Role of N-Glycosylation in Human Angiotensinogen*

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Human angiotensinogen, the specific substrate of renin, is a heterogeneous glycoprotein constitutively secreted by the liver. Different glycosylation levels may be responsible for its heterogeneity. It contains four putative asparagine-linked glycosylation sites (Asn-X-Ser/Thr). Systematic site-directed mutagenesis (Asn replaced with Gln) of these four sites was undertaken, and 11 (single, double, triple, and quadruple (N-4)) mutants were produced in COS-7 and/or CHO-K1 cells and characterized. All of the sites were N-glycosylated with preferential glycosylation of the Asn14 and the Asn271. The suppression of the Asn14 glycosylation site led to 5 times lower $K_m$ and a 10 times lower $k_{cat}$. Angiotensinogen heterogeneity was much lower for the N-4 mutant protein, which produced a single form at 48 kDa. Pulse-chase experiments showed slight intracellular retention (15%) of the deglycosylated protein after 24 h. Interestingly, the N-4 mutant had a higher catalytic efficiency ($k_{cat}/K_m = 5.0$ versus $1.6 \mu mol^{-1} s^{-1}$) than the wild-type protein. The thermal stability of the N-4 protein was unaffected by deglycosylation, suggesting that it was correctly folded. This deglycosylated recombinant human angiotensinogen could be of value for x-ray crystallography studies.

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* The abbreviations used are: Ang I, angiotensin I; AGT, angiotensinogen; PAGE, polyacrylamide gel electrophoresis; hAGT, human AGT; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay.

probably results from the strong linkage disequilibrium between the M235T polymorphism and the G→A substitution in the promoter (5). However, rare missense mutations within the coding region may also affect function. One such mutation at the renin cleavage site, L10F, was first identified in a pre-eclamptic woman and caused a doubling in the catalytic efficiency of the renin angiotensinogen reaction (6). We have studied a family with another rare missense mutation, Y248C, which generates an extra N-glycosylation site leading to the production of an additional 61-kDa form when the cDNA is expressed in CHO cells. The parallelism between the intracellular retention observed in vitro and the decrease in plasma angiotensinogen concentration in affected homozygous individuals highlighted the involvement of glycosylation in the intracellular processing of angiotensinogen.

Plasma human angiotensinogen is a heterogeneous glycoprotein mainly produced by hepatocytes. Pure human angiotensinogen usually gives at least two major bands at about 60 kDa, separated by 3 or 4 kDa in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (for a review, see Ref. 8). A similar level of heterogeneity has been reported for other mammalian species (9–14). Treatment of pure human plasma angiotensinogen with trifluoromethane sulfonic acid or glycopeptidase F reduces its apparent molecular weight by 10 kDa, but angiotensinogen still displays two or three bands in SDS-PAGE (15).

These data suggest that glycosylation plays a predominant role in angiotensinogen heterogeneity.

However, the effect of human angiotensinogen glycosylation on protein secretion, stability, and reaction with renin is not fully understood. Hilgenfeldt et al. (16) reported differences in the half-lives of the two major glycoforms in rats; the highly glycosylated form was secreted faster by the liver and eliminated more slowly by the kidney than the less glycosylated form. Human angiotensinogen has four potential sites for asparagine-linked glycosylation (Asn-X-Ser/Thr): Asn14-Lys15-Ser16, Asn137-Cys138-Thr139, Asn271-Thr272-Thr273, and Asn295-Phe296-Ser297. The first site (Asn14-Lys15-Thr16) is closest to the renin cleavage site (Leu10-Val11) and may play a key role in the recognition of human angiotensinogen by human renin (17). However, it is not known if all four Asn-X-Ser/Thr sequences are actually glycosylated.

We describe herein the site-directed mutagenesis of single N-glycosylation sites and combinations of them in 11 mutants of human AGT (hAGT). Analysis of these mutant proteins, synthesized in eukaryotic cells, indicates that oligosaccharide residues are linked to each site, accounting for the heterogeneity of the protein in vitro. We found that Asn14 and Asn271 were preferentially glycosylated and that glycosylation of Asn14 has a large effect on the kinetics of the reaction of human angiotensinogen with human recombinant renin. We also showed that a fully deglycosylated angiotensinogen is produced efficiently by Chinese hamster ovary (CHO) cells and is a good renin substrate.
Materials—A specific angiotensinogen enzyme-linked immunosorbent assay (ELISA) (18) was provided by Sanofi Recherche (Montpellier, France). Modification enzymes were purchased from New England Biolabs. Neuraminidase and O-glycosidase, cell culture reagents and fetal calf serum were obtained from Boehringer Mannheim (Meylan, France). Phenylmethylsulfonyl fluoride, aprotinin, pepstatin A, antipain, leupeptin, and Triton X-100 were purchased from Sigma. Molecular masses were estimated by comparison with [14C]-methylated proteins from Amersham Pharmacia Biotech and prestained SDS-PAGE molecular mass markers from Bio-Rad.

Site-directed Mutagenesis of hAGT cDNA—The cDNA encoding the entire polyproteidique sequence of hAGT has been described elsewhere (19). The corresponding protein has a Met in position 235, the most frequent variant in Caucasian populations. The cDNA was inserted (7) into the eukaryotic expression vector pECE (20). The human AGT cDNA contains four N-linked potential glycosylation sites (Asn-X-Ser/Thr) at amino acid positions 14, 137, 271, and 295. For single-stranded mutagenesis, the 1.7-kilobase pair insert was inserted between the XbaI and HindIII sites of M13mp19 to produce single-stranded DNA.

Site-directed mutagenesis to convert each Asn into a Gln was performed using Kunkel’s method (21) with the Mutagene M13 in vitro mutagenesis kit (Bio-Rad), recombinant single-stranded M13 DNA as template and an antisense oligonucleotide (Asn14 → Gln, 5′-ACA GGT ACT CTC TTG GTG GAT GAC GAG-3′; Asn137 → Gln, 5′-CCG GGA GGT GCA CTG CTT GTC CCT CCA-3′; Asn271 → Gln, 5′-CAC TGA GGT GCT GTG CTC CAC GCA GAA-3′; or Asn295 → Gln, 5′-AGT CAC CGA GAA GGT GCT GTG CTC GTC-3′) to produce the four mutant proteins with single residue changes: N14Q, N137Q, N271Q, and N295Q. Each mutated M13mp19 clone was sequenced (Sequenase kit, U.S. Biochemical Corp.) and used for a second, third, and fourth round of mutagenesis to obtain double mutants (N14Q/N137Q and N271Q/N295Q) triple mutants (N14Q/N137Q/N271Q, N137Q/N271Q/ N295Q, N14Q/N271Q/N295Q, and N137Q/N271Q/N295Q) and a quadruple mutant, in which all four Asn thought to be involved in N-glycosylation were mutated (N14Q/N137Q/N271Q/N295Q). This mutant was called N-4 (see Fig. 1). Each hAGT mutant was sequenced and inserted into pECE.

Synthesis of N-Glycosylation Mutant Human Angiotensinogen Proteins—The 11 mutant hAGT cDNAs and the wild-type hAGT cDNA were used to transiently transfect COS-7 cells using the DEAE-dextran method (22). COS-7 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 10 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% CO2, 95% air atmosphere. Cell lines producing wild-type angiotensinogen, the four separate single mutant proteins, and the N-4 mutant protein were established so that large amounts (10–40 μg) of proteins could be produced. CHO-K1 cells were transfected with a neomycin-resistant plasmid (pSV2 neo) by the calcium phosphate precipitation procedure (23). Recombinant cells were selected using 500 μg/ml Geneticin® (Life Technologies, Inc.). Pure cell lines overproducing wild-type angiotensinogen or mutant proteins were obtained by the limiting dilution method, as described previously (7). CHO-K1 cells were grown in Ham’s F-12 medium containing 10% fetal calf serum, 10 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Pulse-Chase Experiments in COS-7 Cells—Mutant and wild-type angiotensinogen proteins were metabolically labeled 48 h after transfection. Cells were incubated in 5 x 10-mm dishes in methionine- and cysteine-free Ham’s F-12 medium (Eurobio) for 1 h. A pulse of 50 μCi of [35S]methionine and [35S]cysteine (Amersham Pharmacia Biotech) was given for 10 min in methionine- and cysteine-free Ham’s F-12 medium containing 10% dialyzed fetal calf serum. Various chase times were used in serum-free medium at 37 °C. The cells were solubilized at 4 °C in 20 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1% (v/v) Triton X-100, 0.1% (w/v) bovine serum albumin. Protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml antipain, 1 μg/ml leupeptin) were added. After centrifugation, the supernatants were incubated overnight with 1 μl of normal rabbit serum (Nordic Immunological Laboratories) and 5 μg of protein A-Sepharose (Clone 4B, Pharmacia, Uppsala, Sweden). The protein A-Sepharose was collected by centrifugation to eliminate nonspecific complexes. The resulting supernatants were immunoprecipitated by incubating overnight at 4 °C with 5 μg protein A-Sepharose and a polyclonal human angiotensinogen antibody (dilution 1:1000). The human angiotensinogen antibody, HCL, has been described elsewhere (7, 24). The protein A-Sepharose was washed four times with 20 mM Tris-HCl buffer, pH 7.5, 150 mM NaCl, 0.1% (v/v) Triton X-100, 1 mM EDTA buffer and then with 20 mM Tris-HCl buffer, pH 6.8. The antigen-antibody complex was dissociated by heating (4 min at 95 °C) in Laemmli buffer (25). The [35S]methionine/cysteine-labeled mutant proteins were resolved by 9% SDS-PAGE, and the gel was subjected to autoradiography. The absence of [35S]methionine/cysteine-labeled intracellular proteins in nontransfected COS-7 was checked by the same procedure.
Quantification of Intracellular Retention of the N-4 Mutant Protein—The integrated optical density of each band on the autoradiographs was determined and analyzed using NIH Image, version 1.57 software with a set of 35S radiography standards. The standards were used to produce a calibration curve to convert the optical density values of each pixel of the digitized image into dpm of 35S-labeled protein/mm². The percentage of intracellular retention was calculated by dividing the densities at 6, 24, and 48 h by the densities immediately after the 10-min pulse (t₀) for the wild-type and N-4 mutant protein.

Enzymatic Deglycosylation of the N-4 Mutant Protein and Western Blotting—A COS cell supernatant containing the N-4 mutant protein was denatured by heating at 95 °C for 4 min in the presence of 0.1% (v/v) SDS and 1% (v/v) β-mercaptoethanol. It was then incubated in 50 mM sodium phosphate buffer, pH 6.0, 1% (v/v) Triton X-100 at 37 °C with (i) no addition (mock treatment), (ii) 5 milliunits of neuraminidase (for 18 h), (iii) 5 milliunits of neuraminidase (for 1 h) followed by 2.5 milliunits of O-glycosidase (for 18 h). Enzymatic deglycosylation was stopped by freezing. The samples were resolved by 9% SDS-PAGE and transferred to serum-free medium (UltraCHO, BioWhittaker) by semidy transfer in 25 mM Tris/glycine buffer, pH 7.5, containing 0.5% (v/v) SDS and 20% (v/v) methanol. Human angiotensinogen was detected with HCL antibody (dilution 1:6000). Antigen-antibody complexes were detected by their alkaline phosphatase activity after biotin-streptavidin enhancement.

Determination of Enzymatic Parameters of the Various Glycosylated Human Angiotensinogen Mutant Proteins—Enzymatic parameters of the renin angiotensinogen reaction were determined using different concentrations of various glycosylated angiotensinogen mutant proteins and a fixed amount (20 pM) of recombinant human renin, which was provided by Hoffmann La Roche (Basel, Switzerland). Renin was produced as prorenin in CHO cells, purified, and activated to mature renin to check that no Ang I was produced. Nontransfected CHO cells were transferred to serum-free medium. The supernatant was harvested, concentrated, and incubated with renin to check that no angiotensinogen or Ang I was produced by untransfected CHO cells.

RESULTS

Production of the N-Glycosylation Mutant Proteins in COS-7 Cells—The wild-type hAGT cDNA and 11 N-glycosylation mutant cDNAs were used to transfect COS-7 cells. The intracellular production of the human angiotensinogen proteins was studied by immunoprecipitation after metabolic labeling (Fig. 2A), and extracellular secreted angiotensinogen was studied by Western blotting (Fig. 2B). The wild-type angiotensinogen protein had a complex and reproducible pattern in SDS-PAGE. Intracellular angiotensinogen gave five bands of 58, 55.5, 52, 50, and 48 kDa, the upper band (58 kDa) being the fully N-glycosylated protein. Angiotensinogen secreted into the medium gave a fuzzy band between 50 and 70 kDa.

All of the single mutants lacked the upper intracellular band (58 kDa), and the heterogeneity (2–3 kDa) of secreted angiotensinogen was slightly less, suggesting that each potential

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N-glycosylation site was actually glycosylated. The secreted N137Q single mutant had a different pattern in SDS-PAGE, because the polyclonal antibody did not detect the weakly glycosylated forms of this mutant. The heterogeneity of both intracellular and secreted angiotensinogens was significantly lower when at least three potential N-glycosylation sites were mutated. Deglycosylation of one site gave a 55.5-kDa band, deglycosylation of two sites gave a 52-kDa band, and deglycosylation of three N-glycosylation sites a 50-kDa band in cells. In the medium, the size heterogeneity of secreted angiotensinogen progressively decreased as the number of N-deglycosylated sites increased, although accurate molecular mass determination was difficult due to the fuzziness of the bands. The single 48-kDa band observed with the N-4 mutant protein was a fully N-deglycosylated form. Thus, there was a stepwise decrease in the heterogeneity of angiotensinogen, directly related to the number of deglycosylated sites.

Analysis of triple mutants, in which only one N-glycosylation site was conserved, showed that this site was N-glycosylated in each case. The metabolic labeling of each triple mutant detected two bands, the upper band (50 kDa) demonstrating N-glycosylation of the conserved site and the lower band (48 kDa) corresponding to the N-deglycosylated form of angiotensinogen. The intensity of the two bands was compared for each mutant to estimate the number of different oligosaccharide structures at each site. The Asn14-Lys15-Ser16 (137/271/295 mutant) and Asn271-Ser272-Thr273 (14/137/295 mutant) sites were more glycosylated than the sites of the two other triple mutants, because the 50-kDa form was predominant. The Asn137,Cys138,Thr139 site was less glycosylated, because the two intracellular bands for triple mutant 14/271/295 were of similar intensity. The Asn295-Phe296-Ser297 C-terminal site was also less glycosylated because the deglycosylated form was predominant for the 14/137/271 mutant.

The fully deglycosylated hAGT form (N-4) was correctly synthesized and secreted by COS-7 cells. Metabolic labeling detected a single intracellular band (48 kDa), and Western blot analysis detected two secreted bands: a major product of 48 kDa, in size identical to the intracellular band, and a minor 50-kDa band. Similar data were obtained with pure line CHO cells (data not shown).

Neuraminidase Treatment of the N-4 Mutant Angiotensinogen Protein—Western blot analysis showed that there was an extra N-4 mutant protein (50 kDa) secreted, which was not present in cells. Treatment with endoglycosidase F did not affect this pattern (data not shown), so we treated this form with neuraminidase alone or with neuraminidase followed by O-glycosidase. Neuraminidase treatment resolved recombinant N-deglycosylated angiotensinogen into a single 48-kDa band, which was not further affected by O-glycosidase treatment (Fig. 3), demonstrating that the 50-kDa form contained only sialic acid residues. The 48-kDa form of the N-4 mutant protein produced in the medium was an entirely unglycosylated angiotensinogen with no O-glycosylation.

Intracellular Trafficking of N-Glycosylation Mutant Angiotensinogen Proteins—The effect of N-glycosylation on cellular trafficking and secretion in the culture medium was studied by pulse-chase experiments in transiently transfected COS-7 cells (Fig. 4A). All the mutant proteins were efficiently labeled within 10 min of the pulse (t = 0), giving the pattern shown in Fig. 2A. The wild-type angiotensinogen and the four single mutant proteins were not detectable within cells after 6 h of chase (t = 6). Thus, the mutation of a single N-glycosylation site was not sufficient to affect cellular trafficking. However, a significant amount of the triple and quadruple mutant proteins was retained within the cells. After 24 h of chase (t = 24), it was still possible to immunoprecipitate radiolabeled triple mutant protein.
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**TABLE I**

*Kinetic constants for the hydrolysis of wild type and N-glycosylation mutants of human angiotensinogen by purified recombinant human renin*

| Substrate          | $K_m$ ($\mu$M) | $V_m$ (nmol Ang I·ml$^{-1}$·s$^{-1}$) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (\muM$^{-1}$·s$^{-1}$) |
|--------------------|----------------|-------------------------------------|---------------------|-------------------------------------|
| Wild-type (a)      | 1.7 ± 0.2      | 55 ± 3.5                            | 2.8 ± 0.2           | 1.6 ± 0.1                           |
| N14Q mutant (a)    | 0.3 ± 0.01     | 6.2 ± 2.8                           | 0.3 ± 0.1           | 1.1 ± 0.4                           |
| N137Q mutant       | 1.4            | 54                                  | 2.7                 | 1.9                                 |
| N271Q mutant       | 1.3            | 40                                  | 2                   | 1.5                                 |
| N295Q mutant       | 0.8            | 28                                  | 1.4                 | 1.7                                 |
| N-4 mutant (b)     | 0.7 ± 0.1      | 62 ± 20                             | 3.1 ± 1             | 5.0 ± 2                             |

The maximum velocity of the reaction with N14Q was 10 times lower than that observed with wild-type and other mutated substrates. However, its affinity for renin was 5 times higher than that of the wild-type, resulting in a catalytic efficiency similar to the wild-type $k_{cat}/K_m$. Thus, the asparagine residue at position 14 plays a key role in the kinetics of the renin-angiotensinogen reaction.

In contrast, the kinetic parameters of the N137Q, N271Q, and N295Q hAGT substrates were quite similar to those of the wild-type protein, suggesting that the asparagine residues in positions 137, 271, and 295 had no significant effect on the enzyme reaction.

Deletion of the four N-glycosylation sites (N-4 mutant) resulted in $K_m$ values half those of the wild type (0.7 versus 1.7 \(\mu\)M) and had no effect on $k_{cat}$ (3.1 versus 2.8 s$^{-1}$), increasing catalytic efficiency by a factor of 3.

**Thermostability of the N-4 Mutant Protein—**$N$-Glycosylation may be involved in the protein thermostability, so we studied the effect of various temperatures, (4, 37, 56, and 80°C) on wild-type angiotensinogen and N-4 mutant protein. We used the enzymatic assay (Fig. 6A) and the ELISA test (Fig. 6B) to detect functional changes in the generation of Ang I and epitopic changes in the recognition of the protein. Both methods gave similar results. The N-4 mutant protein had a thermal unfolding transition curve identical to that of wild-type angiotensinogen. The stability of the N-4 mutant and wild-type angiotensinogen proteins after incubation at 37°C for 1, 3, 6,
and 24 h was similar (data not shown). Thus, the deglycosylation of human angiotensinogen did not change the overall structure of the protein.

**DISCUSSION**

Glycosylation of a protein may control its folding and secretion, conformation, and stability and regulate its activity (28). This study demonstrates that (i) each potential N-glycosylation site in human angiotensinogen is linked to oligosaccharide residues, causing the in vitro heterogeneity of the protein; (ii) mutation of the Asn14 residue has a large effect on the kinetics of the renin angiotensinogen reaction; (iii) the absence of N-glycans does not affect the level of production of angiotensinogen in eukaryotic cells; and (iv) a fully deglycosylated angiotensinogen can be expressed efficiently and is a good renin substrate. All of these results were obtained in vitro by producing recombinant angiotensinogens in CHO cells. As previously shown by our group, angiotensinogen synthesized by CHO cells is immunologically similar to the plasma protein and has the correct N-terminal angiotensin I sequence (7, 29). Glycosylation levels may differ between cell types and between tissues (30). However, several arguments suggest that this eukaryotic system is representative of the in vivo situation. Glycosylation of purified plasma angiotensinogen has been reported to be account for about 14% of its apparent molecular mass (31), a level similar to the 16% observed with our in vitro system. This approach has also been widely used to determine the role of glycosylation of secreted proteins, giving results consistent with those expected in vivo (reviewed in Ref. 28).

We have known that the angiotensinogen protein is heterogeneous for over 10 years (for a review, see Ref. 8), but this has not been completely explained. Previous deglycosylation experiments have suggested that N-glycosylation accounts for most, but not all, of this heterogeneity. For example, Campbell et al. (15) found that purified human plasma angiotensinogen gave four bands in SDS-PAGE and that several species were present after enzymatic deglycosylation. However, it was unknown whether there was O-glycosylation and/or incomplete removal of carbohydrate chains. It was also possible that the C-terminal part of the molecule was cleaved, because angiotensinogen is a member of the serpin superfamily (32). We used site-directed mutagenesis of each potential N-glycosylation site in angiotensinogen (mutating Asn to Gln in order to maintain the net charge of the protein). We found that the heterogeneity of the recombinant angiotensinogen produced by COS-7 cells and CHO-K1 cells was completely explained by its N-glycosylation. The N-4 mutant protein, in which all four N-linked sites were mutated, gave a single intracellular 48-kDa band in SDS-PAGE detected by immunoprecipitation after metabolic labeling. Into the medium, this mutant exhibited two bands in Western blot analysis, a major one corresponding to the same unglycosylated 48-kDa form and a minor 50-kDa form with a high proportion of sialic residues. This sialylated form of the N-4 mutant protein was probably secreted too fast into the medium to be detected in cells after the 10-min pulse. Altogether, in vitro N-glycosylation was responsible for about 16% of the apparent molecular mass of angiotensinogen, whereas sialic acids were responsible for 4%. There was no evidence for any additional O-glycosylation.

Our results provide evidence that all four potential N-glycosylation sites were bound to oligosaccharides in vitro and that this complex glycosylation produces the five different glycoforms observed in vitro by SDS-PAGE. This result could not be predicted in advance, because the tertiary structure of the protein is unknown and there are large differences in the number and position of N-glycosylation sites between species (33–35). Comparison of the intracellular wild-type protein with the single and triple mutant proteins and the fully deglycosylated angiotensinogen showed that Asn14 and Asn271 are preferentially glycosylated. N-Glycosylation of the first site was suggested by the absence of any detectable amino acid at position 14 in Edman degradation of purified plasma angiotensinogen (31) and of purified recombinant angiotensinogen obtained in non-denaturing conditions.2 Recombinant sheep angiotensinogen (in which Asn14 is replaced by a Ser) only contains two potential N-glycosylation sites (at positions 271 and 338) and gives a single 56-kDa band in SDS-PAGE (35). This homogeneity probably reflects the presence of N-glycans on Asn271, because the other site (Asn338-Leu339-Ser340) has a Pro at position 341, which should prevent oligosaccharide transfer (36). The conservation of the Asn271 in the mouse and sheep proteins are consistent with our finding that the Asn14 and Asn271 of human angiotensinogen are preferentially glycosylated.

Asn137 was less glycosylated and is followed by a cysteine at position 138. This site was analyzed either by its deletion in the single mutant, N137Q, or by its conservation as the only N-glycosylated site in the triple mutant, N14/271/295Q. The deglycosylated forms of secreted N137Q angiotensinogen were undetectable by Western blotting. The cysteines at positions 138 and 18 are the only cysteine residues conserved in other mammalian angiotensinogen sequences.

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malian species for which the AGT gene has been cloned (rat (33), mouse (34), and sheep (35)), suggesting that there may be a disulfide bridge between these two residues. In this case, the different pattern of the N137Q mutant may be due to an interaction between the correct N-glycosylation of the second site and the correct formation of the disulfide bond during the folding of the molecule. The disulfide bond may exert specific constraints on the structure of the molecule, reducing oligosaccharide attachment at Asn137. Such interactions have been described for the human chorionic gonadotropin β-subunit (36) and tissue-type plasminogen activator (37).

One of the main functions of N-linked oligosaccharides is their involvement in the biosynthetic pathway from the endoplasmic reticulum to the cell surface; therefore, glycosylation alterations may greatly affect the synthesis of the protein (38–40). We previously showed with a rare natural mutant of angiotensinogen, Y248C, that the production of an additional N-glycosylated form led to intracellular retention in vitro in CHO cells, which paralleled an in vivo decrease in plasma angiotensinogen concentration (7). In the present study, the suppression of one or two potential N-linked sites did not affect the intracellular trafficking and secretion of recombinant angiotensinogen. A slightly abnormal pattern of intracellular retention was observed for the triple mutants, which was more pronounced for the less glycosylated N14Q/N137Q/N271Q and N-4. Only about 15% of the N-4 mutant protein was retained in cells after 24 h, and it was efficiently secreted by CHO cells. Thus, N-linked sugars do not play a crucial role in the intracellular trafficking and secretion of angiotensinogen, and the deglycosylated protein appears to be correctly folded. Similar results have been obtained in other N-glycosylation mutagenesis studies with various proteins, such as human lactoferrin (41) or platelet-derived growth factor-B chain (42).

We also investigated the potential effect of N-glycosylation of human angiotensinogen on kinetics with renin. The largest effect on affinity and maximum velocity was observed with the mutation of Asn14. Replacing the Asn at the P4' position of the cleavage site with a Gln resulted in 5 times higher affinity of renin for N14Q angiotensinogen and a decrease in the rate of hydrolysis of this substrate by a factor 10. Oligosaccharides linked to Asn14 may cause a steric hindrance in the recognition of wild-type human angiotensinogen by human renin. The velocity of the reaction may be lower, since there is no ethyl group in the Gln residue, which might lead to inaccurate alignment of the substrate with the renin active site. There is strong species specificity in the renin angiotensinogen reaction. Asn14 is replaced by a Ser in sheep and rat, and human renin has a greater affinity for sheep (κm = 0.31 μM; Ref. 17) than for its homologous substrate. These results and those obtained with synthetic tetradecapeptides, based on different angiotensinogen sequences (17), suggest that the lower affinity of human angiotensinogen for human renin compared with that of sheep and rat angiotensinogen is probably due to N-linked oligosaccharides in position 14 rather than to the intrinsic properties of the Asn14 residue. Our data support this notion and also suggest that N-glycosylation of the other three sites does not affect the enzyme reaction.

Finally, we characterized a completely unglycosylated mutant angiotensinogen protein and found several interesting properties. First, it was produced as a homogeneous protein. Second, small amounts were retained in cells, but the protein was secreted at rates similar to the wild-type protein by CHO cells. Third, it seemed to be correctly folded and heat-stable. Last and unexpectedly, this mutant had a higher catalytic efficiency than the wild-type protein (kcat/Km ratio 3 times higher). As for the N14Q protein, this higher affinity may be due to the absence of branched oligosaccharides, facilitating the protein's recognition by angiotensinogen by renin. Since studies of the structure of human angiotensinogen have been hindered by the protein's heterogeneity, this mutant could be of value for ultrastructural studies.

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