STABILIZATION OF GLCNAC 2-EPIMERASE (RENIN-BINDING PROTEIN) BY NUCLEOTIDES

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Abstract

Renin binding protein (RnBP) is an endogenous renin inhibitor. Recent our studies demonstrated that RnBP was identical with *N*-acetylglucosamine (GlcNAc) 2-epimerase [EC5.3.1.8]. The enzyme catalyzes the conversion between GlcNAc and *N*-acetyl-mannosamine (ManNAc). The purified recombinant GlcNAc 2-epimerases existed as dimers. Nucleotides such as ATP, ADP, dATP, ddATP, and GTP were essential for GlcNAc 2-epimerase activity. Nucleotides stabilized dimeric form of GlcNAc 2-epimerases. The ESI TOF/MS analysis of recombinant rat enzyme showed that one nucleotide bound per monomer enzyme. On the other hand, nucleotides inhibited the formation of a renin-GlcNAc 2-epimerase heterodimer (HMW renin). These results indicate that nucleotides stabilize homodimer of GlcNAc 2-epimerase to form an active site domain of the enzyme and interrupt the heterodimer formation of renin with GlcNAc 2-epimrase (RnBP).

Introduction

The renin-angiotensin-aldosterone system (RAS) plays an essential role in the regulation of blood pressure. Renin is a key enzyme of controlling the activity of RAS [1]. Renin binding protein (RnBP) is a protein that inhibits renin activity with the formation of renin-RnBP hetero complex called high molecular weight (HMW) renin [2-5]. The cDNA cloning of porcine kidney *N*-acetylglucosamine (GlcNAc) 2-epimerase [6] and the expression and characterization of human RnBP [7, 8] showed that RnBP was identical to GlcNAc 2-epimerase. The enzyme catalyzes the conversion between GlcNAc and *N*-acetylmannosamine (ManNAc). The human GlcNAc 2-epimerase specifically inhibited by *N*-ethylmaleimide (NEM), monoiodoacetic acid, and 5, 5'-dithiobis (2-nitorobenzoic acid), and the active site residue of the enzyme was identified as cysteine 380 by site-directed mutagenesis [9]. In our previous studies it was demonstrated that nucleotides, such as ATP, dATP, ddATP, ADP and GTP, enhance human, rat, and porcine GlcNAc 2-epimerase activity [10]. Moreover, nucleotide binding domain and the amino acid residues conferring nucleotides were also determined [11, 12].

In the present study, effects of several nucleotides on the interaction of renin with RnBP (GlcNAc 2-epimerase) were investigated. Our results demonstrated that nucleotides inhibited formation of HMW renin to stabilize the dimeric form of RnBP (GlcNAc 2-epimerase).

Material and Methods

Materials---*N*-Acylhexosamine oxidase (AHOX), ManNAc, and 3-hydroxy 2, 4, 6-triiodobenzoic acid (HTIB) were obtained from Sigma (St. Louis, USA). 4-Aminoantipyrine and horseradish peroxidase were from Nacalai Tesque (Kyoto, Japan) and Wako Pure Chemical (Osaka, Japan), respectively. Restriction enzymes, and dATP, dCTP, dGTP and dTTP were obtained from Takara (Otsu, Shiga, Japan). Porcine kidney renin and recombinant porcine RnBP were purified as described previously [3, 10].

Renin Activity---The renin activity was measured by the rate of formation of angiotensin I from porcine plasma angiotensinogen [13]. After the incubation of renin with partially purified angiotensinogen at 37℃ for 60 min in 0.1 M sodium phosphate buffer, pH 6.5, containing 10 mM EDTA, 1 mM PMSF, angiotensin I produced was determined using a radioimmunoassay [14].

GlcNAc 2-Epimerase Activity---The GlcNAc 2-epimerase activity was measured in a system of AHOX coupled with peroxidase [15]. Samples (20 μl) were incubated at 37℃ with 80 μl of 0.1 M Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM ManNAc and 5 mM ATP. The reaction was terminated by adding 0.9 ml of ice-cold water. The diluted sample (20 μl) was incubated with 0.25 ml of 1 mM 4-aminoantipyrine, 0.5 units/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 2 mM HTIB in 0.1 M sodium phosphate buffer, pH 7.25, containing 0.1 % NaN_3. After incubation at 37℃ for 30 min, the absorbance at 515 nm was measured. One unit of the enzyme activity was defined as the amount of enzyme that produced 1 μmole of GlcNAc per min.

Gel Filtration Chromatography---Samples (0.2 ml) were separated using Superdex 75 HR10/30 (Amersham Bioscience) in 20 mM sodium phosphate buffer, pH 7.0, containing 0.15 M NaCl and 0.02% NaN_3 at a flow rate of 1.0 ml/min and fractionated in 1-ml fractions. Bovine serum albumin (BSA, 67,000), ovalbumin (Oval, 45,000), and soybean trypsin inhibitor (SBTI, 20,100) were used as molecular weight standards.

Electron Spray Ionization Time of Flight Mass Spectrometry (ESI TOF/MS) --- The masses of GlcNAc 2-epimerases were measured with JMS-T100LC (AccuTOF) mass spectrometer (JEOL Ltd., Tokyo, Japan).

Protein Determination---The concentration of protein was determined using a Bio-Rad protein assay kit [16] with bovine serum albumin as a standard. The purified porcine renin concentration was estimated with a coefficient of 9.1 (280 nm) for a 1% solution with a 1 cm path-length [17].

Results

Effects of ATP on Renin Inhibition by RnBP---As shown in Table 1, RnBP inhibited the renin activity in a dose-dependent manner in the absence of ATP. However, the renin inhibition was neutralized by the addition of ATP. On the other hand, ATP had no effects on the renin activity in the absence of RnBP (data not shown). These results indicate that ATP interrupts the interaction of renin with RnBP, GlcNAc 2-epimerase. The following experiments were performed to clarify the effects of nucleotides on the formation of RnBP homodimer and renin-RnBP heterodimer.

Modulation of Renin Inhibition of RnBP by Nucleotides---The effects of other nucleotides on renin inhibition by RnBP are also shown in Figure 1. Deoxy nucleotides, such as dATP, dGTP, dCTP and dTTP, cancelled the inhibition of renin by RnBP. ADP had little effect on the cancellation of renin inhibition by RnBP. However, AMP had no effects of renin inhibition by RnBP.

Table 1 Inhibition of renin activity by RnBP in the presence or absence of ATP

RnBP (pg/ml)	Renin activity (-ATP)	Renin activity (+ATP)
none	100 %	100 %
100	62 %	98 %
200	51 %	94 %
400	39 %	91 %
800	38 %	80 %

The reaction mixture containing 20 mM sodium-phosphate buffer, pH 6.5, 1 mM EDTA, 10 μ M leupeptin, 0.1 % BSA, 65 pg pure renin and RnBP in the absence or presence of 5 mM ATP was incubated at 37℃ for 5 min. Then the residual renin activity was measured.

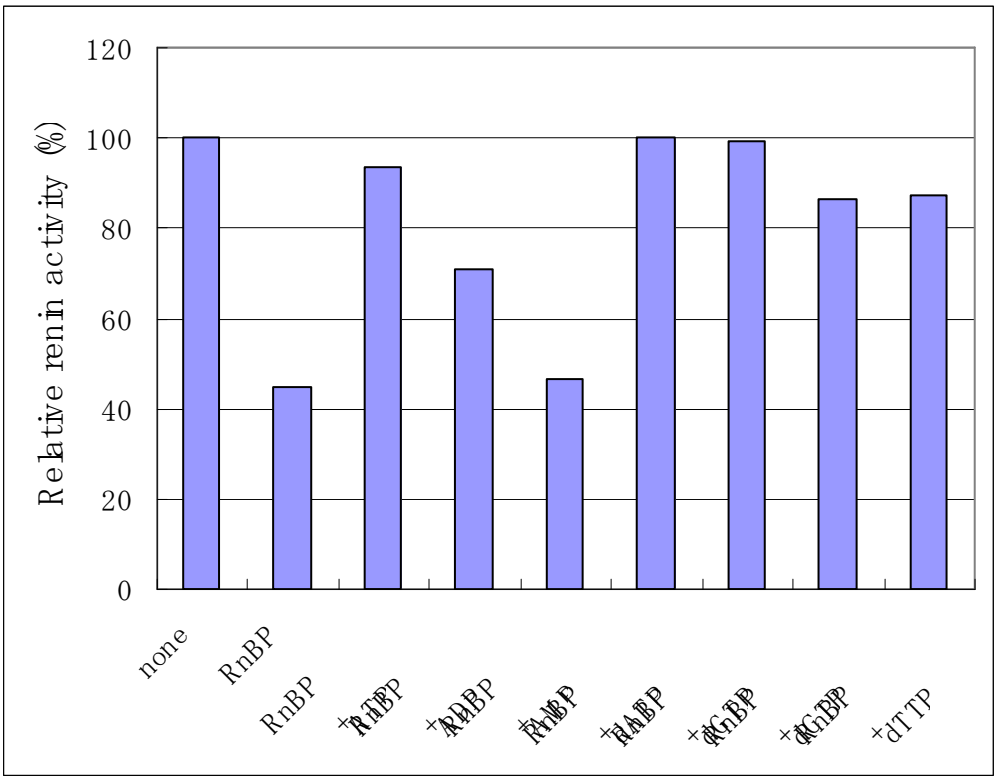

Figure 1 Effects of nucleotides on the renin inhibition by RnBP.
The purified renin (65 pg) was incubated with RnBP (800 pg) in the presence of 5 mM nucleotide. After the incubation at 37℃ for 5 min, the residual renin activity was measured.

Stabilization of Dimeric Form of RnBP by ATP ---It is well known that RnBP binds renin and forms a hetero complex, so-called HMW renin [2-4]. The purified porcine renin showed a single peak with an apparent molecular weight of 40.000. When renin was incubated with RnBP, the molecular weight of renin was shifted to about 60,000. The molecular weight of renin remained of 40,000 in the presence of ATP even after the incubation of renin with RnBP (data not shown). These results indicate that ATP inhibits formation of the renin-RnBP heterodimer.

Determinations of Molecular Weight by ESI TOF/MS ----- The molecular weights of rat GlcNAc 2-epimerase in the presence or absence of ATP were determined by ESI TOF/MS (Figure 2). Human and porcine enzymes showed no clear results because of the low affinity for the nucleotides.

However, rat enzyme showed reasonable signal in the presence or absence of ATP. The molecular weights of nucleotide free and bound form of rat enzyme were determined to be 48,606 and 49,103, respectively. These results indicate that one ATP (Mr. 507.18) bound per monomer.

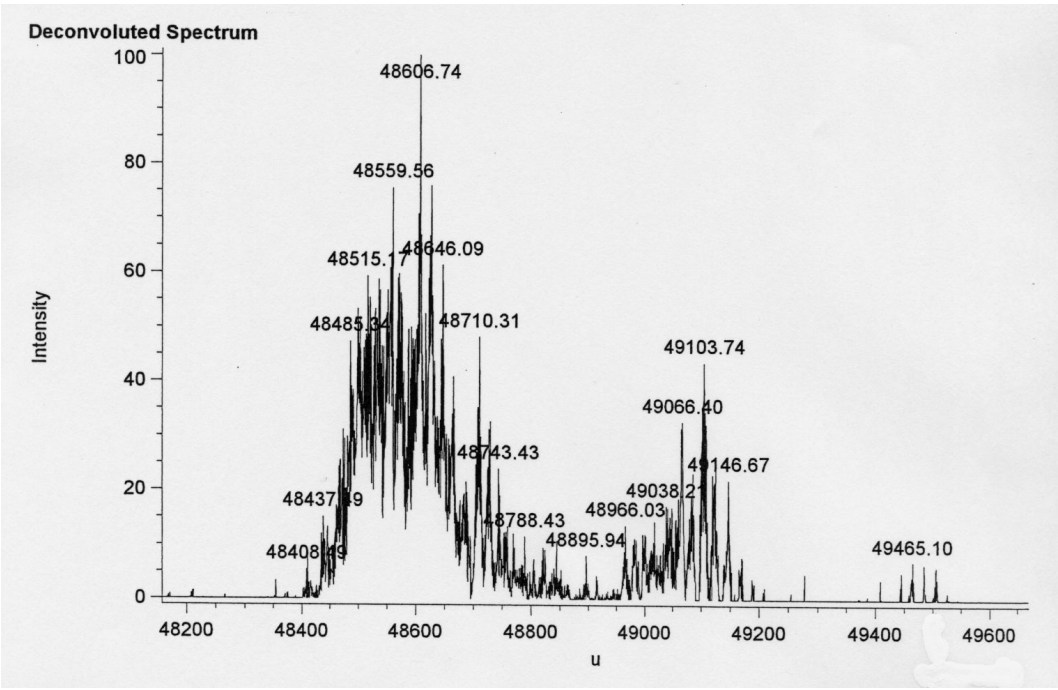

Figure 2 Deconvoluted spectrum of ESI TOF/MS of rat GlcNAc 2-epimerase

Discussion

The RnBP was firstly isolated from porcine kidney as a complex of renin. The protein bound to renin to form HMW renin. Recently, RnBP was identified as the enzyme GlcNAc 2-epimerase by the cDNA cloning of porcine enzyme [6] and the expression and characterization of human RnBP [7, 8]. Nucleotides such as ATP, dATP and ADP, were essential for GlcNAc 2-epimerase activity. However, the effects of nucleotide on the interaction of renin with GlcNAc 2-epimerase had not been understood. In this report, we studied the effects of several nucleotides on GlcNAc 2-epimerase and renin. Our previous studies indicated that RnBP inhibited renin activity in a dose dependent manner [3, 5] (Table 1). Interestingly, the inhibition was modulated in the presence of ATP. Moreover, nucleotides such as ADP, dATP, dGTP, dCTP and dTTP, were effective for the modulation of NEM treatment (data not shown). These nucleotides seem to stabilize GlcNAc 2-epimerase and to form active sites of the enzyme.

Taken together, nucleotides such as ATP, ADP and dATP, stabilize the dimeric form of GlcNAc 2-epimerase (RnBP). In the absence of nucleotide(s), RnBP can easily bind to renin to form a hetero-complex renin called HMW renin. On the other hand, the RnBP homodimer is hard to dissociate into monomers in the presence of nucleotide(s). Thus, nucleotides inhibit the formation of RnBP-renin hetro-complex. In the present results, it is clearly shown that ATP regulates the formation of HMW renin to modulate renin activity *in vitro*. Further elaborate studies eg. *in vivo* and cell experiments will provide the physiological function of RnBP, GlcNAc 2-epimerase.

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