Rapid Communication *J. Biochem.* 126, 639-642 (1999)

Identification of Cysteine-380 as the Essential Residue for the Human *N*-Acetyl-D-Glucosamine 2-Epimerase (Renin Binding Protein)[1]

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Received July 28, 1999; accepted August 25, 1999

Renin binding protein (RnBP) is a proteinous renin inhibitor firstly isolated from porcine kidney. Recently, the protein was identified as the enzyme, *N*-acetyl-D-glucosamine (GlcNAc) 2-epimerase. The GlcNAc 2-epimerase activity of recombinant human RnBP was specifically inhibited by SH-reagents such as *N*-ethylmaleimide, 5,5′-dithiobis-2-nitrobenzoate, and iodoacetic acid, indicating that the most probable reactive site is a cysteine residue. To identify the active site residue(s), we have constructed ten cysteine residue mutants (C41S, C66S, C104S, C125S, C210S, C239S, C302S, C380S, C386S, and C390S) for human GlcNAc 2-epimerase and expressed them in *Escherichia coli* cells. The relative specific activities of C41S, C66S, C125S, C210S, C239S, C302S, C386S, and C390S are nearly the same to that of the wild-type enzyme. The specific activity of the C104S mutant is 26% of that of the wild-type enzyme. The expression of the C380S mutant in *E. coli* cells was detected on Western blotting, whereas GlcNAc 2-epimerase activity was not detected in the extract. These results indicate that Cys380 is essential for the enzymatic activity of human GlcNAc 2-epimerase.

Key words: active site, binding protein, GlcNAc 2-epimerase, renin, site-directed mutagenesis.

Renin [EC 3.4.23.15] is a key enzyme in the renin-angiotensin-aldosteron cascade. The enzyme is mainly synthesized in the kidneys and released into the circulation by means of several stimuli, and controls blood pressure. Renin binding protein (RnBP) is an endogenous renin inhibitor firstly isolated from porcine kidney as a complex of renin, so called high molecular weight renin (*1, 2*). The purified porcine kidney RnBP exists as a dimer (*3*), and dissociates into monomers in the presence of sulfhydryl-oxidizing and -alkylating reagents (*4*). The primary structures of several animal RnBPs have been deduced from the nucleotide sequence of cDNAs and showed that RnBPs have a conserved leucine-zipper motif which plays an essential role in the formation of RnBP homodimers and RnBP-renin heterodimers (*5-9*). 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 (*10*). Moreover, the isolation of human (*11*) and rat (*12*) RnBP genes showed that both genes span about 10 kilobase pairs (kb) and consist of 11 exons separated by 10 introns. On the other hand, the cDNA cloning of porcine kidney *N*-acyl-D-glucosamine 2-epimerase [EC 5.3.1.8] (*13*), and the expression and characterization of human RnBP (*14*) showed that *N*-acetyl-D-glucosamine (GlcNAc) 2-epimerase was identical with RnBP. The enzyme catalyses the interconversion between GlcNAc and *N*-acetyl-D-mannosamine (ManNAc), and ATP is necessary as an effector (*13-15*). However, the catalytic residue(s) of the enzyme has not been identified, although animal RnBPs exhibit high homology with each other (*5, 6, 9*).

In the present study, ten conserved cysteine residues of human RnBP were replaced with serine residues so that we could identify the cysteine residue that is essential for the catalytic activity. The results indicate that cysteine 380 is the active site residue.

The wild-type recombinant human (rh) RnBP and rabbit anti-rhRnBP antiserum were prepared by the method of Takahashi *et al.* (*15*). The oligonucleotide-directed dual amber (ODA) method (*16*) involving a Mutan^R Super Expression system (Takara Shuzo) was used for the construction of ten cysteine-serine mutants of human GlcNAc 2-epimerase (RnBP). Table I shows the primers used for the construction of mutant plasmids. The 1.2 kb *Eco*RI fragment of pUKHRB6 (*14*), the wild-type rhRnBP expression vector, was ligated into the same site of pKF18K to construct pKHRB6. PCR was carried out in a 50 μl reaction mixture comprising 5 pmol selected primer, 5 pmol mutagenized primer, 10 ng of pKHRB6 as a template, 5 μl of 10×LA PCR buffer, 8 μl of 2.5 mM each dNTP mixture,

[1] This study was supported in part by a Research Grant from the Ministry of Agriculture, Forestry, and Fisheries of Japan.

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Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GlcNAc, *N*-acetyl-D-glucosamine; ManNAc, *N*-acetyl-D-mannosamine; NEM, *N*-ethylmaleimide; RnBP, renin binding protein; rhRnBP, recombinant human RnBP.

and 2.5 units of LA Taq™ DNA polymerase. The PCR comprised 30 cycles of denaturation (95°C, 1 min), annealing (55°C, 1 min), and extension (72°C, 3 min). The amplified DNA was used to transform *E. coli* MV1184 cells and the transformants were selected on a LB plate containing 50 μg/ml of kanamycin. Mutations were confirmed by sequence analysis with a Applied Biosystems 373S-1B DNA sequencer. The mutant cDNAs were subcloned into the newly developed *E. coli* expression vector, named pUK223-3 (*17*).

Western blotting was carried out by the method of Towbin *et al.* (*18*). Samples were loaded onto a 5-20% gradient polyacrylamide gel (PAGEL 520, ATTO) and then electrophoresed according to Laemmli (*19*) at a constant current of 20 mA. After the electrophoresis, proteins were transferred to a nitrocellulose membrane. The membrane was immersed in 20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween 20 (Buffer A) containing 5% skim milk, and then incubated for 2 h at room temperature with rabbit anti rhRnBP antiserum (1:1,000 dilution with Buffer A). After the incubation, the membrane was washed three times with Buffer A, incubated for 1 h at room temperature with alkaline phosphatase-conjugated anti-rabbit IgG (Fc) (1:5,000 dilution with Buffer A, Promega), and then washed three times with Buffer A. The membrane was allowed to react with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate for color development.

The purified rhRnBP (4 ng to 4,000 ng) or *E. coli* extracts, that were expressed as mutants or wild-type rhRnBP, were resolved by SDS-PAGE, with color development as described above. The RnBP bands were cut out and put into microtubes. The membranes were washed extensively with Buffer A, and then incubated for 30 min at 25°C with 0.5 ml of 5 mg/ml of *p*-nitorophenyl phosphate in 0.1 M Tris-HCl, pH 9.5, containing 5 mM $MgCl_2$. The reaction product, *p*-nitrophenol, was quantified by measuring the absorbance at 405 nm. The Western blot and standard curve are shown in Fig. 1.

GlcNAc 2-epimerase activity was measured as the rate of formation of GlcNAc from the substrate, ManNAc. The reaction mixture, 100 μl, comprising 0.1 M Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$, 50 mM ManNAc, 5 mM ATP, and 20 μl of enzyme solution, was incubated for 30 min at 37°C. The reaction was terminated by 5 min boiling, and the reaction product was quantified by high performance liquid chromatography with pulsed amperometric detection using a DIONEX Bio-CL gradient pump and a DIONEX Carbopac PA-1 column equipped with a DIONEX Model PAD 2 detector.

Effects of SH-Reagents—Table II shows the effects of sulfhydryl-oxidizing or alkylating reagents on rhRnBP as GlcNAc 2-epimerase activity. *N*-Ethylmaleimide (NEM) (1 mM), monoiodoacetic acid (10 mM), or 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) (0.1 mM) inhibited more than 95% of the GlcNAc 2-epimerase activity. The molecular weights of normal rhRnBP and SH-reagent-treated RnBP were estimated to be 82,000 and 44,000, respectively, by gel filtration (data not shown). Our previous studies (*4, 8*) showed that porcine and rat RnBPs exist as dimers. The dimers dissociate into monomers in the presence of an SH-oxidizing or -alkylating reagent. These results imply that the dimerization is essential for the functional activity of human GlcNAc 2-epimerase. Moreover, the active site residues seem to be cysteine residue(s) in RnBP molecules. The human, porcine, and rat RnBP sequences are highly homologous, the identity being 87, 86, and 83% between the

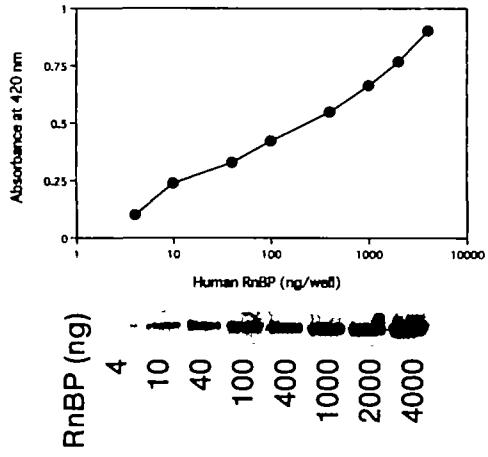

Fig. 1. **Quantitative Western blotting of rhRnBP.** The top panel shows the standard curve for the rhRnBP. The bottom panel is a Western blot. The purified rhRnBP was used as a standard.

TABLE I. **Primers for the mutant RnBPs.**

Mutant	Location of nucleotide change	Nucleotide sequence
C41S	121 T→A	5′TTCTTCACGAGCCTTGGCCG3′
C66S	196 T→A	5′ATGGATGTATAGTCGCCTGTAC3′
C104S	310 T→A	5′TGGCAAGAAGAGTGCCTTTGTG3′
C125S	373 T→A	5′CTTCAGTGAGAGTTTCTACAC3′
C210S	628 T→A	5′GGGACTGGAGCGCCCGGAGGAT3′
C239S	715 T→A	5′CTTCCTGGCAGCCTGGGGAGA3′
C302S	904 T→A	5′GATAACTTCAGCCCCACCCAG3′
C380S	1138 T→A	5′TTTCAAAGGCAGCTTCCACGTG3′
C386S	1156 T→A	5′GTGCCGCGGAGCCTAGCCAT3′
C390S	1168 T→A	5′CTAGCCATGAGCGAGGAGATG3′

*Changed nucleotides.

TABLE II. **Inhibition of recombinant human GlcNAc 2-epimerase activity.**

Reagent	Final conc. (mM)	Residual activity (%)
None	–	100
ICH_2COONa	0.5	73.3
	1.0	49.7
	5.0	12.5
	10.0	5.2
DTNB	0.1	N.D.
	0.5	N.D.
	1.0	N.D.
	2.0	N.D.
NEM	0.5	5.2
	1.0	0.9
	5.0	N.D.
	10.0	N.D.
DFP	10.0	102
NaF	10.0	108
NaN_3	10.0	82

N.D., not detected.

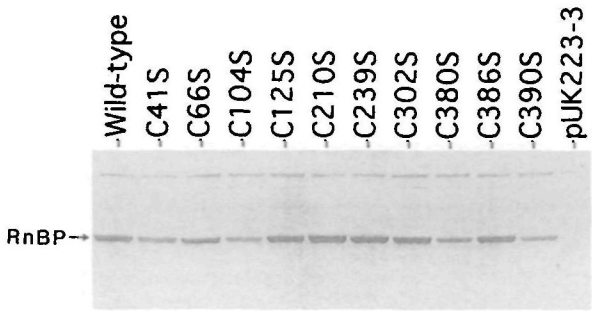

Fig. 2. **Western blotting of mutant RnBPs.** Five microliters of an *E. coli* extract harboring each mutant plasmid was applied on the gel. pUK223-3, expression vector without insert cDNA.

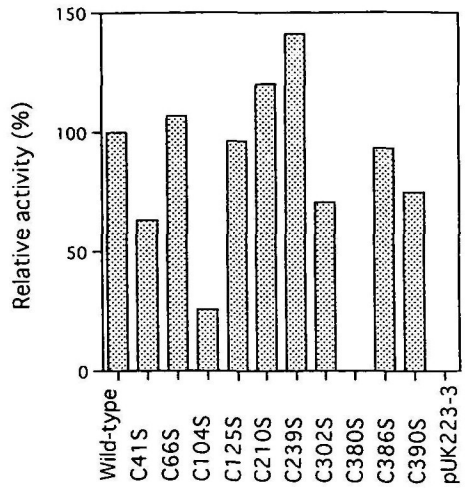

Fig. 3. **Relative specific activities of mutant RnBPs.** The expression of mutant RnBPs was normalized by quantitative Western blotting.

human, and porcine, human and rat, and rat and porcine RnBP, respectively (*6, 9*). In addition to the sequence similarities, 10 cysteine residues are also conserved (*6, 9*). Thus, we constructed 10 cysteine mutants for the identification of the active site residue(s) of human RnBP.

Expression of Mutant RnBPs in E. coli Cells—The *E. coli* expression vector, pUK223-3 (*17*), and *E. coli* JM109 cells were used for the expression of wild-type or mutant rhRnBPs. An overnight culture (1 ml) of JM109 cells harboring a different mutant plasmid was used to inoculate 50 ml of 2×YT medium (1.6% polypepton, 1.0% yeast extract, 0.5% NaCl, pH 7.0) containing 0.1 mg/ml of ampicillin. The culture was incubated at 30°C for 5 h, and then expression of the recombinant protein was induced with 1 mM isopropyl-β-D-thiogalactopyranoside. Cells were harvested 3 h after induction. The cells were sonicated with 5 ml of 20 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA, 10 μM leupeptin, and 0.05% 2-mercaptoethanol, and then centrifuged at 20,000×*g* for 30 min. The supernatant was used for Western blotting or assaying of GlcNAc 2-epimerase. When the extracts of *E. coli* cells harboring different plasmids were used for the Western blotting, only single protein bands corresponding the human RnBP were visible on the immunoblots (Fig. 2). The molecular weights (45,000) of mutated RnBPs expressed in *E. coli* JM109 cells were identical to that of the wild-type enzyme. As the levels of the synthesized recombinant proteins differed from each other, the mutant RnBP concentrations were normalized by quantitative Western blotting (Fig. 1). The relative specific activities of mutant RnBPs are summarized in Fig. 3. The activities of C41S, C66S, C125S, C210S, C239S, C302S, C386S, and C390S are nearly the same as that of the wild-type rhRnBP. The specific activity of the C104S mutant is about 26% that of the wild-type enzyme. The expression of the C380S mutant protein was detected on the Western bolt (Fig. 2). However, the GlcNAc 2-epimerase activity could not be detected even when the *E. coli* extract harboring the C380S mutant plasmid was incubated with the substrate solution for 20 h. These results clearly show that cysteine 380 is essential for the enzymatic activity of human GlcNAc 2-epimerase, RnBP. Cys-104 of human RnBP also seems to be important for the catalytic activity. This residue may participate in the substrate binding or the stabilization of the enzyme. To elucidate the detailed mechanisms of the enzyme reaction, further elaborate studies, *e.g.* detailed kinetic analysis or X-ray diffraction analysis, are necessary. We are now attempting to purify a large amount of rhRnBP expressed in *E. coli* cells and the crystallization of rhRnBP.

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