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Renin Inhibits *N*-Acetyl-D-Glucosamine 2-Epimerase (Renin-Binding Protein)[1]

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Renin-binding protein (RnBP) is a highly specific renin inhibitor first isolated from porcine kidney. Our recent studies demonstrated that the human RnBP is the enzyme *N*-acetyl-D-glucosamine (GlcNAc) 2-epimerase [Takahashi, S. *et al.* (1999) *J. Biochem.* 125, 348–353]. We have developed a new assay method for GlcNAc 2-epimerase activity using a system of *N*-acyl-D-hexosamine oxidase coupled with peroxidase and employed this method to study the effects of renin on GlcNAc 2-epimerase activity. The recombinant human (rh) RnBP existed as a dimer and its GlcNAc 2-epimerase activity was strongly inhibited by the purified renin concomitant with the formation of RnBP-renin heterodimer, so-called high molecular weight (HMW) renin. The renin activity was also inhibited by rhRnBP in a dose-dependent manner. These results indicate that renin is an inhibitor of GlcNAc 2-epimerase, and the renin-RnBP heterodimer HMW renin is an inactive form of both renin and GlcNAc 2-epimerase activities.

Key words: binding protein, dimer, GlcNAc 2-epimerase, high molecular weight, renin.

Renin [EC 3.4.23.15] is a highly specific aspartic proteinase mainly synthesized in juxtaglomerular cells in the kidney. The enzyme plays an essential role in blood pressure control in animals. Renin-binding protein (RnBP) is an endogenous renin inhibitor originally isolated from porcine kidney. This protein exists as a homodimer (*1–3*) and forms a complex with renin, so-called high molecular weight (HMW) renin (*4, 5*). The purified RnBP inhibited renin activity strongly with a dissociation constant of 0.2 nM (*1*).

The primary structures of porcine (*6*), rat (*7*), and human (*7*) RnBPs have been deduced from nucleotide sequences by cDNA cloning and are characterized by the presence of a conserved leucine-zipper motif (*6, 7*). This motif was indicated to mediate the formation of both RnBP-renin heterodimer and RnBP homodimer by a mutational analysis of porcine RnBP (*8*). Co-expression experiments on human renin and RnBP cDNAs in mouse pituitary AtT-20 cells indicated that RnBP regulates active renin secretion from the transformants (*9*).

Recently, human (*10*) and rat RnBP genes (*11*) were isolated from genomic DNA libraries and characterized. Both genes span about 10 kilobase pairs and consist of 11 exons separated by 10 introns. Moreover, the expression of RnBP mRNA was demonstrated in rat. The RnBP mRNA was expressed mainly in the kidney, considerably in the adrenal gland, brain, lung, spleen, and ovary and slightly in the testis and heart (*12*). On the other hand, the cDNA cloning of porcine kidney *N*-acetyl-D-glucosamine (GlcNAc) 2-epimerase [EC 5.3.1.8] and the expression and characterization of recombinant human (rh) RnBP showed that RnBP is the enzyme GlcNAc 2-epimerase (*13–15*). Our recent studies demonstrated that knockout of the RnBP gene did not affect the expression or activity of renin under physiological conditions. However, mice lacking RnBP excrete an abnormal pattern of carbohydrate in the urine, indicating a role of the protein in renal carbohydrate metabolism (*16*).

The human GlcNAc 2-epimerase activity is specifically inhibited by sulfhydryl-oxidizing and -alkylating reagents such as monoiodoacetic acid, *N*-ethylmaleimide, or 5,5′-dithiobis (2-nitrobenzoic acid) (*17*). Moreover, the essential residue for the human GlcNAc 2-epimerase (RnBP) has been identified by site-directed mutagenesis of conserved cysteine residues (*17*). However, the relationship between heterodimer formation and the GlcNAc 2-epimerase or renin activity was not understood.

In the present study, we developed a novel assay method for GlcNAc 2-epimerase and studied the effects of renin on GlcNAc 2-epimerase (RnBP). Our results demonstrate that renin and RnBP inhibit GlcNAc 2-epimerase and renin activities, respectively, in a dose-dependent manner. Thus, renin is an inhibitor of GlcNAc 2-epimerase, and GlcNAc 2-epimerase is a renin inhibitor.

MATERIALS AND METHODS

Materials—*N*-Acyl-D-hexosamine oxidase (AHOX) was from Kikkoman, horseradish peroxidase and *N,N′*-diacetyl chitobiose were from Wako Pure Chemical, and 3-hydroxy 2,4,6-triiodobenzoic acid (HTIB) was from Sigma Aldrich. GlcNAc, *N*-acetyl-D-mannosamine (ManNAc) monohydrate, *N*-acetyl-D-galactosamine, and D-mannosamine hydrochlo-

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Abbreviations: AHOX, *N*-acyl-D-hexosamine oxidase; Dnp, 2,4-dinitrophenyl; GlcNAc, *N*-acetyl-D-glucosamine; HMW, high molecular weight; HTIB, 3-hydroxy 2,4,6-triiodobenzoic acid; ManNAc, *N*-acetyl-D-mannosamine; Nma, *N*-methylanthranyloyl; r, D-Arg; rh, recombinant human; RnBP, renin binding protein.

ride were obtained from Nacalai Tesque. D-Glucosamine hydrochloride and D-galactosamine hydrochloride were obtained from Merk. *N*-Acetylmuramic acid was from ICN Pharmaceutical. The novel fluorogenic substrate for renin *N*-methylanthranyloyl (Nma)-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Lys-2,4 dinitrophenyl (Dnp)-D-Arg (r)-r-NH_2 and reference compound Nma-His-Pro-Phe-Leu were designed and custom-synthesized at the Peptide Institute. rhRnBP (GlcNAc 2-epimerase) was purified as described previously (*15*). The purified rhRnBP was dialysed against 20 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA, 0.05% 2-mercaptoethanol, and 5% sucrose, and stored at –80°C until use. Porcine kidney renin was purified by the method of Takahashi *et al.* (*1*). All other reagents were of analytical grade.

Substrate Specificity of AHOX—A 20-μl portion of GlcNAc or sugar derivative solution was mixed with 0.25 ml of Solution I (1 mM 4-aminoantipyrine, 0.5 unit/ml of AHOX, 5 units/ml of horseradish peroxidase in 0.1 M sodium phosphate buffer, pH 7.25, containing 0.1% NaN_3) and 0.25 ml of Solution II (2 mM HTIB in 0.1 M sodium phosphate buffer, pH 7.25 containing 0.1% NaN_3). After incubation at 37°C for 20 min, absorbance at 515 nm was measured.

GlcNAc 2-Epimerase Activity—GlcNAc 2-epimerase activity was assayed by the rate of formation of GlcNAc from the substrate ManNAc. A reaction mixture contained 80 μl 0.1 M Tris-HCl, pH 7.0, containing 10 mM $MgCl_2$, 50 mM ManNAc, 5 mM ATP, and 20 μl of enzyme solution. After incubation at 37°C for 30 min, the reaction was terminated by adding 0.9 ml of ice cold distilled water. The diluted sample (20 μl) was mixed with 0.25 ml of Solutions I and II, and incubated at 37°C for 20 min. Then the absorbance at 515 nm was measured. The reaction product, GlcNAc, was also measured with a Dionex HPLC system (Dionex Model DX-500) by the method of Takahashi *et al.* (*15*).

Renin Activity—The hydrolysis of the fluorogenic substrate at the Leu-Leu bond was spectrophotometrically determined. The reaction mixture contained 175 μl of 50 mM sodium phosphate buffer, pH 6.0, 0.1 M NaCl, 1 mM EDTA, 5 μl of 1 mM substrate solution in DMSO, and 20 μl of sample solution in a total volume of 200 μl. The reaction mixture was incubated at 37°C for 10 min and the reaction was terminated by adding 800 μl of 10% acetic acid. The increase of fluorescence intensity was measured at an emission wavelength of 440 nm upon excitation at 340 nm using a Hitachi fluorescence spectrophotometer model F-2000. Nma-His-Pro-Phe-Leu was used as a reference compound.

Analytical Methods—SDS-PAGE was performed by the method of Laemmli (*18*) with 5–20% polyacrylamide gel (ATTO). After the electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250. The apparent molecular weights of RnBP, renin, and HMW renin were estimated by gel permeation chromatography on Superdex 200 in a Amersham Pharmacia Biotech FPLC system with UV-1 detector. A sample (200 μl) was chromatographed on a column in 20 mM sodium phosphate buffer, pH 7.0, containing 0.15 M NaCl, 1 mM EDTA, 0.05% 2-mercaptoethanol, and 0.02% NaN_3, and eluted at a flow rate of 0.5 ml per min. Fractions of 0.5 ml were collected and analyzed by SDS-PAGE.

Protein Determination—The concentration of protein was determined using a Bio-Rad protein assay kit (*19*) with bovine serum albumin as a standard. The purified porcine kidney renin concentration was estimated with a coefficient 9.1 for 1% solution in 1 cm path-length (*20*).

(GlcNAc 2-epimerase)

ManNAc ➡ GlcNAc

(*N*-Acyl-*D*-hexosamine-oxidase)

GlcNAc + O_2 + H_2O →

N-Acetylglucosaminic acid + H_2O_2

(Peroxidase)

H_2O_2 + 4-Aminoantipyrine + HTIB →

Quinoneimine dye + 2 H_2O

(Abs. 515 nm)

Fig. 1. **Novel assay method for GlcNAc 2-epimerase activity using a coupled system of AHOX and peroxidase.** GlcNAc 2-epimerase catalyzes the interconversion between ManNAc and GlcNAc. When ManNAc was used as a substrate, the produced GlcNAc could be measured by the two-enzyme coupled system. HTIB*, 3-hydroxy 2,4,6-triiodobenzoic acid.

RESULTS

Substrate Specificity of AHOX—As rhGlcNAc 2-epimerase is highly specific for ManNAc (*15*), ManNAc was used as a substrate for the enzyme. The key point of the GlcNAc 2-epimerase assay is to determine GlcNAc by a rapid and simple method. The possible assay strategy is shown in Fig.

TABLE I. **Substrate specificity of AHOX.**

Substrate*	Specific activity (%)
1. GlcNAc	100
2. ManNAc	2.4
3. GalNAc	99.3
4. D-Glucosamine	26.1
5. D-Mannosamine	n.d.
6. D-Galactosamine	80.8
7. D(+)-Glucose	n.d.
8. D(+)-Mannose	n.d.
9. Galactose	n.d.
10. D(–)-Fructose	n.d.
11. Dulcitol	n.d.
12. D(–)-Mannitol	n.d.
13. D(–)-Sorbitol	n.d.
14. 2-Deoxy-D-glucose	n.d.
15. D(–)-Arabinose	n.d.
16. L(+)-Arabinose	n.d.
17. D(+)-Xylose	n.d.
18. D(–)-Ribose	n.d.
19. *meso*-Erythritol	n.d.
20. Glycerol	n.d.
21. Saccharose	n.d.
22. Maltose	n.d.
23. Trehalose	n.d.
24. Lactose	n.d.
25. Cellobiose	n.d.
26. *N,N'*-Diacetylchitobiose	48.9
27. *N*-Acetylmuramic acid	4.9

*40 nmol of substrates were used. n.d., not detected.

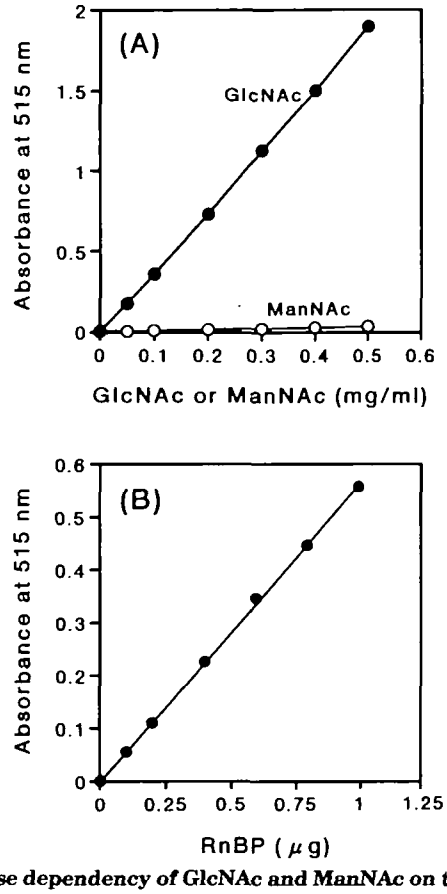

Fig. 2. **Dose dependency of GlcNAc and ManNAc on the AHOX and peroxidase coupled system (A) and the calibration curve of recombinant human RnBP, GlcNAc 2-epimerase (B).** (A) The indicated amount of GlcNAc or ManNAc in 20 μl of solution was mixed with 0.25 ml each of Solutions I and II, and the mixture was incubated at 37°C for 20 min. After the reaction, absorbance at 515 nm was measured. (B) Several concentrations of rh GlcNAc 2-epimerase (RnBP) were incubated with ManNAc at 37°C for 30 min in a total volume of 100 μl as described above. After the incubation, the reaction was terminated by adding 0.9 ml of ice-cold water. A 20-μl aliquot was incubated with 0.25 ml each of Solutions I and II at 37°C for 20 min. After the reaction, absorbance at 515 nm was measured.

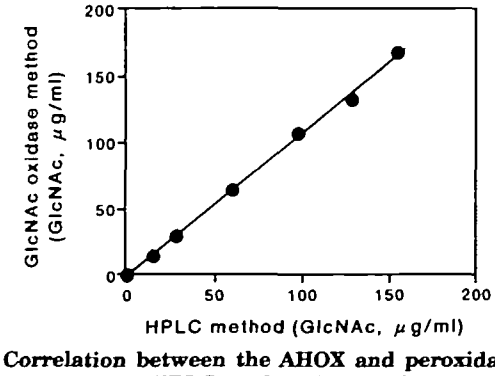

Fig. 3. **Correlation between the AHOX and peroxidase coupled system and an HPLC method for the determination of GlcNAc 2-epimerase activity.** Various concentrations of rhRnBP were incubated with ManNAc at 37°C for 30 min and the GlcNAc produced was measured by the AHOX and peroxidase coupled system and by the Dionex HPLC method (*15*).

1. The enzyme activity might be measured using a system of AHOX coupled with peroxidase. GlcNAc formed through the GlcNAc 2-epimerase reaction can be measured as the quinoneimine dye formed, which absorbs at 515 nm. First, we checked the substrate specificity of commercially available AHOX. Forty nanomoles of substrate in 20 μl of distilled water was incubated with 0.25 ml of Solutions I and II. After the reaction at 37°C for 20 min, absorbance at 515 nm was measured. As shown in Table I and Fig. 2A, AHOX was specific for GlcNAc, *N*-acetyl-D-galactosamine, D-galactosamine, and *N,N'*-diacetylchitobiose. The enzyme reacted very slightly on *N*-acetylmuramic acid and ManNAc. These results indicate that the coupled system of AHOX and peroxidase is applicable for the GlcNAc 2-epimerase assay.

Novel Assay Method for GlcNAc 2-Epimerase Activity—The dose-dependent curve for rhGlcNAc 2-epimerase (RnBP) is shown in Fig. 2B. The absorbance at 515 nm correlated linearly with the concentration of the GlcNAc 2-epimerase (rhRnBP). The sensitivity of the assay method is about 50 ng of the enzyme.

Correlation between HPLC and Enzyme Coupled System—To evaluate the coupled system of AHOX and peroxi-

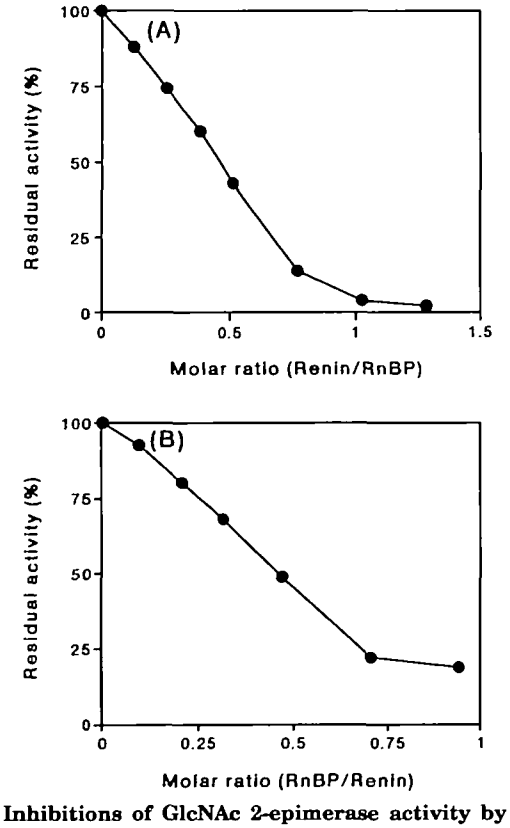

Fig. 4. **Inhibitions of GlcNAc 2-epimerase activity by renin (A) and renin activity by GlcNAc 2-epimerase (B).** (A) The rhGlcNAc 2-epimerase (220 pmol) was incubated with various concentrations of purified porcine kidney renin (0–300 pmol) in a total volume of 0.1 ml at 37°C for 30 min and the residual GlcNAc 2-epimerase activity was measured by the AHOX and peroxidase coupled system. (B) The purified porcine kidney renin (240 pmol) was incubated with varying concentrations of rhGlcNAc 2-epimerase (0–220 pmol) in a total volume of 0.1 ml at 37°C for 30 min and the residual renin activity was measured using Nma-His-Pro-Phe-His-Leu*Leu-Val-Tyr-Lys(Dnp)rr-NH_2 (-Leu*Leu-, scissile peptide bond) as a substrate.

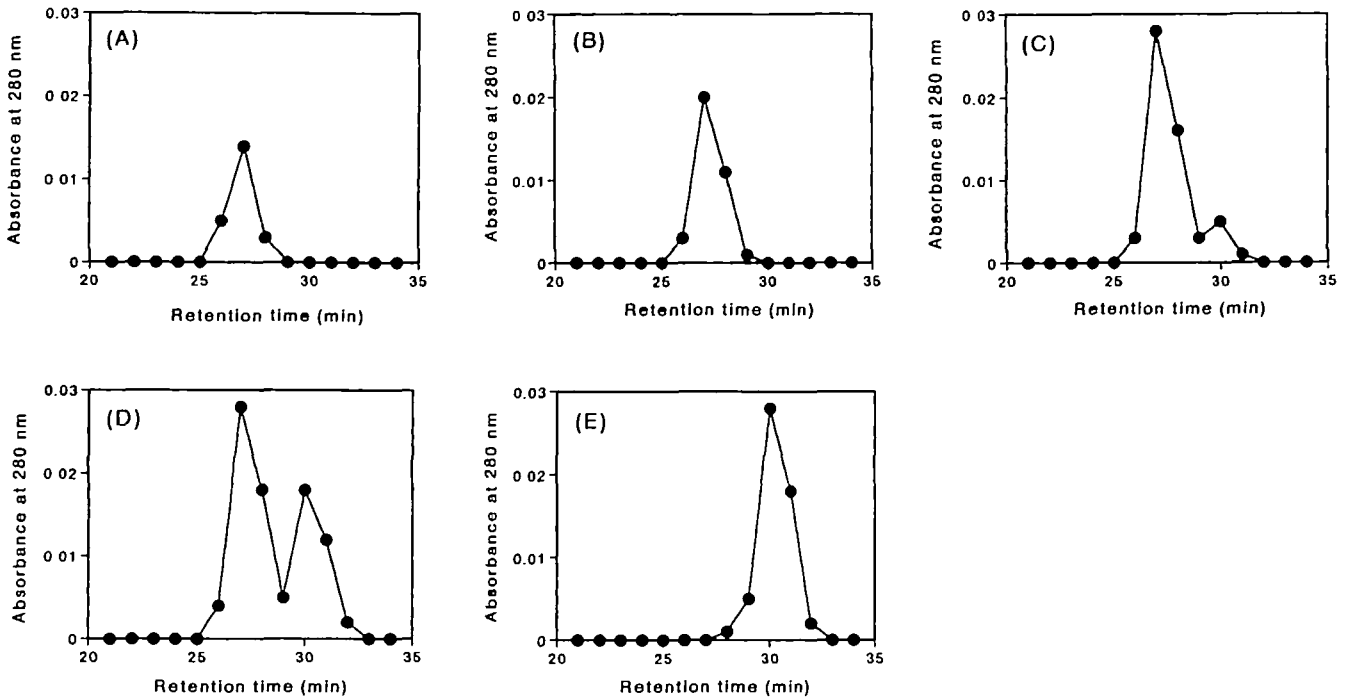

Fig. 5. **Interaction of GlcNAc 2-epimerase with renin.** Samples used were: (A), pure rhGlcNAc 2-epimerase (440 pmol); (B), rhGlcNAc 2-epimerase (440 pmol) incubated with renin (240 pmol) for 30 min at 37°C; (C), rhGlcNAc 2-epimerase (440 pmol) incubated with renin (480 pmol) for 30 min at 37°C; (D), rhGlcNAc 2-epimerase (440 pmol) incubated with renin (960 pmol) for 30 min at 37°C; (E), renin (960 pmol). Samples (0.2 ml) were resolved on a Superdex 200 column. Fractions of 0.5 ml were collected and absorbance at 280 nm of each fraction was measured. Each fraction was then subjected to SDS-PAGE. The retention times of the standards were as follows: Blue dextran 2000, 16.5 min; bovine serum albumin, 27.0 min; ovalbumin, 29.5 min; and chymotrypsinogen, 33.5 min.

dase for the measurement of GlcNAc 2-epimerase activity, we compared the values measured by this method with the results of HPLC assay with pulse amperometric detection (*15*). As shown in Fig. 3, the two assay systems correlated very well with each other, the equation as expressed by [HPLC method] = −1.247 + 1.0702 × [AHOX method], and the correlation factor of 0.999.

Effects of Renin on GlcNAc 2-Epimerase Activity—The purified GlcNAc 2-epimerase (220 pmol) was mixed with purified porcine renin (0 to 300 pmol) in sodium phosphate buffer, pH 7.0, containing 1 mM EDTA, 0.05% 2-mercaptoethanol in a total volume of 100 μl. After incubation at 37°C for 30 min, the residual GlcNAc 2-epimerase activity was measured by the AHOX and peroxidase coupled method. The GlcNAc 2-epimerase activity decreased with the increase of renin concentration (Fig. 4A) and the 1:1 renin/GlcNAc 2-epimerase complex had only 2% of the activity of free GlcNAc 2-epimerase.

Effects of GlcNAc 2-Epimerase on Renin Activity—The effect of GlcNAc 2-epimerase (RnBP) on renin activity was also investigated. The purified porcine kidney renin was incubated with rh GlcNAc 2-epimerase and the residual renin activity was measured using the newly developed fluorescence quenching substrate as described in "MATERIALS AND METHODS." As anticipated, renin activity was strongly inhibited by GlcNAc 2-epimerase RnBP (Fig. 4B).

Size Exclusion Chromatography of Renin-GlcNAc 2-Epimerase RnBP Complex—To determine whether the loss of activity of GlcNAc 2-epimerase and renin can be attributed to the formation of a heterodimer, we performed size exclusion chromatography using Superdex 200. The obtained

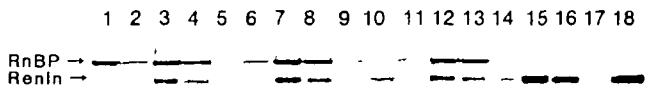

Fig. 6. **SDS-PAGE of renin, GlcNAc 2-epimerase, and HMW renin.** Fractions of 30 μl in Fig. 5 were resolved on SDS-polyacrylamide gel (5–20% polyacrylamide gradient gel), and the gel was stained with Coomassie Brilliant Blue R-250. Samples used were: lane 1, Fr. 27 in Fig. 5A; lanes 2–5, Fr. 26–29 in Fig. 5B; lanes 6–10, Fr. 26–30 in Fig. 5C; lanes 11–17, Fr. 26–32 in Fig. 5D; lane 18, Fr. 30 in Fig. 5E.

fractions were also analyzed by SDS-PAGE. When GlcNAc 2-epimerase or purified renin was resolved on the column, proteins were eluted as single peaks with the apparent molecular weight of 65,000 for GlcNAc 2-epimerase and 40,000 for renin (Fig. 5, A and E, and Fig. 6, lanes 1 and 18). When 440 pmol of GlcNAc 2-epimerase was incubated with 240 pmol of purified renin, no free renin was detected (Fig. 5B and Fig. 6, lanes 2–5), indicating that almost all added renin forms a complex with GlcNAc 2-epimerase. The free form of renin appeared with increase of renin concentration (Fig. 5, C and D, and Fig. 6, lanes 6–17).

DISCUSSION

Human RnBP has recently been identified as the enzyme GlcNAc 2-epimerase (*14, 15*). At that time we used a Dionex HPLC system with pulse amperometric detection for the determination of GlcNAc 2-epimerase activity. The

HPLC method was easy to perform and was useful for the separation and quantification of GlcNAc and ManNAc (*15*). However, it took a relatively long time of 40 min per sample. Other assay methods for GlcNAc 2-epimerase were complicated because of the use of commercially unavailable enzymes (*22, 23*) or derivatization of the reaction products (*13, 24*). For these reasons, a simple and rapid assay method for GlcNAc 2-epimerase activity was developed.

Our previous studies showed that the human GlcNAc 2-epimerase was highly specific for ManNAc, and the initial velocity of ManNAc substrate was about eighth-times higher than that of GlcNAc (*15*). The enzyme did not react with *N*-acetyl-D-galactosamine, mannosamine, glucosamine, mannose, and glucose. On the other hand, Horiuchi (*21*) reported the purification and characterization of AHOX from *Pseudomonas* sp. This enzyme was a flavoprotein and was highly specific for GlcNAc. Indeed, the commercially available AHOX was highly specific for GlcNAc and poorly reacted with ManNAc (Table I and Fig. 2A). These results suggested the novel assay method of GlcNAc 2-epimerase using a system of AHOX coupled with peroxidase (Fig. 1). The novel assay method is rapid and easy and requires only aspectrophotometer. One cycle of GlcNAc 2-epimerase assay takes approximately 60 min. Theoretically, several hundred samples could be measured in one day. The values obtained by the novel GlcNAc 2-epimerase assay method correlated well with those of the HPLC method reported previously (*15*) with the correlation coefficient of 0.999 (Fig. 3). Thus, the AHOX and peroxidase coupled system is applicable for the assay of GlcNAc 2-epimerase.

We have already pointed out that RnBP is an endogenous renin inhibitor first isolated from porcine kidney as a complex of renin, so-called HMW renin (*1–4*). The protein strongly inhibited renin activity with a dissociation constant of 0.2 nM (*4*). However, the effects of renin on GlcNAc 2-epimerase (RnBP) had not been understood, because of the difficulties of preparing large amounts of purified renin and the unknown enzymatic properties of RnBP. In the present study, we have purified porcine kidney renin by the reported method (*1*). Approximately 5 mg of purified preparation was obtained from 10 kg of porcine kidney cortex. The purified preparation showed a single protein band on SDS-PAGE with the molecular weight of 36,000 (Fig. 6, lane 18). The purified renin inhibited GlcNAc 2-epimerase activity in a dose-dependent manner with the formation of renin-GlcNAc 2-epimerase (RnBP) complex (Figs. 4A and 5, B–D). On the other hand, renin activity was inhibited by GlcNAc 2-epimerase (RnBP) (Fig. 4B). From these results, we propose the model of renin-GlcNAc 2-epimerase (RnBP) heterodimer formation shown in Fig. 7. GlcNAc 2-epimerase (RnBP) and renin exist as a dimer and a monomer, respectively. Both species are enzymatically active forms. When the dimeric form of GlcNAc 2-epimerase was incubated with renin, the dimer dissociated, and the monomer formed a complex with renin monomer. The resulting renin-GlcNAc 2-epimerase (RnBP) heterodimer is an inactive form of both renin and GlcNAc 2-epimerase. In connection with this hypothesis, we have checked the GlcNAc 2-epimerase activity of HMW renin preparation isolated from porcine kidney (*4*). The GlcNAc 2-epimerase activity could not be detected in the purified HMW renin. These results also support our scheme.

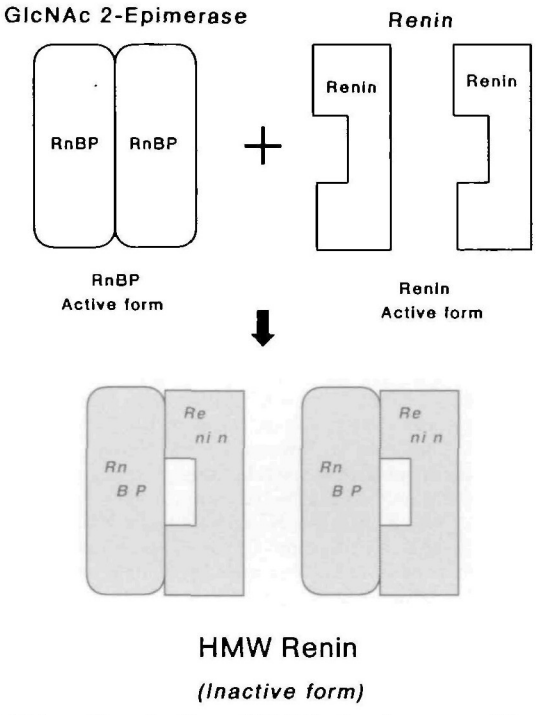

Fig. 7. **Heterodimerization of GlcNAc 2-epimerase with renin.** GlcNAc 2-epimerase exists as a homodimer and forms a heterodimer with renin, so-called HMW renin. HMW renin is an inactive complex of renin and GlcNAc 2-epimerase.

Our previous studies showed that the renin activity of HMW renin increased gradually upon incubation at acidic pH (*4*). During the course of incubation, RnBP forms an insoluble precipitate as the free form of active renin. The conditions under which the renin-GlcNAc 2-epimerase heterodimer can reassociate to form active GlcNAc 2-epimerase dimer and active renin monomers are not yet understood. Further studies, *e.g.*, X-ray crystallography of GlcNAc 2-epimerase or renin-GlcNAc 2-epimerase heterodimer, should provide an insight into the structure and function relationship of renin and GlcNAc 2-epimerase.

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