Two N-Linked Glycosylation Sites (Asn18 and Asn106) Are Both Required for Full Enzymatic Activity, Thermal Stability, and Resistance to Proteolysis in Mammalian Deoxyribonuclease I

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Deoxyribonuclease I (DNase I) is known to be a glycoprotein, and two potential N-linked glycosylation sites (N18 and N106) are known for mammalian enzymes. In the present study, N18 and N106 were mutated in order to investigate the biological role of N-linked glycosylation in three mammalian (human, bovine, and equine) DNases I. The enzyme activities of N18Q and N106Q were lower than that of the wild type, and that of the double mutant (N18Q/N106Q) was lower than those of the single mutants, in accord with the sugar moiety contents in the three mammals. In addition, all mutant enzymes were unstable to heat, suggesting that both sites are required for heat stability. Moreover, in human and equine enzymes, the N18Q and N106Q mutant enzymes were less resistant to trypsin, while N18Q/N106Q was the most sensitive to trypsin. As for bovine DNase I, the trypsin resistance of N18Q and N106Q was similar to that of the wild type, but that of N18Q/N106Q decreased in a time-dependent manner. On the other hand, N-linked glycosylation was not related to pH sensitivity. The results of the present study suggest that N18 and N106 are both necessary for (i) full enzymatic activity, (ii) heat-stability, and (iii) trypsin resistance.

Key words: cow; horse; human; N-glycosylation; recombinant DNase I

Deoxyribonuclease I (DNase I, EC 3.1.21.1) is a divalent cation-dependent enzyme that cleaves double-stranded DNA to produce oligonucleotides with 5′-phospho and 3′-hydroxy termini.1) In the past, it was regarded as simply a digestive enzyme secreted by the exocrine glands, including the pancreas and/or parotid, into the alimentary tract,1–3) but other functions in vivo have recently been found. For example, endogenous DNase I has been suggested as a candidate endonuclease responsible for internucleosomal DNA degradation during apoptosis.4) In addition, recent studies have pointed out the relevance of DNase I to disease. Kumamoto et al. found that the prevalence of DNASE1*2 was significantly higher in patients with myocardial infarction than in healthy subjects.5) A significant association has been found between gastric and colorectal carcinoma and a high frequency of DNASE1*2.6,7) Serum DNase I activity has recently been highlighted as a potential diagnostic marker for the detection of acute myocardial infarction,8–11) and has been shown to be lower in patients with systemic lupus erythematosus (SLE) than in healthy subjects,12,13) suggesting that maintenance of DNase I activity in the serum is essential for the prevention of SLE. However, to date, the mechanisms of the involvement of DNase I in the onset of the above-mentioned diseases remain unknown.

DNase I is known to be a glycoprotein, and two potential N-linked glycosylation sites are known for mammalian enzymes. Asn18-Ala/Asp-Thr and Asn106-Asp-Thr/Ser are well conserved in almost all mammalian enzymes.14–21) We have found that N-linked carbohydrate moieties differ among rat, rabbit, and porcine DNase I: one is composed of high-mannose/hybrid and complex-type carbohydrate moieties,22) and the other is a high-mannose/hybrid-type moiety fully sensitive to endoglycosidase H digestion.22) Moreover,
using Peptide N-glycosidase F (PNGase F), we have found that DNase I activity was decreased by enzymatic deglycosylation.\textsuperscript{22,23} Nishikawa et al. have shown that the number of sugar chains in bovine DNase I differed with the tissue. DNase I isolated from parotid glands and submaxillary glands was found to have two sugar chains, in contrast to the single sugar chain in pancreatic DNase I,\textsuperscript{24} but the roles of N-linked sugar moieties at the glycosylation sites (Asn\textsuperscript{106} and Asn\textsuperscript{18}) in DNase I\textsuperscript{22} were not fully understood. In mammals, DNase I is primarily localized in the salivary gland or pancreas, and mammalian DNases I can be classified into three types, pancreas, parotid, and pancreas-parotid (mixed), based on the tissue with highest activity. 3) Differences in the biochemical properties of DNase I among the three mammalian DNases I can be classified into three types, primarily localized in the salivary gland or pancreas, and submaxillary glands was found to have two sugar chains, in contrast to the single sugar chain in pancreatic DNase I,\textsuperscript{24} but the roles of N-linked glycosylation in the biochemical properties of human, bovine, and equine DNase I are shown in Fig. 1. Underlined sequences are N-linked glycosylation sites. After double-digestion with \textit{Bam}HI and \textit{Not}I, the fragments obtained were ligated into pcDNA3.1 (+) vector (Invitrogen, San Diego, CA) to construct the expression vector.\textsuperscript{25} Each N-glycosylation site at the aa position 18 (Asn\textsuperscript{18}) residue was replaced with Gln (N18Q) and site-directed mutagenesis are shown in Table 1.

### Materials and Methods

**Materials.** Superscript II reverse transcriptase (RT), LipofectaminPlus, all oligonucleotide primers, and the 3'- and 5'-rapid amplification of cDNA end (RACE) systems were from Life Technologies (Gaithersburg, MD) and the Expanded High Fidelity PCR system was from Roche Diagnostics (Mannheim, Germany). Recombinant Peptide N-glycosidase F (PNGase F) was purchased from Prozyme (San Leandro, CA). All other chemicals used were of reagent grade and are commercially available.

**Construction of expression vector inserting human, bovine, and equine DNases I cDNA and site-directed mutagenesis.** Following previous methods,\textsuperscript{14–16} a DNA fragment containing the entire coding sequence of mammalian DNase I cDNA was prepared from total RNA derived from the pancreas by reverse transcriptase PCR amplification using Superscript II reverse transcriptase and an expanded high-fidelity PCR system. The entire amino acid sequences of human, bovine, and equine DNase I are shown in Fig. 1. Underlined sequences are N-linked glycosylation sites. After double-digestion with \textit{Bam}HI and \textit{Not}I, the fragments obtained were ligated into pcDNA3.1 (+) vector (Invitrogen, San Diego, CA) to construct the expression vector.\textsuperscript{25} Each N-glycosylation site at the aa position 18 (Asn\textsuperscript{18}) residue was replaced with Gln (N18Q) and constructed by site-directed mutagenesis based on the PCR method\textsuperscript{26} using WT constructs as a template. The primer sequences used in site-directed mutagenesis are shown in Table 1.

**Transient expression of the constructs in COS-7 cells.** COS-7 cells were grown on 6-well plates in Dulbecco’s modified Eagle’s medium containing 1 mm L-glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 10% (v/v) fetal calf serum (Life Technologies) at 37 °C under an atmosphere of 5% CO\textsubscript{2} in air. The cells were transiently transfected with each vector using the LipofectaminPlus reagent, according to the method described previously,\textsuperscript{27} except that fetal calf serum

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<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences</th>
<th>Comment</th>
</tr>
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<tbody>
<tr>
<td>Hum-N18Q-1</td>
<td>5'-GTTCAGGCACGCTGACGCT-3'</td>
<td>Sense, used in N18 substitution</td>
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<tr>
<td>Hum-N18Q-2</td>
<td>5'-AGCTGAGTGGTGGTAGCTGAGAGACGCT-3'</td>
<td>Antisense, used in N18 substitution</td>
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<tr>
<td>Hum-N106Q-1</td>
<td>5'-CCTGCGGCAGACGCTGAGTGGTGGTAGCTGAGAGACGCT-3'</td>
<td>Sense, used in N106 substitution</td>
</tr>
<tr>
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<td>5'-GAAGGTGTGGTGGTGGTAGCTGAGAGACGCTGAGGAGACGCT-3'</td>
<td>Antisense, used in N106 substitution</td>
</tr>
<tr>
<td>Bov-N18Q-1</td>
<td>5'-GTCCAGCTGACGCTGACGCT-3'</td>
<td>Sense, used in N18 substitution</td>
</tr>
<tr>
<td>Bov-N18Q-2</td>
<td>5'-AGCTGAGTGGTGGTAGCTGAGAGACGCT-3'</td>
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</tr>
<tr>
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<td>Bov-N106Q-2</td>
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<td>5'-TAGAAACACAGTGGAG-3'</td>
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Nucleotide residues underlined correspond to those for the replacement of Asn with Gln.
was not added to the cells during the interval of transfection. A mixture of 2 μg expression vector and 600 ng pSV-β-galactosidase vector (Promega, Madison, WI) was introduced into the cells to estimate transfection efficiency. After 2 d, the medium and cells were recovered for analysis. The DNase I secreted into the medium was used for subsequent analyses, and the cells were subjected to β-galactosidase activity assay as previously described.28 Each transfection was performed in triplicate with at least two different plasmid preparations. The recovered medium was purified according to our previous method.28 The active fractions were collected and used as the particular purified enzymes in subsequent analyses. Purification was confirmed by electrophoresis, and only one band was observed.

**DNase I activity assay.** DNase I activity was assayed by the previously described test-tube method or by single radial enzyme diffusion.22,23 A 25-μl sample was added to a test tube containing 0.35 ml of 1% saltin testis DNA in 125 μl of 0.2 M Tris/HCl buffer (pH 7.5) containing 0.2 M MgCl₂ and 0.02 M CaCl₂. Following incubation at 37 °C for 20 min, the reaction was stopped by adding 500 μl of 0.1 N HCl and 2 ml 99% ethanol. After 5 min incubation in crushed ice, the mixture was centrifuged at 1540 × g for 10 min, and the absorbance at 260 nm of the supernatant was determined. One unit of DNase I activity was defined as the amount of enzyme that increased by the absorbance of 1.0 at 260 nm. Also, based on the test tube method, one unit was determined by the SRED method.2 The single radial enzyme diffusion (SRED) method was carried out according to Nadano et al.2 The reaction buffer was placed in a test tube, 1% salmon testis DNA and 1% ethidium bromide were added to the tube, and the mixture was vortex mixed. Two percent molten agarose GP-36 was added to this mixture, which was immediately poured onto a horizontal Agafix MSL sheet (Wako Pure Chemical Industries, Tokyo). Following congelation, rows of cylindrical sample wells with centers 15 mm apart were punched in the gel. Then 5-μl samples were placed in the wells and incubated in a moist chamber at 37 °C. DNase I activity was observed under UV (312 nm). The radius of each dark circle was measured, and DNase I activity (units per mg of protein) was calculated by comparison with the standard curve.

**Analytical methods.** Activity staining for DNase I was performed by the DNA-casting native PAGE (12.5%) method.22,23,26 The sugar moiety content of each enzyme was assayed using a glycoprotein carbohydrate estimation kit (Pierce, Rockford, IL). Protein concentrations were determined with a protein assay kit (Bio-Rad, Richmond, CA) using bovine serum albumin as the standard.

DNase I was used in the analysis of proteolysis by the proteases as described previously.16 Each DNase I (0.2 unit) was incubated at 37 °C with 0.05% trypsin in PBS for various periods. Control reactions were performed by incubating the enzyme without each protease. Then the activities were assayed as described above, and the remaining activities were determined by comparison with those of the controls. Deglycosylation using PNGase F was performed as previously described.23

**Examination of heat and pH sensitivity.** The WT and substitution mutant of DNase I were incubated for 5, 10, 20, 30, 40, and 60 min at 50 °C to survey the heat sensitivity, as described previously.29 Following incubation, the remaining activity was measured. pH sensitivity was investigated by the SRED method using sodium acetate (pH 5.0), MES (pH 6.0, 6.75), and Tris-HCl (pH 7.5, 8.0, and 8.8) buffers containing 10 mM MgCl₂ and 1 mM CaCl₂, as described previously.31

**Statistical analysis.** Differences between the mean values of activity were analyzed by Student’s t-test using the STATCEL program (OMS Publishing).

**Results and Discussion**

**Expression of WT and mutant DNases I in COS-7 cells.** In the present study, human (pancreas type), bovine (pancreatic-parotid type), and equine (parotid type) recombinant DNases I were used to investigate the role of N-linked glycosylation. To evaluate the role of two N-linked glycosylations, the recombinant N18Q, N108Q, and N18Q/N106Q mutants of the human, bovine, and equine DNases I were constructed by site-directed mutagenesis. Human and equine DNases I contain the same aa sequences at both the 18 and 106 glycosylation sites, but in bovine DNase I, Thr¹⁰⁸ is replaced by Ser¹⁰⁸ (Fig. 1). Almost all mammalian DNases I have two potential N-linked glycosylation sites, Asn¹⁸-Ala¹⁹/Glu¹⁰⁷-Thr²⁰ and Asn¹⁰⁶-Glu¹⁰⁷-Thr¹⁰⁸/Ser¹⁰⁸, except for the rabbit enzyme, which has only one potential site at Asn¹⁸-Ala¹⁹-Thr²⁰.14–21

The entire coding region in each DNase I cDNA was cloned into a mammalian expression vector and transiently expressed in COS-7 cells. The sugar contents of the recombinant enzymes were investigated. Figure 2 shows the % content of the sugar moiety of human, bovine, and equine recombinant DNases I as compared with double mutant. Elimination of the glycosylation site resulted in a loss of sugar. The sugar moiety contents of the single human mutants (N18Q and N106Q) were significantly lower than those of human WT (n = 6, p < 0.01), and those of the double mutant (N18Q/N106Q) were much lower than those of the single mutants (n = 6, p < 0.01) in the three mammals. In the human enzyme, the sugar moiety contents of the single mutant (N18Q and N106Q) decreased by about 60% and that of the double mutant decreased by 80%. On the other hand, the sugar moiety contents of the bovine and equine single mutants did not decrease...
significantly, and that of the double mutant remained at about 50%. It is unclear whether the double mutant of bovine and equine contains the sugar moiety. To our knowledge, no research report on the presence of O-glycosylation in DNase I has been published. Hence this result indicates that further investigation is required to confirm the presence of O-glycosylation in mammalian DNase I.

Each mammalian recombinant enzyme was subjected to the DNA-casting native PAGE (12.5%) method, followed by activity staining (Fig. 3). In the three mammals, N18Q and N106Q showed similar mobility bands that were lower than those of WT, and N18Q/N106Q showed lower mobility bands than those of N18Q or N106Q. Enzymatic deglycosylation using PNGase F was also performed to confirm N-linked glycosylation: the WT and single mutant (N18Q and N106Q) enzymes migrated to a lower position with the same mobility as that of N18Q/N106Q following PNGase F treatment (data not shown). These results may be due to a loss of the N-linked sugar moiety, as shown in Fig. 2. Our previous report on human DNase I supports these data.23) In addition, Figs. 1 and 2 suggest two potential N-linked glycosylation sites in these three mammalian DNases I are glycosylated to similar extents. The presence of N-linked glycosylation site does

Fig. 1. Alignment of Amino Acid Sequences of DNases I from Human, Cow, and Horse.
GenBank accession numbers for these amino acid sequences are as follows: Homo sapiens (human) (M55983), Bos Taurus (cow) (AB048832), Equus caballus (horse) (AB162819). Potential N-linked glycosylation sites are shown in bold, and underlining indicates sequence.

Fig. 2. Sugar Moiety Content of Recombinant DNase I of Human (a), Cow (b), and Horse (c), Followed by Transient Expression in COS-7 Cells. Each bar represents the average of six independent transfections (mean ± S.D.). % Sugar moiety of DNase I is given as the percentage activity of WT. **p < 0.01 as compared with WT by t-test.
not always lead to glycosylation. In the present study, it was confirmed that both potential N-linked glycosylation sites (N18 and N106) are glycosylated in the three mammals.

Only in the bovine enzyme, the triple bands were observed in WT and double bands were observed in N18Q (Fig. 3). Lower bands are considered to be non-glycosylated proteins that perhaps result in low N-glycosylation efficiency. N-glycosylation efficiency differs with aa at the N-glycosylation motif. Nishikawa et al. 24) have found that the extent of glycosylation increased from 70% to almost 100% when Ser108 in the second glycosylation site of DNase I was changed to Thr. 108 Asn18-Ala-Thr is the same in human, equine, and bovine enzymes. The Thr (Asn106, Asp-Thr) found in human and equine enzymes is replaced by Ser in the bovine enzyme (Asn106, Asp-Ser) (Fig. 1). 14–16)

Role of N-linked glycosylation in DNase I activity

Several studies have produced results showing that the enzyme activity of mutants lacking a N-glycosylation site was reduced, suggesting that N-glycosylation is important for full activity. Loriol et al. found decreased enzyme activity in the substitution mutant (N65L/N and N63L/N) of bovine protein O-fucosyltransferase 1. 30) Saito et al. 31) found that Asn23 is necessary for full enzymatic activity among three potential N-linked glycosylation sites (Asn13, Asn16, and Asn23) in a rat Melanin-concentrating hormone. We have found that human recombinant DNase I activity was decreased by PNGase F treatment. 23) In the present study, the enzyme activities of the mutants were compared with those of WT. The activities of WT in each mutant enzyme are shown in Fig. 4. The DNase I activities of mutant enzymes were lower than those of WT. The activities of N18Q, N106Q, and N18Q/N106Q in humans, N18Q/N106Q in cows, and N18Q and N18Q/N106Q in horses were significantly lower than those of WT. These decreases in activity were in accord with the various sugar moiety contents of the mutant enzymes (Figs. 1 and 4). Based on deglycosylation analysis using PNGase F and Endo H, we have suggested that N-linked glycosylation is important for enzymatic activity. 22,23) In the present study, both N-glycosylated sites of N18 and N106 were found to be essential for full enzymatic activity in these three mammals.

Fig. 3. Electrophoretic Patterns of DNase I of Human (a), Cow (b), and Horse (c) Expressed in COS-7 Cells.
DNase I was subjected to the DNA-casting native PAGE (12.5%) method, followed by activity staining. Lane 1, WT; lane 2, N18Q mutant; lane 3, N106Q mutant; lane 4, N18Q/N106Q mutant. Electrophoresis were carried out using 0.5 U enzymes.

Fig. 4. Recombinant DNase I Activity of Human (a), Cow (b), and Horse (c), Followed by Transient Expression in COS-7 Cells.

Each bar represents the average of six independent transfections (mean ± S.D.). % DNase I activity is given as the percentage activity of WT. **p < 0.01 as compared with WT by t-test.
Role of N-glycosylation in heat stability and pH sensitivity

Previous studies have found that recombinant enzymes that lose N-glycosylation sites are less thermostable than the WT enzyme. Koseki et al.32) found considerably reduced thermostability of N79A and N79Q mutant enzymes in Aspergillus awamori feruloyl esterase. Terashima et al.33) found that elimination of the N-glycosylation site in rice alpha-amylase reduced the thermostability of the enzyme. To determine whether N-glycosylation is essential for the heat sensitivity of DNases I, both mutant and WT enzymes were incubated at 50°C for 5 to 60 min, and the remaining activity was evaluated (Fig. 5). The activities of human, bovine, WT DNases I were stable for up to 60 min. In contrast, all mutation enzymes were unstable under heat. The heat sensitivities of the equine WT DNases I were less stable under heat than the human enzyme. The heat sensitivities of the equine mutant enzymes were lower than those of WT, and N18Q/N106Q was most unstable under heat.

Post-translational glycosylation can be involved in acid stability. Takeshita et al.29) and Mogi et al.34) found that the DNases I of amphibians and fish have no glycosylation sites and are sensitive to low pH. However, in the present study, the pH sensitivities of the mutant enzymes (N18Q, N106Q, and N18Q/N106Q) were similar to those of WT in the three mammals (Fig. 6). Hence pH stability is considered to be related to factors other than N-linked sugar moieties.

Effect of trypsin treatment on the activities of human, bovine, and equine DNases I

As mentioned above, DNase I is known to be a digestive enzyme secreted by the exocrine glands. In mammals, it is primarily localized in the salivary gland or pancreas, and mammalian DNases I can be classified into three types pancreas (human), pancreas-parotid (cow), and parotid (horse), based on the tissue with the highest activity.35) Trypsin is localized in the pancreas, and it is important to determine whether DNase I is resistant to trypsin proteolysis.

Recently, Chen et al. found that a mutant, C101A, was inactivated by trypsin, indicating that the disulfide bond in DNase I plays a role in stabilizing the structure.35) Ueki et al.16) recently explained that equine and bovine DNases I were much more sensitive to proteolysis by trypsin than the human enzyme. However, to date, the role of the N-glycosylated moiety in the trypsin resistance of DNase I has not been determined. Therefore, the inhibitory effects of trypsin on human DNase I from WT and mutants were assayed (Fig. 7). As
reported previously. WT human DNase I was more resistant to trypsin than the equine and bovine enzymes were. In the human and equine enzymes, N106Q and the N18Q mutant enzyme were less resistant to trypsin and N18Q/N106Q was most sensitive to trypsin (Fig. 7a, c).

As for bovine DNase I, N18Q and N106Q showed slight decreases in trypsin resistance, and it is of interest that N18Q/N106Q showed a decrease to about 20% in only 30 min (Fig. 7b). In the bovine enzyme, both N18 and N106 are required for trypsin resistance. According to a previous report, human DNase I is resistant to trypsin, while bovine and equine enzyme are sensitive. The decrease in the activity of the human enzyme might have been due to a loss of the sugar moiety. In the equine enzyme, activity decreased gradually. Perhaps this may be due to its resistance, trypsin. N-glycosylation plays important roles not only in protein folding but also in its function. This study is the first to indicate that the N-linked and N carbohydrate chains may be required for trypsin resistance, and it is the first to show the difference in roles between N18 and N106.

**Conclusion**

In summary, the present study indicates that both N-linked glycosylation sites (Asn\textsuperscript{18} and Asn\textsuperscript{106}) are glycosylated in human, bovine, and equine enzymes. In the bovine enzyme, non-glycosylated DNase I was present due to the difference in N-linked glycosylation (Asn\textsuperscript{106}-Asp-Thr), which result in low glycosylation efficiency. This study is the first to indicate that Asn at aa positions 18 and 106 of mammalian DNases I is required for full enzymatic activity, acquisition of thermal stability, and trypsin resistance. The function of N-linked glycosylation did not differ among the three types (pancreas type, pancreatic-parotid type, and parotid type). Further comparative studies of the structure of the N-glycosylated moiety in DNase I are needed to clarify the physiological role of N-linked glycosylation in DNase I and the differences in the DNase I of the three types.

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