Molecular Properties of Recombinant Ovine Angiotensinogen

AKIO EBIHARA¹, TAKAO KONDOU², SATOKO MIZUNO², TSUTOMU NAKAGAWA², UDDIN MOHAMMAD NASIR³, YOSHITO INUI², AKIYOSHI FUKAMIZU^{1,4}, FUMIAKI SUZUKI⁵, YUKIO NAKAMURA² and KAZUO MURAKAMI¹

¹Institute of Applied Biochemistry, University of Tsukuba, Tsukuba, Ibaraki 305-8572, ²Department of Biotechnology, Faculty of Agriculture and ³United Graduate School of Agricultural Science, Gifu University, Gifu 501-1193, ⁴Center for Tsukuba Advanced Research Alliance, University of Tsukuba, Tsukuba, Ibaraki 305-8577, ⁵Department of Animal Science and Technology, Faculty of Agriculture, Gifu University, Gifu 501-1193, Japan

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ABSTRACT

Recombinant ovine angiotensinogen (oANG) was purified from conditioned medium of Chinese hamster ovary cells. Mass spectroscopic analysis showed that oANG has a molecular weight (M_r) of 52,126, although the M_r calculated from the amino acid sequence is 49,000. Dynamic light scattering analysis showed that oANG is a typical globular protein (M_r 49,100) and a monomer in solution. Little free thiol was present in oANG, indicating that the only two cysteine residues (Cys¹⁸ and Cys¹³⁷) form an intramolecular disulfide bond. A single deglycosylated form (M_r 49,000) was produced from intact oANG (M_r 52,000) by digestion with peptide-*N*-glycosidase F and any other deglycosylated form was not observed. This result suggests that oANG has a single site for *N*-glycosylation. Deglycosylated oANG had similar properties to intact oANG in pH stability, thermal stability and kinetic parameters to human renin. Three-dimensional structure model of oANG (Ala⁷⁰-Leu⁴⁴⁹) was built by a homology modeling method. In the model, the cleavage site by renin (Leu¹⁰-Leu¹¹) is estimated to localize far from the *N*-glycosylation site and near Cys¹³⁷, because the glycan attached did not affect reactivity of oANG to renin and a disulfide bond was formed between Cys¹⁸ and Cys¹³⁷.

Angiotensinogen is a plasma glycoprotein and is specifically cleaved by an aspartyl protease, renin (EC 3.4.23.15) to release the N-terminal decapeptide, angiotensin I (AI). AI is further hydrolysed

by angiotensin-converting enzyme (EC 3.4.15.1) to produce the octapeptide, angiotensin II, which regulates blood pressure and electrolyte balance (13).

Abbreviations: AI, angiotensin I; oANG, ovine angiotensinogen; CHO, Chinese hamster ovary; PNGase F, peptide-*N*-glycosidase F; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; T_{50} , temperature at which 50% of activity is lost

Ovine angiotensinogen (oANG) has two potential *N*-linked glycosylation sites (Asn-Xaa-Ser/Thr) at Asn²⁷¹ and Asn³³⁸ (23). Recently, we showed that recombinant oANG expressed in Chinese hamster ovary (CHO) cells is sialylated (6). As approximately 60% of *N*-linked glycans of a glycoprotein expressed in CHO cells are sialylated (1), glycans are probably attached to the glycosylation sites. *N*-Linked glycosylation influences biochemical properties of proteins (14, 20, 32). For example, human protein C mutant in which a specific glycosylation site is eliminated

Correspondence to: Dr Yukio Nakamura at the above address.

Fax: +81-58-293-2907;

Email: n-yukio@cc.gifu-u.ac.jp

by site-directed mutagenesis is activated by thrombin-thrombomodulin complex faster than the wild-type protein (14). These facts suggest that stability and reactivity of oANG can be affected by *N*-linked glycan. Furthermore, oANG has two cysteine residues at positions 18 and 137 (23), which are highly conserved in rat (26), murine (2) and human (18) angiotensinogens. These two cysteine residues form a disulfide bond in human angiotensinogen (11, 28). These findings raise the possibility that Cys¹⁸ and Cys¹³⁷ of oANG also form a disulfide bond and this information would be a good clue to assume location of cleavage site by renin (Leu¹⁰-Leu¹¹) in tertiary structure.

In this study, we purified recombinant oANG from conditioned medium of CHO cells, examined the formation of disulfide bond, the number and location of glycosylation sites and the function of *N*-linked glycan and built a three-dimensional structure model of oANG.

MATERIALS AND METHODS

Materials

Beta-n-octylglucoside was obtained from Sigma-Aldrich, Inc., Saint Louis, MO. Peptide-*N*-glycosidase F (PNGase F) from *Flavobacterium meningosepticum* (EC 3. 5. 1. 52) was obtained from Boehringer Mannheim GmbH, Germany. 5, 5'-Dithiobis-(2-nitrobenzoic acid) was obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. All other reagents were of pure grade. Human renin used in this study was produced as prorenin in CHO cells and activated by trypsin (25).

Expression and purification of recombinant oANG

Recombinant oANG was expressed in CHO cells (24) and purified by ammonium sulfate precipitation and CM-Toyopearl column chromatography (6). The purified oANG was used in all experiments.

Mass spectroscopic analysis

Recombinant oANG (2.5 pmol) was treated with a matrix solution containing sinapic acid, 0.1% trifluoroacetic acid and 30% acetonitrile. Spectrum was recorded on a Voyager RP (Perseptive Biosystems, Framingham, MA). The accelerating

voltage was 30 kV. The apparatus was calibrated in linear mode with single ($m/z=66,431$)- and double ($m/z=33,216$)-charged ions of bovine serum albumin as internal markers.

Dynamic light scattering analysis

Recombinant oANG (1 mg/mL in 25 mM imidazole-HCl buffer, pH 7.4) was analysed on a DynaPro 801-TC&MS instrument (Protein Solutions, Inc., Charlottesville, VA). Hydrodynamic radius was calculated from the result of light scattering with a software supplied by the manufacturer. Based on the value, molecular weight (M_r) was also estimated from a standard curve of M_r versus hydrodynamic radius for a number of well-characterized globular proteins.

Ellman assay

Recombinant oANG (20 μ M) was unfolded in 100 mM sodium phosphate buffer (pH 7.3) containing 7 M guanidine-HCl and 1 mM EDTA in a final volume of 90 μ L. Subsequently, 10 μ L of Ellman's reagent containing 2 mg/mL 5,5'-dithiobis-(2-nitrobenzoic acid) was added. After 15 min at room temperature, the absorbance at 412 nm was measured. Concentration of free thiol was determined using the molar extinction coefficient of 13,700 M⁻¹ cm⁻¹ (3).

Deglycosylation with PNGase F

Sodium dodecyl sulfate (SDS)-denatured oANG (25 μ g) was incubated at 23°C with 0.5 units of PNGase F in 50 μ L of 250 mM sodium phosphate buffer (pH 7.0) containing 0.1% (w/v) SDS, 0.2% (w/v) β -n-octylglucoside and 15 mM β -mercaptoethanol (8, 30). Intact oANG (100 μ g) was incubated at 23°C for 24 h with 40 units of PNGase F in 20 μ L of 200 mM sodium phosphate buffer (pH 7.0)(30). Progress of deglycosylation was monitored by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 19) followed by Coomassie blue staining.

AI-generating activity

Recombinant oANG (0.4 μ M) was incubated with human renin at 37°C for 30 min in 200 mM sodium phosphate buffer (pH 6) containing 7.4 mM diisopropyl fluorophosphate and 7.4 mM EDTA (22). The amount of AI generated was

measured by AI-enzyme-linked immunosorbent assay (AI-ELISA; 29).

Kinetic analysis

Various concentrations (0.0–1.0 μM) of recombinant oANG were incubated with human renin at 37°C for 30 min in a buffer containing 7.4 mM diisopropyl fluorophosphate and 7.4 mM EDTA (22). The rate of AI generation was determined by AI-ELISA. Michaelis constant (K_m) and maximum velocity (V_{max}) were estimated with a software, UltraFit (Biosoft, Cambridge, UK) with Michaelis-Menten equation as a fitting equation and were expressed as 95% confidence limits.

Modeling of three-dimensional structure

Angiotensinogen is a member of serine protease inhibitor superfamily (5). In amino acid sequence, oANG has 40% similarity to ovalbumin, a member of the superfamily (5, 23). Based on the atomic coordinate of ovalbumin (PDB code, 1OVA, Gly²⁴-Pro³⁹¹), three-dimensional structure model of oANG was built by a homology modeling method (Insight II with

homology 95.0, Biosym/MSI).

RESULTS

Mass spectroscopic analysis of recombinant oANG

The mass spectrum of recombinant oANG showed a single peak at m/z of 52,126 (Fig. 1A) and the peak at m/z of 26,063 corresponds to double-charged recombinant oANG. The theoretical M_r calculated from the amino acid sequence is 49,000 (23) and the difference in M_r of 3,126 is probably due to posttranslational modifications in CHO cells, mainly due to glycosylation.

Dynamic light scattering analysis of recombinant oANG

Recombinant oANG showed a narrow monomodal size distribution (Fig. 1B), as judged by the polydispersity of less than 15%, the baseline of 0.999 and the sum of squares of 0.579. The hydrodynamic radius of recombinant oANG was calculated to be 3.21 nm. The M_r was estimated to be 49,100, which is similar to the theoretical M_r of oANG (23).

Fig. 1 Physicochemical analysis of recombinant oANG. *Panel A*, matrix-assisted laser desorption ionization time-of-flight mass spectrum. Peaks at m/z of 22,145, 33,216 and 66,431 are derived from bovine serum albumin. *Panel B*, distribution of hydrodynamic radius determined by dynamic light scattering.

Ellman assay of recombinant oANG

The molar ratio of free thiol to oANG was 0.11 mol thiol per mol oANG.

Deglycosylation of recombinant oANG

SDS-denatured oANG was digested with PNGase F for different times of incubation to produce a series of partially deglycosylated molecules (Fig. 2A). During the digestion, a single band additionally appeared at the position of M_r 49,000 (Fig. 2A, lanes 2 to 5), whereas undigested oANG gave a single band at M_r of 52,000 (Fig. 2A, lanes 1 and 7). Any band did not appear between these two bands during the deglycosylation. Even in the presence of 10 times higher concentration of PNGase F, the migration distance was at the position of M_r 49,000 (Fig. 2A, lane 6) and the M_r is same as the theoretical M_r calculated from the amino acid sequence of oANG (23). These results indicate that SDS-denatured oANG is completely deglycosylated under the condi-

tions.

Intact oANG was completely deglycosylated to give also a single band at M_r of 49,000 (Fig. 2B, lane 2).

Stability of deglycosylated oANG

Deglycosylated and intact oANGs were stable between pH 4.4 and 9.4 in reactivity to human renin (Fig. 3A). The activities at pH 8.0 for deglycosylated and intact oANGs were 0.50 and 0.47 nM AI/min, respectively. Thermal stability of deglycosylated oANG was measured at pH 7.0 and above a temperature around 50°C, the activities of both oANGs gradually decreased (Fig. 3B). The maximal activities for deglycosylated and intact oANGs were 0.39 and 0.38 nM AI/min, respectively. The T_{50} for deglycosylated and intact oANGs were 53 and 54°C, respectively, where T_{50} means the temperature at which 50% of activity is lost.

Fig. 2 Deglycosylation of recombinant oANG with PNGase F. *Panel A*, partial digestion of oANG. SDS-denatured oANGs were incubated with 0.5 units of PNGase F for 5 min (lane 2), 20 min (lane 3), 60 min (lane 4) and 18 h (lane 5). In addition, denatured oANGs were incubated for 18 h either with 5 units of PNGase F (lane 6) or without the enzyme (lane 7). These preparations and intact oANG (lane 1) were subjected to SDS-PAGE. Each lane contained 1 μ g of oANG. M_r of marker protein was shown to the left of the panel. *Panel B*, deglycosylation of intact oANG. Intact oANG and SDS-denatured oANG were incubated with PNGase F (lanes 2 and 3, respectively). The preparation in lane 3 is the same as that in lane 6 of panel A. In addition, intact oANG was incubated without PNGase F (lane 4). These preparations and intact oANG (lane 1) were subjected to SDS-PAGE. Each lane contained 1 μ g of oANG.

Fig. 3 Stability of deglycosylated oANG. *Panel A*, pH stability. One micromolar of oANG was incubated at 37°C for 1 h in a universal buffer at the indicated pHs. The buffer used was 20 mM citric acid-20 mM potassium phosphate-20 mM boric acid-20 mM barbital-20 mM sulfuric acid (17). Treated oANG was assayed for AI-generating activity. Activities of intact oANG (open square) and deglycosylated oANG (closed triangle) are both expressed as the percentage of their values at pH 8.0. *Panel B*, thermal stability. One micromolar of oANG was incubated in the universal buffer, pH 7.0 for 1 h at various temperatures from 37 to 70°C. Treated oANG was assayed for AI-generating activity. Activities of intact oANG (open square) and deglycosylated oANG (closed triangle) are expressed as the percentage of their values at 50 and 45°C, respectively.

Reactivity of deglycosylated oANG to human renin

Deglycosylated and intact oANGs had a similar pH dependence of the reactivity to human renin and showed two pH optima, 5.7 and 8.4 (data not shown). The presence of two pH optima for reactivity of oANG to human renin is consistent with the previous reports by Do *et al.* (4) and Nasir *et al.* (25). At the two pH optima, kinetic parameters of deglycosylated oANG to human renin were measured and at both pHs, K_m and V_{max} for deglycosylated oANG were similar to those for intact oANG (Table 1).

Three-dimensional structure model of oANG

Fig. 4 shows the three-dimensional structure model of oANG. A structure of 69 N-terminal residues could not be modeled, as the atomic coordinates of counter residues could not be obtained in the Protein Data Bank. Two potential N-linked glycosylation sites (Asn²⁷¹ and Asn³³⁸) and Cys¹³⁷ are located on the protein surface, indicating that Asn²⁷¹ and Asn³³⁸ can be glycosylated and Cys¹³⁷ can participate in a disulfide bond.

DISCUSSION

Dynamic light scattering analysis showed that recombinant oANG is a typical globular protein

Table 1 Kinetic parameters of deglycosylated oANG

oANG	pH 5.7		pH 8.4	
	K_m (μ M)	V_{max} (nM/min)	K_m (μ M)	V_{max} (nM/min)
Intact	0.26 +/− 0.05	0.45 +/− 0.03	0.31 +/− 0.11	1.1 +/− 0.1
Deglycosylated	0.22 +/− 0.04	0.47 +/− 0.03	0.33 +/− 0.06	1.2 +/− 0.1

Fig. 4 Three-dimensional structure model of oANG. The backbone of α -carbon is shown. The structure model has Ala⁷⁰ to Leu⁴⁴⁹ and positions of these residues are indicated by the letters, N and C, respectively. Positions of Cys¹³⁷, Asn²⁷¹ and Asn³³⁸ are shown in the model.

and exists as a monomer in the solution. This finding provides physicochemical evidence that oANG reacts with renin as a monomer.

Ellman assay showed that little free thiol is present in recombinant oANG preparation. This result indicates that two cysteine residues of oANG form an intramolecular disulfide bond, because recombinant oANG was a monomer in solution and has only two cysteine residues (Cys¹⁸ and Cys¹³⁷). Such disulfide bond was reported in human angiotensinogen at the residues between Cys¹⁸ and Cys¹³⁸ (11, 28). These disulfide bonds are, therefore, thought to have a common function in the angiotensinogen molecule.

During the deglycosylation of recombinant oANG, any band did not appear between two bands with M_r of 52,000 and 49,000 (Fig. 2A) and the decrement in M_r after the deglycosylation was 3,000. The absolute value of the decrement is same as that of the increment in M_r after addition of a single *N*-linked glycan to oANG in CHO cells (16). Previously, we deduced the amino acid sequence of oANG from cDNA and showed that oANG has Asn²⁷¹-Thr²⁷²-Thr²⁷³-Ser²⁷⁴ and Asn³³⁸-Leu³³⁹-Ser³⁴⁰-Pro³⁴¹ (23). In the glycosylation site of Asn-Xaa₁-Ser/Thr-Xaa₂, Pro residue either at the position Xaa₁ or Xaa₂ usually blocks the glycosylation at Asn residue (9, 21, 27). These facts indicate that Asn²⁷¹ is the candidate site for the glycosylation with higher probability rather than Asn³³⁸ in recombinant oANG.

Deglycosylated oANG was similar to intact

oANG in pH stability, thermal stability (Fig. 3) and kinetic parameters to human renin (Table 1). This indicates that stability and reactivity of recombinant oANG are retained after removal of the glycan attached. Recently, Gimenez-Roqueplo *et al.* (10) produced various mutants of human angiotensinogen in which glycosylation sites are eliminated by site-directed mutagenesis, expressed the mutants in CHO cells, and investigated the function of *N*-linked glycan. They suggested that *N*-linked glycans at Asn¹³⁷, Asn²⁷¹ and Asn²⁹⁵ do not affect reactivity of human angiotensinogen to human renin, although the glycan at Asn¹⁴ significantly decreases the affinity to renin. Moreover, thermal stability of the completely deglycosylated mutant is identical to that of the wild-type. Our results in this study agree with those of human angiotensinogen reported by them (10). In the three-dimensional structure model of oANG (Fig. 4), a structure of 69 *N*-terminal residues could not be modeled and location of the cleavage site by renin (Leu¹⁰-Leu¹¹) and Cys¹⁸ remained unclear. However, several observations give a good clue to assume location of the cleavage site by renin in tertiary structure. Firstly, Fig. 1B and Ellman assay showed that Cys¹⁸, which localizes near the cleavage site by renin, was found to form a disulfide bond with Cys¹³⁷ in oANG. Secondly, the glycan attached to recombinant oANG did not affect reactivity of this protein to renin (Table 1). Thirdly, Gimenez-Roqueplo *et al.* (10) and Inui *et al.* (16) reported that a glycan near the cleavage site by renin significantly affects the reactivity of oANG to renin. These observations suggest that the cleavage site by renin localizes far from Asn²⁷¹ and near Cys¹³⁷ in the structure model.

Plasma oANG exhibits a higher affinity for human renin than human angiotensinogen and has contributed as a better renin substrate in a clinical assay (12). We showed that recombinant oANG has little free thiol and exists as a monomer in solution. On the other hand, two free thiols in human angiotensinogen are shown to form disulfide bonds with another angiotensinogen molecule and other protein (11). The complex of angiotensinogen with other protein is hydrolysed by renin slower than angiotensinogen (11). Since the complex formation of human angiotensinogen seems to decrease the reactivity to renin, the absence of free thiol may be advantageous to oANG as the renin substrate. Furthermore, recombinant oANG had a single *N*-linked

glycan and showed a narrow monomodal size distribution. A protein with free thiols on the surface (31) or a heavily glycosylated protein (15) sometimes fails to crystallize and a protein with a monomodal distribution is likely to crystallize (7). Recombinant oANG used in this study may be useful to obtain crystals for X-ray diffraction analysis.

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