Brief Review

Purification of Renin and Prorenin
Tadashi Inagami

Introduction

In this issue of the journal, we feature two articles—one by Tadashi Inagami and the other by Pierre Corvol and Joel Menard—relating the story of the purification of renin. These investigators received the 1985 CIBA Award for Hypertension Research for biochemical studies leading to the isolation, amino acid structural and three-dimensional configuration, and the physiochemical and immunochemical characterization of renin in humans and other species. We are pleased to feature the views of two distinguished groups on this subject, and hope the readers of the journal find their reminiscences and thoughts for the future of interest.

Alyn L. Mark
Editor-in-Chief

For more than 70 years, since a “pressor substance” was discovered in kidney extract and the term “renin” was coined by Tigrstedt and Bergman in 1898, renin remained a mysterious entity until it was purified in stable forms in the 1970s. During this 70-year period, the pathophysiological importance of renin in renovascular hypertension was identified by Goldblatt et al. Identification of renin as an enzyme rather than a pressor hormone and evidence for the existence of angiotensin by Page and Helmer and Braun-Menendez et al laid the most important foundation for the future development of research on the renin-angiotensin system. This led to extensive studies on angiotensins and their formation from angiotensigen by renin and angiotensigen converting enzymes.

In the meantime, numerous attempts were made to purify and characterize this enzyme, which has been considered to play a crucial role in hypertension. In addition to documented reports of partial purification (Peart et al from hog kidneys, Haas et al from pig, and human kidneys, Skeggs et al from dog kidneys, Lucas et al from human kidneys, and Rubin from hog kidney), attempts were made by numerous investigators to isolate this enzyme without reportable results. As a protease biochemist, I personally observed my friends make the attempt and soon abandon their venture. Researchers approached this problem by thinking that a protease like renin would not be very difficult. Proteases, such as trypsin and pepsin, were among the proteins that became amenable to purification from the pancreas and stomach at very early stages of the development of protein biochemistry.

However, it is intriguing that renin did not possess the general proteolytic action. It did not seem to belong to a known type of proteases since it was refractory to known types of group-specific protease inhibitors such as diisopropyl fluorophosphate (for serine protease), metal chelators (for metalloenzymes), or the thiol-specific reagents p-chloromercuribenzoate (for cysteine proteases). It looked as if it had very narrow substrate specificity limited to one specific leucyl peptide bond present only in the angiotensigen molecule. Such unusual properties made renin a highly tantalizing subject for a biochemist. In addition, specific enzymes catalyzing the prohormone-to-hormone peptide conversion had not been identified except for kallikrein in its kinin-generating function from kinogen.

However, biochemists who were interested in renin studies and attempted its purification had to face a set of insurmountable technical problems: its exceedingly low content in the kidney, lack of a simple biochemical assay procedure, and highly unstable nature as it approached a higher degree of purity, were but a few of the barriers. Nevertheless, new protein purification techniques were applied every time they became available.

We were blessed with a fortunate combination of favorable circumstances in approaching this difficult problem. Due to a generous Center of Excellence grant from the National Science Foundation in support of basic biomedical research at Vanderbilt University, we were able to invite numerous scientists as visiting professors from all over the world in the 1960s. The late Professor Franz Gross of Heidelberg was invited by the late Professor Grant Liddle as a visiting Professor at Vanderbilt several times.
Since Werle's group in Munich reported the presence of renin-like enzyme activity in the submaxillary gland of mice in 1963, hypertension researchers in Europe (Bing in Copenhagen\(^\text{15}\) and Gross in Heidelberg\(^\text{16}\)) were interested in this renin-like enzyme in mouse submaxillary gland. Stanley Cohen of our department had pure epidermal growth factor and nerve growth factor preparations from male mouse submandibular gland. I had the privilege to collaborate with him in the determination of the amino acid sequence of epidermal growth factor.\(^\text{17}\) We also purified kallikreins from the same gland. Franz Gross tested these protein preparations for their possible renin-like activity in nephrectomized, anesthetized, and ganglion-blocked rats in Andrew Michelakis' laboratory in Clinical Pharmacology at Vanderbilt. These preparations elicited prolonged pressor responses, which is typical of renin. However, amounts of these purified submandibular gland proteins needed to elicit the pressor responses were in microgram quantities, which were several orders of magnitude greater than what would be expected from pure renin. Thus, the activity should have been due to contamination of renin in these protein preparations. However, it also indicated that the gland contains a sizable amount of renin and that it might be feasible to purify it by a conventional column chromatographic procedure. Cohen, Michelakis, and I huddled together to work out renin purification from submandibular glands of male albino mice, even though the gland is an unusual and unexpected source of renin.

Although column chromatographic methods on ion exchange and gel filtration gels had been well developed by then, the major obstacle to this endeavor was the lack of a simple assay method for biochemical purification. Bioassay using nephrectomized, ganglion-blocked, and anesthetized rats experienced by numerous investigators should have been preventable by the addition of permanent protease inactivators such as diisopropylfluorophosphate and sodium tetrathionate. Although the fact that renin was present in male mouse submaxillary gland (renin content in the submaxillary gland was approximately 100-fold as high as in the kidney) came out in five peaks on ion exchange chromatography, but the structural basis for multiplicity is not clear even now, because it is not a glycoprotein,\(^\text{21}\) and thus is without identifiable structural element for microheterogeneity. This protein had no general protease activity even at a 1,000-fold excess of the quantity used for renin assay. We were able to produce good antibodies that were useful in localizing renin in the juxtaglomerular cells of mouse kidney by immunohistochemical methods.\(^\text{22}\)

Pure renin and antibodies permitted us to construct a direct radioimmunoassay of renin for the first time.\(^\text{23}\) By this method we learned that submaxillary gland renin is not secreted into the plasma in large measure under normal conditions in spite of its enormously high concentration in male mouse submandibular gland (renin content in the salivary gland was approximately 100-fold as high as in the kidney). These studies on mouse renin provided us with several important pieces of information and built impetus for major developments in renin research in the future. It permitted the identification of the active site of renin as discussed later, thus clarifying one of the most mysterious aspects of renin, and opened the way for the development of renin inhibitors for therapeutic and research purposes. Another finding was that the purified renin molecule is not unstable as had been thought, and the renin can be obtained in a stable preparation. As a biochemist with experience in proteases, it indicated to me that the instability of partially purified renin renin experienced by numerous investigators should have been due to contamination of other proteases and that it should be preventable by the addition of permanent protease inactivators such as diisopropyIfluorophosphate and sodium tetrathionate. Although the fact that renin was present in male mouse submaxillary gland was mysterious and its physiological role intrigued us, I decided to seize this opportunity to purify and investigate renal renin since its pathophysiological roles were well defined and demanded more immediate attention.

In view of the far lower content of renin in the kidney than in the salivary gland, we decided it was absolutely essential to use an affinity chromatographic technique, which had become a state-of-the-art technique by then. Umezawa and his group\(^\text{24}\) had reported isolation of pepstatin from culture filtrate of actinomycetes and reported it in the Journal of Antibiotics. As the circulation of this journal was limited, I learned about it indirectly in a review article written...
in Japanese. I noticed that the structure of pepstatin contains a structure similar to the critical sequence of hog renin substrate (Leu-Leu-Val). This peptide was originally screened for pepsin inhibitory activity as a potential antigastric ulcer drug. Subsequently, Aoyag et al.\(^{25}\) reported that although pepstatin inhibits renin, the inhibition of renin was several orders of magnitude weaker than the inhibition of pepsin or cathepsin D, typical aspartyl acid proteases. Thus, there was a doubt as to whether renin was an aspartyl protease; this doubt was strengthened because renin functions in a neutral pH rather than an acidic pH range like pepsin or cathepsin D. Some structural features similar to renin substrate and extensive inhibition of mouse renin at a rather high concentration of \(10^{-7}\) M provided a strong basis for considering pepstatin as an affinity ligand for affinity chromatography of renin no matter whether renin is an aspartyl protease. Other investigators also reported the inhibitory action of pepstatin on renin.\(^{26-29}\) The rather weak inhibitory activity of micromolar ID\(50\) indicated that a mild condition can be used for releasing renin from an affinity column and seemed to be ideal for an affinity ligand.

In response to our request for this inhibitor peptide, Drs. Aoyagi and Umezawa were kind enough to send us 50 mg and later 100 mg of pepstatin. Since pepstatin had a blocked amino terminal and free carboxy group, we thought it would be easy to couple it to aminoalkyl gels. However, its very low solubility in water and bulky hydrophobic residue made it almost impossible to make it react with amino alkyl groups on agarose at a good yield. Use of organic solvents such as alcohol or dimethylformamide collapsed the agarose or even Sepharose gels of the 1970s, but we found that the gels resisted dioxan.

At this point my experience obtained from dabbling with peptide synthesis was of some use. We decided to use an activated ester method in which pepstatin was converted to its \(N\)-hydroxysuccimide ester in 100% dimethylformamide and then was allowed to react with aminoalkyl Sepharose in a 1:2 (vol/vol) dimethylformamide/dioxane mixture. This two-step method seemed to alleviate much of the difficulty. Pure submandibular renin already at hand was of great use in evaluating the usefulness of affinity columns with spacer arms of various lengths. Kazuo Murakami, who joined me in 1969 as a research associate, tried various venues in this synthetic work. Although his background was in a totally unrelated field of nutrition, his effort and perseverance were valuable in producing a useful affinity gel for renin.

In 1973 the American Chemical Society had a timely symposium on affinity chromatography in Charleston, S.C. Meir Wilchek came from Israel as a plenary speaker and personally gave us a great deal of advice. At this time Israel was in the middle of the Yom Kippur War; Wilchek was a tank battalion commander and was defending his country in life-or-death battles. Nevertheless the Israeli government thought it was more important for him to contribute to science as a pioneer and world authority on affinity chromatography\(^{30}\); they called him back to Tel Aviv one day before the symposium and sent him to Charleston at a moment's notice. He knew of our attempt to prepare a pepstatin column because he was a former graduate student of Abraham Patchornick with whom I had collaborated earlier, and his advice was very valuable for our effort in the development of the affinity column.

Now the major barrier to our work was the limited supply of pepstatin. Although 150 mg from Aoyagi and Umezawa were generously supplied, we needed a lot more for the numerous trials and finally for preparing a large amount of the affinity gel. Again, good luck fell on me; a postdoctoral fellow about to come to my laboratory wrote me to ask if he could bring something from Japan with him. He might have had a Japanese food in mind. I wrote back that we needed pepstatin very badly. It turned out he had access to an unlimited quantity of pepstatin from its manufacturer. It allowed us to make numerous trials in the preparation of the affinity column and to prepare several hundred milliliters of the affinity gel required for processing liters of kidney extract.

After numerous trials with various spacers with different lengths, reasonable affinity gels were prepared.\(^{26-31}\) A similar affinity gel was prepared by Corvol et al.\(^{29}\) in Paris by an almost identical method. We and the French group both realized that there was a competition for renal renin purification. Both groups chose to purify hog renal renin because of the reason given below.

A still unexplainable thing happened. Although our affinity gel\(^{31}\) and the gel of Corvol et al.\(^{29}\) were prepared by similar but not quite identical methods and contained similar amounts of pepstatin (1.8 \(\mu\)mol/ml versus 1.6 \(\mu\)mol/ml, respectively), we could elute renin with 0.1 M acetic acid at pH 3.2, whereas elution of renin from Corvol's column required 6 M urea. In acetic acid, renin could be recovered readily by neutralization, but in 6 M urea it was at least partially denatured. Later we found an even more favorable condition for elution of renin in 0.1 M Tris-Cl buffer at pH 7.5.\(^{32}\) We extended the application of pepstatin-aminohexyl agarose to various renin studies and received wide acceptance by many investigators.

Since Haas et al.\(^{33}\) showed that renin content in hog renin had the highest amount of all species, we targeted hog kidney as the source of renal renin in our first trial. (However, later studies by my colleagues Teruyoshi Matoba\(^{34}\) and Amintas Figueiredo\(^{32}\) showed that rat kidney had a much greater renin concentration.) In view of the minute quantities of renin, we had to process a large quantity of kidney—one on the order of tens of kilograms. In my small laboratories there were no facilities to process such quantities of the kidney as there were in Haas' laboratory at Mt. Sinai Hospital in Cleveland, which was equipped with a gigantic extraction tank and a large filter press. Again, we were lucky. In 1972, Rubin\(^{35}\)
had developed a very interesting method for the selective extraction of renin from dry and defatted powder prepared from ground kidney by freeze-drying. She reported that a methoxy-ethanol/water (1:2, vol/vol) mixture was most appropriate to minimize coextraction of cathepsin D and other unwanted proteins while extracting 90% of renin activity. Since the substrate specificity of pepstatin was not specifically directed to renin, a single affinity chromatographic step was not sufficient to produce pure renin. Additional steps of conventional chromatography were necessary. At the end of 1974, chromatographically and electrophoretically pure renin was isolated. Starting with 6 kg of frozen hog kidney, it took more than a 100,000-fold purification to obtain 0.6 mg of pure hog renal renin in a stable form. By this time the radioimmunoadsays of Haber et al had become widely available, which greatly facilitated our study. The result was presented in the FASEB Meetings and the Japanese Biochemical Society Meeting in 1975. It was received with great enthusiasm by investigators in a wide range of specialty. In 1977, Corvol et al reported the purification of hog renin by elution from the pepstatin column with urea. Presumably due to the use of 6 M urea, the specific activity of their preparation was less than half of the hog renin eluted at pH 3.2 from the affinity column.

As the foregoing historical background indicates, the successful purification of renin was supported by numerous timely scientific and technical innovations that occurred in the late 1960s just before our work. These include discovery of renin-like activity in the male mouse submandibular gland, pepstatin, affinity chromatography, selective extraction methods, fluorogenic renin substrate, radioimmunoadsays, and introduction of protease inhibitors. We were blessed with much good luck. If we had started our work even 5 years earlier, the purification might have been infeasible and we might have had to abandon it. Precious help from many of the investigators such as Drs. Roth, Reinhart, Rubin, Aoyagi, and Umzewa was indispensable. Above all, however, the singleminded and concentrated effort of a very small team, myself and Kazuo Murakami, with the support of three very able technical assistants, Edward Price Jr., Abdul Qureshi, and Yuriko Murakami who were completely devoted to this work, were major factors to the success.

Once the method was established, it was applied, with variations, to rat renin in our laboratory; human kidney renin by Yokosawa in our laboratory (first reported in 1978 then a report of characterization studies in 1980), by Slater et al, Hiroki et al, and Do et al; dog kidney renin by Dzau et al; and bovine pituitary Hirose et al. In every case the pepstatin gel worked well at pH 3.2 without 6 M urea.

The late Dr. Erwin Haas' contribution was essential for our purification of human renin. At a High Blood Pressure Research Council meeting in Cleveland in 1975, Dr. Haas offered his partially purified human kidney renin preparation for our affinity chromatographic purification. We had tried to acquire autopsied human kidneys; however, the number of autopsies were small and we were getting nowhere. Thus, we were very happy to accept Dr. Haas' offer. Close to 5 g partially purified human renin derived from about 200 kidneys was provided. The preparation apparently was more crude than the standard human renin prepared by Haas, Goldblatt, and Gipson. When Yokosawa applied the solution of this preparation to the pepstatin column and eluted it with 0.1N acetic acid according to our established procedure, to our dismay, less than 1% of the original renin activity was recovered and even this activity disappeared in a few hours in the cold. It became clear that the original preparation contained an enormous amount of cathepsin D that was copurified with renin due to their similar molecular properties. Without an appropriate method for its elimination we could not make any progress. Since cathepsin D and renin are very similar in their properties, the separation could not be accomplished by a conventional method.

We had been talking with H.J. Chou in Bob Greggerman's Gerontology Laboratory at the National Institutes of Health in Baltimore. They were using a hemoglobin-Sepharose column for the purification of cathepsin D. By passage through this column, most of cathepsin D in the crude renin preparation was removed from renin fractions, although other proteases were still present. This device permitted us to proceed to the next affinity chromatographic step on the pepstatin-aminohexyl gel. By a 480,000-fold purification, pure and stable renin with a specific activity of about 830-1,050 Goldblatt units/mg was obtained as reported in 1978. This specific activity range was confirmed by other investigators and has become a gold standard for future studies including human renin produced by recombinant DNA technology.

A similar technique for the affinity chromatographic separation of renin from cathepsins turned out to be useful when Hirose et al in our laboratory used casein-Sepharose to separate a large amount of cathepsin D from specific renin in the extract of porcine brain. This work resolved the raging controversy as to whether "iso-renin" in dog brain reported by Ganten et al is renin. Day and Reid and Hackenthal et al published data indicating that it was the nonspecific action of cathepsin D. Use of the casein-Sepharose column clearly demonstrated that although the great majority of the iso-renin is due to the nonspecific action of cathepsin D, a small amount of specific renin exists in the brain. This was another very important application of affinity purification of renin. This result was supported by an independent approach by Ganten and Speck in 1978 validating Ganten's concept of brain renin.

At about the same time Dorer et al showed that pseudorenin is identical with cathepsin D. Thus, it had become clear that the renin-like activities, iso-
renin and pseudorenin, in extrarenal tissues determined at pH below 7 with partially purified angiotensinogen or tetradecapeptide renin substrates are not renin but nonspecific actions of acid protease. Frequently the nonspecific activity comprised the majority of the renin-like activity of certain tissue, specific renin activity being only very small portions, thus requiring reevaluation of “tissue renin” activity reported earlier. While these findings negated the concept that isorenin was renin, it provided a more reliable methodological approach to identify renin in various tissues. Thus, since 1978 the terms isorenin and pseudorenin lost their scientific meaning and dropped from the literature. A combination of this affinity chromatography and selective inhibition of renin by monospecific antirenin antibodies was used in establishing a methodology to distinguish specific renin from isorenin in renal and extrarenal tissues. While specific renin was present in much smaller quantities in these tissues than reported previously, it permitted us to demonstrate the presence of renin in various tissues, including neuronal, endocrine, vascular, cardiac, hepatic, digestive, and immune tissues.54,55 These studies have been developed further to the demonstration of intracellular action of renin and local generation of angiotensins in neuronal, endocrine, and vascular tissues introducing a new concept that might lead to an explanation of the etiology of essential hypertension. This concept was strengthened further as the localization of renin messenger RNA (mRNA) demonstrated in these tissues later.56-59

Another example of the combination of luck, ideas, and diligence was seen in the purification of human renin from human renin-secreting tumors by Galen et al.60 Only a 40-fold purification by simple chromatographic procedures was needed to obtain pure human renin without using an affinity column. More sophisticated purification methods were developed later using renin-specific inhibitor peptide (H-77) as affinity ligands that had been synthesized by Szelke et al.61 Human renin was purified by a single step by McIntyre et al62 and rat renin in two affinity steps by Kim et al.63 Monoclonal antibodies used as affinity ligand was also effective in simplifying the affinity chromatographic purification of renin and inactive prorenin. Dorer et al64 used it for the complete purification of hog renin and McIntyre et al65 and Higashimori et al66 used monoclonal antibody against human renin to obtain pure inactive human prorenin from the kidney and chorion, respectively.

The advent of pure and stable renins permitted several important developments in renin research. These included the production of specific antibodies, characterization of its active site, structural determination, and distinguishing specific renin and nonspecific renin-like action of proteases (as described above).

Uses of monospecific anti-renin antibodies were expected to be incalculably great; however, they did not come cheap. In contrast to mouse renin, which was available in a reasonable quantity, renal renins were available only in limited quantities making it difficult to raise antibodies to renins from hog, rat, and human kidneys. A more serious problem was that rat and hog renins were similar to rabbit renin and were not recognized readily as a non-self protein in rabbits. Talwar et al67 reported that when a human protein is conjugated to tetanus toxoid, the protein gains antigenicity in humans, and applied it for raising autoantibodies against human chorionic gonadotrophin in men as a means for male contraception. We conjugated hog68 and rat renin69 to tetanus toxoid and were able to raise high affinity antibodies suitable for its immunohistochemical localization. Later renin-bovine thyroglobulin conjugates were found to be appropriate as a strong immunogen. Since antibodies against renin available before this work had been raised against partially pure renin, its specificity was not reliable, although some of them had been used to stain the juxtaglomerular renin containing cells. Using these antibodies, we were able to localize renin in various specific organs including brain,70,71 LH-gonadotrophs of anterior pituitary,72 and adrenal cortex (outer layer for the rat73 and inner layer for the mouse74) and testicular Leydig cells.75 Slater et al76 showed it in various neuronal cells of human brain. Re et al77 showed renin in dog aorta. This discovery opened an interesting development of studies on vascular renin. These antibodies were also useful for confirming Goormaghtigh’s hypothesis78 that the epithelioid cells of the afferent arterioles (juxtaglomerular cells) were the site of renin production and storage.79-84

While trying to demonstrate intraneuronal production of angiotensin II by coexisting renin in collaboration with Marco Celio, we stumbled on an interesting discovery that rat renal juxtaglomerular cells contain a strong angiotensin II immunoreactivity85 as kidney sections were routinely used as a reference for the validation of our immunohistochemical methodology. We interpreted this coexistence to indicate the intracellular formation of angiotensin II. Taugner and Hackenthal85 reported a similar observation, and postulated this may be due to internalization of angiotensin II. Our subsequent finding of both angiotensins I and II in juxtaglomerular cells86 was in consonance with the intracellular formation mechanism. We obtained further support to this hypothesis by the demonstration of the coexistence of renin angiotensins I and II in cultured juxtaglomerular cells.87 The immunogold technique showed angiotensin II exists in the renin granules.88,89 Simultaneously with studies on juxtaglomerular cells, neuroblastoma cells were used as models of neuronal cells to examine the hypothesis of intraneuronal formation of angiotensin II. Okamura et al90 demonstrated the coexistence of renin, angiotensin I and II, and angiotensin converting enzyme in several lines of neuroblastoma cells. Fishman et al91 reported a similar observation with a neuroblastoma x glioma hybrid cell line. Based on these observations, we were
led to the new concept of intracellular formation of angiotensin II. This concept was generalized since similar coexistence was found in other types of cells, which included pituitary gonadotrophs, testicular Leydig cells, adrenocortical cells, vascular endothelial cells, and vascular tissues.

The antibodies permitted direct immunooassay of hog68 and rat69 renin. However, the thresholds of detection were 40 pg/ml for hog renin and 100 pg/ml for rat renin, which were an order of magnitude less sensitive compared with renin activity assay. Since plasma renin consists of active and inactive renin, specific assay for the active renin had to await specific monoclonal antibody for the active human renin of Galen et al.97 which was incorporated into a sandwich method, greatly increasing the sensitivity of assay. However, even this method did not cover the lower ranges of renin activity in human plasma.

The exceedingly stringent substrate specificity of renin cast a shroud of mystery on the identity of renin. Its insensitivity to known type-specific protease inhibitors such as diisopropyl fluorophosphate,12-13 p-chloromercuri-benzoate,13 or EDTA,12 precluded the possibility of renin being serine-, cysteine-, or metallo-protease. However, its affinity to pepstatin (albeit weak) suggested the possibility that it belongs to the acid (aspartyl) protease family. However, the fact that the optimal pH of renin action is in a neutral range mitigated against this hypothesis. Temporarily reports appeared on specific inhibitors of aspartyl (acid) protease, diazoacetyl amino acid esters, by Rajagopal et al.98 in 1966, and 1,2-epoxy-3-p-nitrophenoxypropane by Tang69 in 1971. Application of the former reagent in 1974 in my laboratory produced definitive evidence that human and mouse renins are aspartyl proteases.100 A similar finding with human renin was reported by McKown and Greggerman101 in the following year. More systematic and quantitative studies by Misono and Inagami102 revealed involvement of two aspartyl residues, two tyrosyl residues, and one arginyl residue in the active site of a renin molecule.

The obvious next step in renin research was to determine its structure and to clone its complementary DNA (cDNA). Another problem came up, our studies had been supported by the Specialized Center of Research (SCOR) in Hypertension Program. In view of its emphasis on clinical aspects in the early 1970s, we were not permitted to use the SCOR grant for studies on renin structure or cDNA cloning. We had to acquire an independent investigator-initiated grant. It caused a few years of delay in our sequencing work. This is an interesting contrast with the current National Heart, Lung, and Blood Institute policy in the late 1980s. Science and science policies changed rapidly.

The determination of the amino acid sequence of mouse submandibular gland renin by Edman chemistry became feasible because of the development of a large scale purification method by Misono et al.103 We realized that mouse renin consists of two chains linked by a disulfide bridge. The complete amino acid sequence reported in 1982105 provided unequivocal evidence that it is an aspartyl protease with 48% amino acid identity with porcine pepsin.

In the cDNA cloning studies, a major breakthrough was made by Rougeon et al.106 in 1981 toward renin structure determination through cloning its cDNA. This ingenious cloning made use of differential expression of renin gene in the submandibular glands of male and female mice. After cloning a full length cDNA, Panthier et al.107 deduced the amino acid sequence of mouse preprorenin, a very important accomplishment in renin biology.

Deduction of the primary structure of a protein from the base sequence of its cDNA does not provide information on its posttranscriptional modification. I presume that by partial amino terminal sequence analysis, investigators in Paris realized possible posttranscriptional cleavage of the single chain translation product to a two-chain mature product. We had separated two chains, determined the amino acid sequence of the light chain and localized it at the carboxyl terminal of the renin molecule.104 The identification of the two chain form is an important step toward the recognition of maturation process of renin.

The renin sequence paper by the recombinant technique from the Paris group107 and our results by the Edman methods105 appeared at about the same time in the summer of 1982. We were shocked to find that there were major discrepancies between the two results involving a segment encompassing four amino acid residues. Comparison of their nucleotide sequence and our amino acid sequence in this segment readily indicated that the discrepancy was due to their erroneous reading of base sequence by a frame shift due to an extra cytidine base. They reinvestigated their sequence and published a corrected sequence in the Corcoran Lecture paper the following year.108 The sequence analyses confirmed the molecular weight of active renin (36,000) and prorenin (43,000).

The recombinant work clarified the amino acid sequence of the prepro segment of renin, whereas our work clarified the site of cleavage needed for the activation of prorenin and identified the position of disulfide bridges in addition to the overall sequence and the structure of active renin. The site of peptide bond cleavage for the activation of prorenin was not readily predictable and it required direct determination for each species. The amino terminal sequences of active renins showed that activation of mouse prorenin occurs through the cleavage of the Arg-Ser bond of -Lys65-Arg66-Ser67-Ser68,105,108 Human renal prorenin is activated by the cleavage of a similar Arg-Leu65 bond in -Lys65-Arg66-Leu67-Thr68.109,110 However, rat kidney renin has the amino terminal sequence of Ser37-Pro38-Leu39,40,42 rather than Ser45-Ser46-Phe47 predicted from the presumed activation sequence -Lys65-Lys66-Ser67-Ser68-Phe69.111 The activation of prorenin may not always proceed by the
cleavage of the peptide involving the carboxyl group of double basic sequence. Different types of activating enzyme may be used in different organs. Small differences in the amino terminal structure of active renin may not affect the enzyme activity, which is compatible with the reversible activation of prorenin by an acid treatment without the cleavage of the activation peptide as reported by Hsueh et al.\textsuperscript{112} Leckie et al.\textsuperscript{113} and McIntyre et al.\textsuperscript{114}

It was interesting that native mature renins from all species examined have an internal nick. The mouse submandibular gland renin\textsuperscript{89,107} has the gap near the carboxy terminus, human renal renin in the middle,\textsuperscript{42,44} and pig renal renin presumably both in the middle and a terminal region.\textsuperscript{64} However, human chorion renin\textsuperscript{60} or the one chain form of mouse submandibular gland renin,\textsuperscript{114-116} do not have such a gap. These renins are considered to be secreted by a constitutive pathway.\textsuperscript{114} The hypothesis that the nicking occurs only with the renin in the storage granules seems to be plausible.\textsuperscript{114,115} The nicking of human renin in the middle split the molecule into two separate lobes, which are held together by a noncovalent force and the enzyme activity of such a dimer is maintained.

The recombinant technique was quickly applied to the determination of human prorenin by Imai et al.\textsuperscript{109} Hobart et al.\textsuperscript{110} and rat prorenin by Burnham et al.\textsuperscript{111} Gene analysis led to the finding that mammals other than the mouse have only one gene for renin whereas high renin mouse strains have two genes.\textsuperscript{117,118}

In 1971, an interesting discovery was reported. Lumbers observed that an activatable form of renin (or inactive renin) exists in human amniotic fluid.\textsuperscript{119} Similar inactive renin was found in human plasma,\textsuperscript{120} rabbit,\textsuperscript{121} and hog kidney.\textsuperscript{122} That it is activated by proteases,\textsuperscript{123-125} acidification,\textsuperscript{119} and cold exposure\textsuperscript{124,125} suggested that either it is a renin zymogen or renin-inhibitor complex. The situation was further complicated by the finding of "big renin" with a molecular weight difference of 20,000.\textsuperscript{120-122} Looking back, much confusion arose because it was difficult to recognize the presence of two distinct entities, inactive prorenin (renin zymogen) and big renin (high molecular weight renin, renin-renin binding protein complex).

We thought our experience with affinity chromatography might, at least, clarify some of the molecular problems, namely, 1) whether the plasma renin is a mixture of fully active renin and completely inactive renin; 2) if so, whether the completely inactive renin was prorenin; and 3) are big renin and prorenin different?

In the study of plasma proteins we had to remove albumin, which constitutes the greatest bulk of plasma proteins. Takahashi in our laboratory used an Affi-gel Blue column that had an ability to bind and remove albumin. He stumbled on some interesting properties of inactive renin. Inactive renin binds to Affi-gel Blue but active renin does not.\textsuperscript{126} Similar observations were made by Hsueh et al.\textsuperscript{127} and Atlas et al.\textsuperscript{128} Furthermore, pepstatin-Sepharose gel also discriminated inactive renin from active renin. Combining these techniques, Yokosawa and Takahashi produced evidence that there was completely inactive renin that can be activated by proteolysis.\textsuperscript{126} Takii and Inagami\textsuperscript{129,130} purified the completely inactive renin to homogeneity by the combination of these affinity methods and hydrophobic chromatography on octyl-Sepharose from hog kidney and showed that it was a prorenin with a single 50,000 d polypeptide chain that can be activated by limited proteolysis by several proteases. A similar but much smaller inactive prorenin was purified from hog kidney by Takahashi et al.\textsuperscript{131} as a 39,000 d protein without using an affinity column. This small molecular weight was not in immediate agreement with that prorenin predicted by its cDNA.\textsuperscript{108}

McIntyre et al.\textsuperscript{135} purified a 48,000 d prorenin from human kidney by affinity chromatography on a gel with monoclonal antibody to human renin. Eagan et al.\textsuperscript{131} purified prorenin from culture medium of human chorion cells. Higashimori et al.\textsuperscript{136} purified inactive renin from human chorion as well as cultured medium of chorion cells using an affinity column—bearing monoclonal antibodies. A single chain inactive renin with a molecular weight of 47,000 was isolated. They were activated by trypsin. Interestingly, the prorenin from the chorion membrane and cultured chorion cells had an amino terminal sequence Leu-Pro-Thr-Asp, which was three residues shorter than predicted from the preprorenin sequence, suggesting the site of cleavage of the signal peptide is the Gyl-Leu peptide bond rather than the Cys-Thr of prorenin. These results obtained from a series of purification studies of inactive renin combined with information on its structure produced solid evidence that inactive renin (45,000–50,000 d) is prorenin. The molecular weight of the renin precursor was in agreement with those estimated by the Western blot analyses of in vitro translation products of renin mRNA from mouse submandibular gland,\textsuperscript{133,134} mouse kidney,\textsuperscript{135,136} and human kidney.\textsuperscript{137}

What about big renin of 60,000 d? The relation of its size (60,000 versus 40,000 d) to activity is not yet completely clear. Leckie and McConnell\textsuperscript{138} reinterpreted her original observation of possible inactive zymogen and postulated that big inactive renin is a complex of renin and renin inhibitor on the basis of the observation of dissociable renin inhibitor from inactive renin. Boyd’s discovery of inactive big renin (60,000 d) and active small renin (40,000 d) in hog kidney\textsuperscript{121} suggested that the activation was dependent on the molecular weight reduction by 20,000. On the other hand, Yamamoto et al.\textsuperscript{139} showed reversible conversion of big and small renin with the retention of renin activity. We were able to maintain active renin in hog and rat kidney extract in the 60,000 d form in the presence of sodium-tetrathionate or N-ethylmaleimide,\textsuperscript{140} and such a big active renin could be isolated in a pure form from porcine.
kidney. Furthermore, we and Atlas et al. could purify 56,000 d inactive renin from human plasma and then activated it with various proteases without a recognizable change in molecular weight. These observations can be explained by a renin binding protein that binds inactive prorenin or active renin without affecting appreciably the enzyme activity of renin. Funakawa et al. demonstrated such a binding substance that binds 40,000 d renin reversibly. It was present in dog renal cortex but not medulla. Tanaka et al. reported an enzyme that may catalyze such a conversion.

Takahashi et al. purified a renin binding protein from hog kidneys. It showed a strong affinity to active renin with a dissociation constant of 0.2 nM. While the binding protein has a molecular weight of 42,000, its complex with 40,000 d renin resulted in a 60,000 d high molecular weight renin as examined by gel filtration. This behavior, not explainable by simple arithmetics, may be due to an anomaly of the gel filtration method for molecular weight estimation. The structure of the renin binding protein was reported by Inoue et al. It is an inhibitor of renin. Its physiological function is not clear. There may exist other renin binding proteins.

As soon as the amino acid sequences of mouse and human renin were determined, the sequence homology between renin and fungal or mammalian aspartyl proteases was recognized. Prompted by Blundell et al., Carlson et al., and Akahane et al. to build three-dimensional models of mouse or human renin using coordinates of the fungal enzymes. Although the accuracy of these models based on homology was not clear, it was informative in providing some idea of the active site of renin, which no doubt assisted the design of renin inhibitors. Unfortunately, renin isolated from mammalian tissue did not produce the crystals, despite repeated attempts in our laboratory and presumably others, due to microheterogeneity.

The x-ray crystallographic determination of the three-dimensional structure had to await until large scale production of recombinant human renin expressed in Chinese hamster ovary cells. Sielecki et al. finally produced the three-dimensional picture. Although precise atomic coordinates were not permitted for publication due to restriction by a patent right, their results demonstrated a bilobal structure with a catalytically essential aspartyl residue on each of the lobes and sticking into the substrate-binding cleft. It is gratifying that the purification, structure analysis, and gene cloning have provided great impetus to the vigorous progress on renin studies in recent years.

In 1976 Mohinder Sambhi held a symposium on renin in Murietta Hot Springs outside of Los Angeles. Some seasoned renin scientists predicted that it would be the last of symposia on renin research. Fortunately, this prediction did not materialize. Recent conceptual breakthroughs have contributed to the extraordinary longevity and vigorous development of renin research. These include the demonstration of important roles of the renin-angiotensin system not only in renovascular hypertension but also in essential hypertension by Gavras and Brunner and spontaneous hypertension in rat by Laffan et al. and Muirhead et al. and subsequent realization of intracellular function of renin and production and release of angiotensin II from many types of cells. Furthermore, recognition of the efficacy of the inhibition of the renin-angiotensin system in the treatment of congestive heart failure or prevention and treatment of cardiac hypertrophy will provide further impetus to the study of renin and angiotensin in the future.

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References


142. Takahashi S, Ohtsawa T, Miiura R, Miyake Y: Purification of high molecular weight (HMW) renin from porcine kidney and direct evidence that the HMW renin is a complex of renin with renin binding protein (RnBP). J Biochem 1983;93:265–274
146. Takahashi S, Ohtsawa T, Miiura R, Miyake Y: Purification and characte-.
Purification of renin and prorenin.
T Inagami

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