Analysis of inactive renin by renin profragment monoclonal antibodies

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Two peptides were synthesized, corresponding to the sequences (−19 to −7) and (−26 to −17) of the prorenin prosegment. Monoclonal antibodies were raised to these sequences and used to characterize human plasma inactive renin. Only anti (−19 to −7) reacted with inactive renin, as measured by direct assay or affinity chromatography. The data were used to evaluate two possible inactive renin stuctures: (i) plasma inactive renin is a truncated prorenin lacking the prosegment N-terminal portion; (ii) its spatial conformation masks the N-terminal extremity, preventing interaction of this region with specific antibodies.

(Human) Renin Preprorenin Prorenin Monoclonal antibody Synthetic peptide Region specific

1. INTRODUCTION

Renin (EC 3.4.23.15) is a key enzyme in the regulation of blood pressure and electrolyte metabolism. This enzyme is synthesized in the juxtaglomerular cells of the kidney as an inactive biosynthetic precursor: preprorenin, prorenin and renin are then secreted [1–3]. Two forms of human renin exist in plasma, kidney and chorionic cells: an inactive form and an active form [1,2]. Inactive renin can be activated by limited proteolysis or acidification [1,2].

The amino acid sequence of the plasma inactive renin is unknown. But, much information has been provided by the primary structure of human preprorenin determined by cloning and sequencing of the cDNA and gene [4,5]. Recently, Hirose et al. [6] demonstrated that the precursor of human renin contains a 23 residue signal peptide and a prosegment of 43 residues (fig.1). An immunologic similarity between inactive renin and prorenin has been determined by Bouhnik et al. [7] and Kim et al. [8] using polyclonal antibodies directed against synthetic peptides having the sequence of the prosegment of the renin.

With a view to determine the structure of inactive human plasma renin and the length of the prosegment, two overlapping peptides have been synthesized. In this approach the analysis of the chorionic or plasma inactive renin has been executed by direct assay of this enzyme using region specific monoclonal antibodies raised against the two synthetic peptides.

Abbreviations: GU, Goldblatt Unit; RIMA, radioimmunometric assay; PRA, plasma renin activity; EIMA, enzymoimmunometric assay

2. MATERIALS AND METHODS

2.1. *Human renin sources*

2.1.1. Chorionic inactive renin

Inactive renin released into the medium of cultured human chorionic cells was used [9]. The concentration of active renin was 6 mGU and of inactive renin was 40 mGU/ml medium.

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2.1.2. Plasma renin

Human blood was collected at 4°C on heparin, centrifuged and the plasma stored at −20°C. A number of plasma samples were pooled before use.

2.2. *Renin monoclonal antibodies*

The two human antirenin monoclonal antibodies used (3E8 and 4G1) have been described [10]. Antibody 3E8 recognizes prorenin and renin while 4G1 is specific for active renin.

2.3. *Preparation of F(ab)'2 antibodies*

Samples of antibody 3E8 were dialysed against sodium formate buffer, pH 2.8, followed by a dialysis against sodium acetate buffer, pH 4.2 [11]. The F(ab)'2 fragments were then prepared by digestion with pepsin (1 mg/ml) for 5 h at 37°C.

2.4. *Immunogen preparation*

Hirose et al. [6] have determined that the prosegment contains 43 residues by sequential Edman degradation of ^{35}S Met-radiolabelled human prorenin. These 43 residues are numbered negatively from the activation site (−43 to −1) in the present study.

The peptide (−26 to −17) was synthesized by the same method used to synthesize peptide (−19 to −7) [6]. An extra tyrosyl residue was added at the N-terminus to permit iodination. The two peptides are called: (−26 to −17) and (−19 to −7). Pure peptides as established by amino acid analysis were obtained by preparative HPLC. Each peptide was coupled to bovine serum albumin using glutaraldehyde [12]. The coupling efficiency was 65% (2 mol peptide/mol BSA) as evaluated after dialysis by adding ^{125}I (peptide) to the reaction mixture.

2.5. *Immunization, cellular fusion, screening and cloning*

(i) High responder Biozzi mice were immunized by subcutaneous injection with a mixture containing 10 μg of each peptide [14]. After three injections, selected mice received 5 μg (−26 to −17) and 5 μg (−19 to −7) intravenously.

(ii) Spleen cells were fused with the myeloma cell line P3-x 63-Ag8-653 according to Di Pauli [14] modified by Galen et al. [10].

(iii) Antibodies to the peptides were detected in mouse serum, culture medium, ascites and purified IgG fractions by RIA. The binding to iodinated peptides was determined after separation of bound and free peptide using polyethylene glycol [15].

(iv) Antibody producing hybridomas were cloned by limiting dilution.

2.6. *Mass production and purification*

Ascites were produced by intraperitoneal injection of cloned hybridoma cells into pristane treated Balb/C mice. Monoclonal antibodies were purified by affinity chromatography on protein A-Sepharose [16].

2.7. *EIMA of inactive renin*

The different monoclonal antibodies were tested by EIMA for inactive renin in chorionic cells [9] and plasma (fig.2). This assay measures inactive renin in a 'sandwich' between two monoclonal antibodies. The F(ab)'2 fragments of the first monoclonal antibody (3E8) [10] (anti total renin monoclonal antibody) were coated on polyvinyl microtitration plates (0.1 mg/ml) in 10 mM phosphate buffer (pH 7.4) for 18 h at 4°C. Medium from cultured chorionic cells or plasma was then added in 10 mM phosphate buffer containing 1% bovine gamma globulin (BGG) and 0.1% Tween 20, and incubated for 2 h at 4°C. The different anti-peptide monoclonal antibodies were then added and incubated for 18 h at 4°C. The wells were washed in phosphate buffered saline (PBS)-Tween 20 and inactive renin identification by the second monoclonal antibody was determined by adding mouse IgG anti Fc peroxidase antibodies (Jackson Immunoresearch Laboratories).

Controls were performed by replacing the monoclonal antipeptide antibodies by the monoclonal active renin antibody 4G1, and incubating, as above with inactive renin activated by acidification to pH 3.3 for 18 h at 4°C.

2.8. *Analytical affinity chromatography*

Two monoclonal antibodies were purified for this experiment: the (−19 to −7) antibody and the (−26 to −17) antibody. The antibodies were coupled to cyanogen bromide activated Sepharose 4B [17] using 5 mg IgG/ml moist gel. 90% of the IgG were effectively coupled to the gel. Each coupled antibody was then incubated with the pooled human renin-rich plasma in a ratio of 3:1 (3 ml plasma/ml gel) for 18 h at 4°C with shaking.

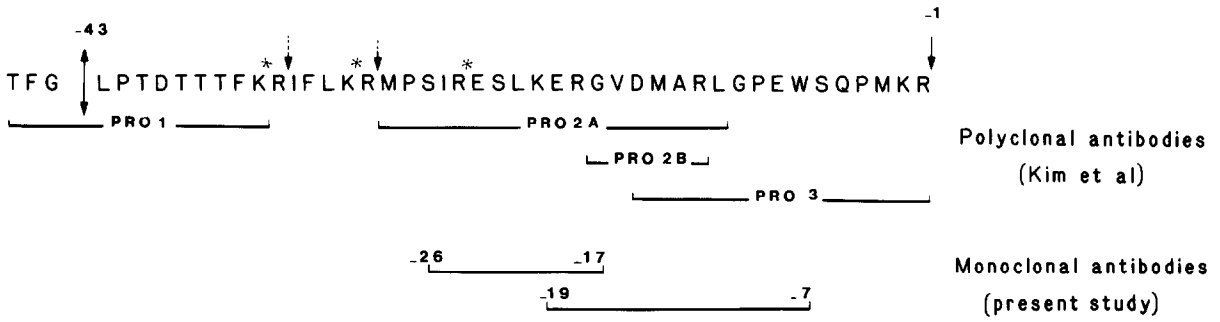

Fig.1. Primary structure of the renin profragment. (⟵⟶) Cleavage of preprorenin/prorenin suggested by Hirose et al. [6]; (⟶) cleavage of prorenin/renin according to the renin model; (- - -→) potential cleavage (dibasic pair); (*) AA which potentially react with the molecule of renin (according to the pepsinogen model).

The Sepharose was then packed into a plugged Pasteur pipette and the supernatant plasma was collected and retained for renin assay. Renin was measured by both its enzymatic activity (PRA) [18] and RIMA ([10] and Simon et al., in preparation) before and after activation by different concentrations of trypsin (0–1 mg/ml).

3. RESULTS

3.1. *Production of monoclonal antibodies*

After fusion, 11 hybridomas directed against the two synthetic renin prosegment peptides were obtained. 10 of them produced monoclonal antibodies directed against the (−26 to −17) peptide whereas a single hybridoma produced an antibody against the (−19 to −7) peptide. These results are in accordance with the humoral immunity of the mouse. Indeed on the fusion day, the titer of the immune serum was 1/200 for (−19 to −7) and 1/3200 for (−26 to −17). This difference in immunity response was probably due to an antigenic competition phenomenon. The two monoclonal antibodies used for affinity chromatography had association constants (K_a) measured by Scatchard analysis [15] of 3.9×10^8 and 4×10^9 M^{-1} for the (−19 to −7) and (−26 to −17) antibody, respectively. Isotypes were determined by immunoelectrophoresis with anti subclass mouse antibodies. The two antibodies were IgGs, the (−19 to −7) antibody was an IgG2a and the (−26 to −17) antibody was an IgG1.

3.2. *Immunoenzymometric assay*

The ability of the 11 monoclonal antibodies to recognize inactive renin was tested on a pool of human plasma and on the medium of cultured chorionic cells (fig.2). None of the 10 antibodies directed against the peptide (−26 to −17) recognized inactive renin, since an enzymatic signal equal to the background was observed. We reversibly activated the inactive renin by lowering the pH in order to try to alter the conformation of

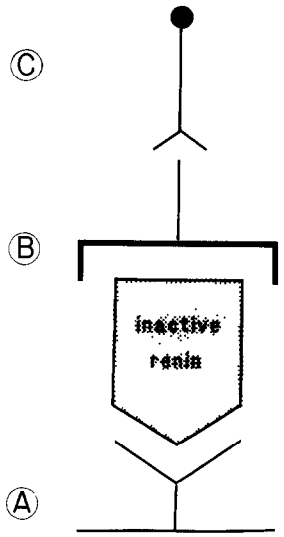

Fig.2. Immunoenzymometric assay. This assay measures inactive renin in a sandwich between two monoclonal antibodies. (A) The F(ab)'2 fragment of an anti total renin monoclonal antibody (3E8) were coated on microtitration plates. (B) The (−19 to −7) monoclonal antibody was then incubated. (C) The identification of inactive renin was determined by mouse IgG anti Fc peroxidase antibodies.

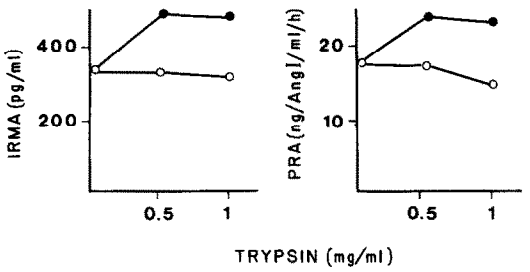

Fig.3. Analytical affinity chromatography. The monoclonal antibodies: (○) the (−19 to −7) antibody and (●) the (−26 to −17) antibody were coupled to cyanogen bromide activated Sepharose 4B. A pool of human renin rich plasma was then added for 18 h at 4°C. The filtrate from each column was analyzed by (A) IRMA and (B) PRA before and after activation by different concentrations of trypsin.

the prosegment and to expose the sequence (−26 to −17) at the surface of the molecule. No binding was observed. Only the (−19 to −7) monoclonal antibody directed against the C-terminal part of the prosegment specifically recognized the inactive renin. This inactive renin was not recognized by this antibody after trypsin activation, but was recognized after activation by acidification.

3.3. *Analytical affinity chromatography*

The results of analytical affinity chromatography of human plasma on (−19 to −7) antibody-gel and (−26 to −17) antibody-gel are shown in fig.3A and B. The filtrate of the 4C7-gel contained inactive renin which was detectable after activation by trypsin: the value for active renin by RIMA was 340 pg/ml before activation and 514 pg/ml after activation of inactive renin. In contrast, 340 pg/ml of inactive renin were detected in the filtrate of the (−19 to −7) antibody-gel, both before and after activation by trypsin. Thus the inactive renin was bound to the column. These results were confirmed by assay of PRA in the two filtrates (fig.3B).

4. DISCUSSION

Region specific monoclonal antibodies raised against synthetic peptides having the sequence of the prosegment of human renin can be useful tools for establishing the structure of inactive renin. In the present study, 11 such antibodies were prepared and used to analyze the structure of plasma inactive renin before and after activation. A single antibody recognized the (−19 to −7) peptide, the remaining 10 reacted with the (−26 to −17) peptide. The (−19 to −7) peptide corresponds to the C-terminal region of the prosegment while the (−26 to −17) peptide corresponds to the middle region (see fig.1).

The ability of these monoclonal antibodies to recognize inactive renin was evaluated by RIMA and analytical immunoaffinity chromatography. Only the anti (−19 to −7) peptide antibody reacted with inactive renin. Hirose et al. [6] obtained similar results using polyclonal antibodies to prosegment peptides. They found that a single antibody (antipro 3), directed against the (−15 to −1) peptide, identified inactive renin. This strongly suggests that the prosegment of human renin contains at least the (−19 to −1) sequence.

These data may be used as the basis of at least two hypotheses for the activation of the renin precursor. The first possibility, as suggested by Hirose et al. [6], is that plasma inactive renin lacks a large portion of the NH_2-terminal part of the profragment and may represent a proteolyzed activation intermediate. The second hypothesis is that the antibodies directed against the center part of profragment (−26 to −17) cannot recognize inactive renin because this part of the molecule is buried on the inside of renin or because it represents a discontinuous epitope.

These two hypotheses can be discussed in the light of the pepsinogen-pepsin model. There is a high degree of homology between the profragments of renin and pepsin, at the NH_2-terminal region where most of the basic amino acids are conserved in similar position. The three dimensional structure of prorenin is not known and no molecular modeling has yet been reported. However, the three dimensional structure of pepsinogen has been recently described by James and Sielecki [19]. The 44 residues of pepsinogen are numbered between Leu-44 and Leu-1. After a large N-terminal extension formed by the first 8 residues, Leu-44–Arg-36, three short helical stretches are seen: Ser-33–Asp-25, Lys-23–Thr-16 (these sequences are in an approximately orthogonal position to each other and contain hydrophobic residues) and Pro-11–Tyr-7. The chain from His-15 to Asn-12 has an extended con-

formation. From Pro-6 in the profragment to Asn-8 in pepsin, the polypeptide chain forms a large loop into the center of which fits the tip of the flap. The peptide bond between Leu-1 and Ile-1 is exposed at the surface of the molecule. The conformation of pepsinogen is stabilized at neutral pH by electrostatic binding forces. At acidic pH, a conformational change occurs due to the disruption of the electrostatic forces by protonation of the carboxylate groups. In the case of pepsinogen, an intramolecular activation occurs leading to an activation intermediate.

By analogy, it is tempting to speculate a similar conformation for prorenin. Indeed, renin is reversibly activated by exposure to acidic pH and it is likely that acidification provokes a disruption of electrostatic forces between basic amino acids of the profragment and renin itself. The irreversible activation of renin might occur after proteolytic cleavage after one of the two dibasic pairs (Lys-35–Arg-34, Lys-30–Arg-29).

The inability of (−26 to −17) monoclonal antibody to recognize plasma inactive renin activated by acidification is consistent with (i) plasma inactive renin having lost residues −43 to −20 of the renin profragment or (ii) a spatial structure of the synthetic peptide in inactive renin completely different from its original structure in solution (where both peptides were recognized by monoclonal antibodies). Clearly this point merits further investigations. X-ray crystallographic studies and the isolation and sequencing of the plasma renin prosegment would help to establish the validity of one of these hypotheses. It would then be possible to decide whether inactive renin is a proteolyzed activation intermediate or whether its spatial conformation masks its N-terminal extremity which would result in the absence of recognition by specific N-terminal antibodies.

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