' renin with a molecular weight higher than 150 000 [31] were found, either in the present study or in that of Shulkes et al. [4]. It therefore seems likely that

non-activated and activated renin molecules are present in the liquor amnii and have similar molecular weights, ±5000, which is the sensitivity limit for gel filtration techniques. A similar molecular weight (45 000) for active and inactive renin forms was also found in plasma by Boyd [14] and Shulkes et al. [4]. These data contrast with those of Day et al. [6] who found a molecular weight of 63 000 for renin in plasma and normal amniotic fluid. Renin activation in kidney samples is not always accompanied by a change in molecular weight, and renal 'big' renin might be a protein-bound form of renin [17] related to the stored form of renin in kidney. In our experiments, isoelectric focusing showed that human amniotic renin was resolved into 5 or 6 different peaks. No obvious difference between the isoelectric focusing pattern of activated and non-activated renin was found. The same isoelectric points were obtained whether activation occurred before or after focusing. The absence of difference in isoelectric points found by the present technique cannot exclude a small difference in the charges of the molecules at neutral pH, since both Shulkes et al. [4] in amniotic plasma, and Boyd in human plasma [14] found that inactive renin had a lower negative charge than active renin at pH 7.5. An interesting difference between the isoelectric points of plasma and amniotic fluid should be noted: both enzymes focused in several peaks, but one major amniotic renin peak focused at pH 6.6. No similar renin peak was found in normal human kidney. The enzymic data obtained with non-activated and activated renin respectively argue in favor of similarity between the original amniotic enzyme and the activated enzyme. In addition, we obtained approximately the same optimum pH curve and the same K_m on hog angiotensinogen for both types of renin and a similar pepstatin concentration producing 50% inhibition. Although the main physicochemical properties of activated and non-activated human amniotic renin now seem well-established, the activation mechanism is not yet clear.

Three main types of mechanism may be envisaged: (1) renin secretion in the form of zymogen, which is then converted into an active enzyme by acidification or partial proteolysis; (2) renin activation by an intramolecular mechanism (autolysis) at acidic pH which can be mimicked by partial proteolysis, and (3) removal or destruction of a renin-bound inhibitor, by acidification or proteolysis.

As regards the first possibility, the presence of an activating enzyme is still hypothetical, since, contrary to the results obtained with kidney renin [30], we were unable to inhibit activation with the protease inhibitors tested. On the other hand, it has been proved that exogenous proteolytic enzymes can produce activation: thus, in the present study, trypsin activated renin at neutral pH, and Morris [16] activated it at acidic pH, using pepsin and cathepsin D. These results therefore support the existence of an activating enzyme. If such an enzyme is present in amniotic fluid, its molecular size and isoelectric point must be similar to those of renin, since renin activation was obtained both in gel eluates and in isoeletric focusing column eluates.

In connection with the second possible mechanism listed above, autocatalytic renin activation at acidic pH cannot be excluded. However, [28] no activation or autocatalysis was detected in highly purified tumor renin extracted without acidification [28].

As far as the third possibility is concerned, any renin inhibitor present in amniotic fluid must have a small molecular weight otherwise it would affect the elution pattern of renin during gel filtration. Furthermore, it must be irreversibly destroyed at acidic pH, since renin activation is obtainable by direct acidification, without dialysis of a low molecular weight inhibitor.

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