Molecular Cloning and Identification of N-Acyl-D-glucosamine 2-Epimerase from Porcine Kidney as a Renin-binding Protein*

(Received for publication, January 22, 1996, and in revised form, March 27, 1996)

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N-Acetylnearaminic acid (NeuAc) is an important molecule in biological recognition systems. NeuAc is known to be biosynthesized either from UDP-N-acetyl-D-glucosamine by an action of UDP-N-acetyl-D-glucosamine 2-epimerase or from N-acetyl-D-glucosamine by N-acetyl-D-glucosamine 2-epimerase (GlcNAc 2-epimerase). However, the physiological function of the GlcNAc 2-epimerase in NeuAc biosynthesis has not been fully evaluated. To clarify the role of GlcNAc 2-epimerase in NeuAc biosynthesis, the enzyme and its gene were isolated from porcine kidney cortex. Escherichia coli cells transformed with the gene expressed the GlcNAc 2-epimerase having the same properties as those of the GlcNAc 2-epimerase from porcine kidney. Sequence analysis indicated that the gene was capable of synthesizing a 46.5-kDa protein (402 amino acids) with a conserved leucine zipper motif. Homology search for the cloned gene revealed that the GlcNAc 2-epimerase was identical with renin-binding protein (RnBP) in porcine kidney (1). 

To clarify reaction mechanism and function of the GlcNAc 2-epimerase, we have isolated from porcine kidney and analyzed their properties. Surprisingly, the GlcNAc 2-epimerase activity is modulated by the catalytic amount of ATP. Datta (7) also reported that the GlcNAc 2-epimerase possesses two distinct interaction sites, a catalytic site for substrate and an allosteric site for ATP.

In this paper, we report purification and molecular cloning of GlcNAc 2-epimerase from porcine kidney, and demonstrate the renin-binding ability of GlcNAc 2-epimerase.

EXPERIMENTAL PROCEDURES
Materials
Porcine kidney was purchased from Sigma. DEAE-cellulose DE-52 was from Whatman. Q Sepharose FF, Superose 12 HR 10/30, and Mono Q H R 5/5 were from Pharmacia Biotech Inc. Butyl-Toyopearl 650M was from Tosoh. Hydroxylapatite and lysyl endopeptidase were from Wako Pure Chemical Industries. The ZAP-cDNA synthesis kit, Gigapack II Gold packaging extract, and the ExaMung

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GlcNAc 2-Epimerase Assay

GlcNAc 2-epimerase was assayed by measuring interconversion of GlcNAc and ManNAC. The reaction mixture (0.1 ml) consisted of 100 mM Tris-KCl, pH 7.4, 40 mM ManNAC, 0.01 M MgCl₂, 20 μl of enzyme. After incubation at 37°C for 30 min, the reaction was stopped by boiling for 3 min. The reaction products were treated with 1-phenyl-3-methyl-5-pyrazolone, and the derivatives were analyzed by HPLC (18). One unit of enzyme activity was the quantity that produced 1-phenyl-3-methyl-5-pyrazolone, and the derivatives were analyzed by HPLC (18). One unit of enzyme activity was the quantity that produced 1-phenyl-3-methyl-5-pyrazolone.

Purification of GlcNAc 2-Epimerase

Unless otherwise noted, all operations were carried out at 0–4°C. The potassium phosphate buffer, pH 7.6 (KPB), was used, always contained 1.0 mM EDTA and 0.05% 2-mercaptoethanol. Centrifugation was carried out at 16,000 x g for 30 min, and dialysis was for 16 h against 20 mM KPB.

GlcNAc 2-Epimerase in Porcine Kidney—Kidney cortex (5.6 kg) was homogenized in 12 liters of 3.0 mM KPB. The supernatant (11.7 liters) obtained after centrifugation was dialyzed with 11.7 liters of cold water followed by adding 705 ml of 2.0% progamine sulfatone. Precipitated materials were removed, and the supernatant (22.9 liters) was treated again with 2.3 liters of 2.0% progamine sulfatone. Precipitates were washed three times with 3.0 mM KPB containing 150 mM KCl, and then the enzyme was incubated for 10 min with 58 g of bentonite, which was suspended in 580 ml of 1 mM EDTA. The mixture was centrifuged, and the supernatant was dialyzed. The dialysate (6.5 liters) was put on a DEAE-cellulose DE-52 column (25 x 13 cm). After washing the column with 50 mM KPB containing 100 mM KCl, the GlcNAc 2-epimerase was eluted with 200 mM KPB containing 300 mM KCl. Ammonium sulfate (6.7 kg) was added to the eluate (12 liters) to 80% saturation. The precipitates were dissolved in 200 ml of 20 mM KPB and dialyzed. The dialysate (280 ml) was put on a hydroxyapatite column (2.6 x 9.5 cm) equilibrated with 10 mM KPB, and the enzyme was eluted with 324 ml of the same buffer. The enzyme was treated with ammonium sulfate (80%) and dialyzed. The dialysate (40 ml) was put on a Q Sepharose column (2.6 x 10 cm), and then the enzyme was developed with a linear gradient (1 liter) of 0.1 M–4 M KCl in 20 mM KPB. The GlcNAc 2-epimerase was eluted at 180–220 mM KCl. Active fractions, which were eluted between 180 and 220 mM KCl, were concentrated with ammonium sulfate (80%) and used throughout this study after dialysis.

GlcNAc 2-Epimerase Expressed in E. coli...E. coli XL-1 Blue were transformed with a plasmid pEP114 (see "cDNA Library, Subcloning, and Nucleotide Sequencing") for 30 min in the presence of or absence of the GlcNAc 2-epimerase (140 pmol) in the mixture (0.1 ml) as described above. After incubation, the mixture was put on a Superoxose 12 HR 10/30 column equilibrated with 50 mM KPB containing 150 mM KCl and incubation products were eluted with the same buffer at 1.0 ml/min. Enzyme activity in each fraction (0.5 ml) was determined as described above.

cDNA Library, Subcloning, and Nucleotide Sequencing

Total RNAs were extracted from porcine kidney cortex in acid guanidium thiocyanate-phenol-chloroform mixture (20), and poly(A)+ RNA was fractionated on oligo(dT)-cellulose column chromatography. The cDNA library was constructed as described by Gubler and Hoffman (21) by using a DNA phage (λ ZAP) vector and E. coli SURE host. The cDNA library of 1.2 x 10⁸ clones was screened by immunostaining using a monoclonal antibody against the protein of the GlcNAc 2-epimerase and the alkaline phosphatase-conjugated anti-rabbit IgG (goat). One of the positive clones was screened to phagemids carrying cDNA insert (1.4 kilobase pairs) in the sense orientation between EcoRI and Xhol sites of pBluescript SK(−) by in vivo excision in E. coli XL-1 Blue host with R408 helper phage, and plasmid thus constructed was designated pEP11. Sequential unidirectional deletion of the recombinant plasmid pEP11 was carried out by deavage at a unique SacI site of multicloning site, followed by digestion with exonuclease III and mung bean nuclease. These deletion fragments were self-ligated with T4 DNA ligase, and the recombinant plasmids were used to transform E. coli XL-1 Blue. One of the recombinant plasmids thus generated had a deletion of about 60 base pairs of nucleotide sequence localized 5’ terminus in cDNA 5’-noncoding region and was designated pEP114. The nucleotides of cDNA were sequenced in both strands by the dideoxy sequencing method of Sanger et al. (22).

Preparation of Peptide and Amino Acid Analysis

GlcNAc 2-epimerase (0.5 mg) from porcine kidney cortex was digested with 5 μg of lysyl endopeptidase in 0.5 ml of 100 mM Tris-HCl, pH 8.0, containing 4 x 10⁵ urea at 37°C for 12 h. After reaction, the peptides were separated with a reversed-phase HPLC column (µBondapak 5 μ C18–300 Å, 3.9 x 150 mm, Waters). N-terminal amino acids of protein or peptide were sequences by automated Edman degradation (23) on an Applied Biosystems protein sequencer (model 477A). C-terminal amino acids of protein were sequenced using carboxypeptidase Y by Klemm et al. (24). Free amino acids and hydrolyzed peptides were constant-boiling hydrochloric acid at 112°C for 24 h were analyzed with a Hitachi amino acid analyzer (model L8500).

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done with 10–20% gradient gel as described by Laemmli (25). Proteins were stained with Coomassie Brilliant Blue R-250.

Computer Analysis

Homology analyses with other nucleotide and protein sequences were done using the FASTA comparison program (26) with the GenBank/EMBL data base of nucleotide sequence and PIR data base of amino acid sequence.
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RESULTS AND DISCUSSION

Properties of GlcNAc 2-Epimerase—We have purified the GlcNAc 2-epimerase from porcine kidney cortex (Table I). Overall purification achieved was approximately 630-fold with 3% recovery of activity. Specific activity of the enzyme was 21 units/mg of protein, which was about 3.5-fold higher than that of the preparation of Datta (7). The molecular mass of the enzyme was determined to be 45 kDa on SDS-PAGE (Fig. 1) and 93 kDa by sedimentation equilibrium (data not shown). This result suggested that the enzyme consists of two identical subunits of 45 kDa. The purified enzyme could be stored without loss of activity at −20°C for at least 6 months in 20 mM potassium phosphate buffer, pH 7.6, containing 1.0 mM EDTA, 0.05% 2-mercaptoethanol, and 5.0% sucrose. The optimum pH and temperature were 6.8 and 47°C, respectively, and catalyzed the interconversion of GlcNAc and ManNAc with apparent K_m values of 7.4 mM for GlcNAc, 6.3 mM for ManNAc, and 0.18 mM for an effector, ATP. ATP was not essential for the GlcNAc 2-epimerase reaction, but the activity of the enzyme was enhanced about 20-fold in the presence of ATP or deoxy-ATP.

Properties of GlcNAc 2-Epimerase Gene—A gene for the GlcNAc 2-epimerase was cloned by immunoscreening from a cDNA library for porcine kidney cortex. The plasmid with cDNA for the GlcNAc 2-epimerase was isolated, designated pEP114 and used for the structure analysis (Fig. 2a). Fig. 2b shows the 1372-nucleotide sequence of cDNA in pEP114. Examination of the nucleotide sequence showed an open reading frame starting at position 68 and ending at position 1273. The 1206-nucleotide reading frame encoded 402 amino acids with a hydropathy plot analysis (data not shown). C-terminal amino acid sequence was determined to be -Leu-Ala by carboxypeptidase Y digestion, which corresponded to the sequence at positions 86–102, respectively to the sequences at positions 4–12 and 86–102, respectively. Peptides (B and C) had sequences of Glu-Arg-Lys and Ala-Thr-Leu-Glu-Arg with pEP114, respectively, under the HPLC conditions. The recombinant plasmid pEP114 constructed contains a 1.4-kilobase pair cDNA fragment between EcoRI and XhoI sites of a vector pBluescript SK(−). When the host cells were transformed with the plasmid, the cells apparently showed the GlcNAc 2-epimerase activity, although the specific activity of the GlcNAc 2-epimerase was almost the same as that of the enzyme in porcine kidney homogenate. To enhance the expression level of the clone cDNA, the cDNA was expressed in E. coli XL1-Blue having no GlcNAc 2-epimerase activity (Table III). The recombinant plasmid pEP114 was purified from porcine kidney cortex (Table II).

Expression of GlcNAc 2-Epimerase in E. coli—In order to confirm that the GlcNAc 2-epimerase is the product of the cloned cDNA, a 1.4-kilobase pair cDNA fragment between EcoRI and XhoI sites of a vector pBluescript SK(−). When the host cells were transformed with the plasmid, the cells apparently showed the GlcNAc 2-epimerase activity, although the specific activity of the GlcNAc 2-epimerase was almost the same as that of the enzyme in porcine kidney cortex (Table II). The recombinant plasmid pEP114 was purified approximately 57-fold from cell extracts with 19% of activity yield (Table IV). The purified GlcNAc 2-epimerase was homogeneous on SDS-PAGE (Fig. 1) and was identical with the GlcNAc 2-epimerase purified from porcine kidney cortex in molecular size (45 kDa on SDS-PAGE), affinity for substrates GlcNAc (K_m = 7.5 mM) and ManNAc (K_m = 7.5 mM), and catalysis of enzymatic activity.

| Steps                    | Total protein | Total activity | Specific activity | Purification | Yield |
|-------------------------|---------------|----------------|-------------------|--------------|-------|
| Crude extract           | 284,000       | 9,500          | 0.033             | 1            | 100   |
| Protamine concentration | 29,000        | 4,300          | 0.15              | 5            | 45    |
| Bentonite adsorption    | 5,640         | 4,270          | 0.76              | 23           | 45    |
| DEAE-cellulose          | 391           | 1,150          | 2.9               | 88           | 12    |
| Hydroxyapatite          | 76            | 620            | 8.2               | 248          | 7     |
| Q Sepharose             | 23            | 463            | 20.1              | 609          | 5     |
| Mono Q                  | 16            | 332            | 20.8              | 630          | 3     |

**Table I**

Purification of GlcNAc 2-epimerase from porcine kidney cortex.

**Fig. 1. SDS-PAGE of purified GlcNAc 2-epimerase.** Purified GlcNAc 2-epimerases (2 μg) were purified from porcine kidney cortex (lane 2) and E. coli XL1-Blue carrying pEP114 (lane 3) analyzed on SDS-PAGE. Standard proteins in lane 1 were (from top): phosphorylase b (97.4 kDa), bovine serum albumin (66.3 kDa), carbonic anhydrase (30.0 kDa), soybean trypsin inhibitor (20.1 kDa), and lysozyme (14.4 kDa).
FIG. 2. Nucleotide and deduced amino acid sequences for GlcNAc 2-epimerase gene. To attain accurate comparison with the reported nucleotide and amino acid sequences for RnBP gene from porcine kidney (7), the nucleotide sequence of GlcNAc 2-epimerase (b) was determined extensively according to the strategies shown in (a). Nucleotides are numbered to the right, beginning with the first nucleotides of the cDNA insert preceded by EcoRI site. The predicted amino acid residues are indicated below the nucleotide triplet. Thin underlined amino acid residues were determined by amino acid sequencing of porcine GlcNAc 2-epimerase prior to cloning. The polyadenylation signal (AATAAA) is indicated by a double underline. The potential asparagine-linked glycosylation site is indicated by a broken underline. The leucine residues included in the leucine zipper motif are indicated by a bold underline.
for Arg, and Glu-318 for Gln, respectively. These results strongly imply that, in porcine kidney, GlcNAc 2-epimerase is the RnBP.

This conclusion was further evidenced by the following facts. The molecular mass of 45 kDa (SDS-PAGE) or 46.4 kDa (calculated from deduced amino acid sequence; Fig. 2b) determined for the GlcNAc 2-epimerase was closely similar to the reported molecular mass for porcine kidney RnBP (42 kDa) (9). The nucleotide sequence of porcine kidney RnBP contains four leucine residues at positions 185, 192, 199, and 206 comprising a proposed leucine zipper motif region (10, 28), which is believed to play an essential role in the formation of RnBP homodimer and RnBP-renin heterodimer (28). The same arrangement for leucine zipper motif was also recognized in the GlcNAc 2-epimerase (Fig. 2b). Furthermore, the amino acid sequence of the porcine kidney GlcNAc 2-epimerase was highly homologous to that of RnBPs from human and rat kidneys (29), with identity of 87.8% and 83.1%, respectively.

Inhibition of Renin by GlcNAc 2-Epimerase—To directly confirm that the GlcNAc 2-epimerase is a RnBP, the purified porcine kidney enzyme was incubated with porcine kidney renin and renin activity was determined (Fig. 3). As anticipated, the renin (1.4 pmol) activity was apparently inhibited, and the inhibition was 50% in the presence of 10 pmol of the enzyme. Similar results were also confirmed when the GlcNAc 2-epimerase purified from E. coli XL1-Blue carrying pEP114 was used in place of the enzyme purified from porcine kidney cortex. Although the inhibition was lower level, the porcine kidney renin activity was also inhibited by rat kidney GlcNAc 2-epimerase, which was isolated by the same methods for the purification of porcine kidney enzyme (Fig. 3). This was presumably due to the low affinity of rat kidney GlcNAc 2-epimerase toward porcine kidney renin.

Formation of Higher Molecular Mass Renin with GlcNAc 2-Epimerase—By the incubation of porcine kidney GlcNAc 2-epimerase with porcine kidney renin, higher molecular mass protein complex was formed (Fig. 4). Similar result was also obtained when rat kidney GlcNAc 2-epimerase was incubated with porcine kidney renin (data not shown). The molecular mass of the complex was determined to be about 70 kDa. Higher molecular mass renin (60 kDa) has been isolated from porcine kidney (12), and it was shown to be a heterodimer of renin and RnBP subunit (9, 19). The formation of higher molecular mass renin, although the molecular mass of the complex (70 kDa: Fig. 4) was slightly low compared with that calculated for Arg, and Glu-318 for Gln, respectively. These results strongly imply that, in porcine kidney, GlcNAc 2-epimerase is the RnBP.

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The GlcNAc 2-epimerase purified from porcine kidney was incubated with renin from porcine kidney. a, renin; b, complex between renin and GlcNAc 2-epimerase. Arrows indicate the elution positions of standard proteins: (from left) thyroglobulin (TG, 669 kDa), ferritin (FE, 440 kDa), catalase (CA, 232 kDa), aldolase (AL, 158 kDa), bovine serum albumin (BS, 67 kDa), ovalbumin (OV, 43 kDa), chymotrypsinogen A (CH, 25 kDa), and ribonuclease A (RI, 13.7 kDa). Vo, void volume estimated with blue dextran 2000 (200 kDa).

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from the molecular masses of renin (36 kDa) (30) and the GlcNAc 2-epimerase (45 kDa, SDS-PAGE). The similar discrepancy in molecular mass has also been indicated in the case of renin and RnBP from porcine kidney (9), and the reason for this discrepancy is attributed to the unique hydrodynamic features such as leucine zipper motif and hydrophobic domain of the RnBP molecule (10, 29). It was suggested that hydrodynamic features in the RnBP molecule mediate the formation of both the RnBP-renin heterodimer (higher molecular mass renin) and the RnBP homodimer (28).

All the data including sequence similarity confirmed that GlcNAc 2-epimerase was the RnBP. However, there is a discrepancy between the tissue sources of GlcNAc 2-epimerase and RnBP. The GlcNAc 2-epimerase has been found in kidney, liver, spleen, brain, intestinal mucosa, thymus, pancreas, and in salivary gland (4, 5), but RnBP was very slightly in liver or salivary gland (14). This question should be elucidated with further investigations.

In conclusion, we have shown that, in porcine and rat kidneys and possibly in other tissues, the RnBP is the enzyme GlcNAc 2-epimerase. The identification of the RnBP as an enzyme GlcNAc 2-epimerase may open new insight into the physiological function of the RnBP, since no definitive conclusion as to the intrinsic function of RnBP has been elucidated.

**Fig. 4. Binding activity to renin of GlcNAc 2-epimerase from porcine kidney.**