Role of Carbohydrate Moiety in Carboxypeptidase Y: Structural Study of Mutant Enzyme Lacking Carbohydrate Moiety

Hiroyuki SHIMIZU, Hiroshi UENO, and Rikimaru HAYASHI†

Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Sakyo, Kyoto 606-8502, Japan

Received January 11, 1999; Accepted February 12, 1999

To study the roles of the carbohydrate moiety in the function of carboxypeptidase Y, asparagine residues at 13, 87, 168, and 368, the four-consensus N-linked glycosylation sites, were altered to alanine with site-directed mutagenesis. The mutant enzyme of 51 kDa completely lost the carbohydrate moiety which was present in the 61-kDa wild-type enzyme. Structural studies of the mutant enzyme showed that it maintained the native-like structure; hydrolytic activity, and substrate specificity of the mutant enzyme analogous to those of the wild-type enzyme. Susceptibility of the mutant enzyme toward proteolysis and pressure denaturation was reduced by 10–20%. It is concluded that the carbohydrate moiety functions to maintain the structural integrity of the enzyme under stressed.

Key words: carboxypeptidase Y; serine carboxypeptidase; site-directed mutagenesis; glycoprotein

Carboxypeptidase Y of Saccharomyces cerevisiae is an N-linked glycoprotein in which Asn-13, Asn-87, Asn-168, and Asn-368 are glycosylated. All four sites have the consensus glycosylation sequence, Asn-Xaa-Thr. Two N-acetylglucosamine molecules and 14 to 18 mannose molecules are attached to these glycosylation sites except for Asn-87, at which 9 to 11 mannose molecules are located. No phosphorylation occurred on the mannose molecules attached to Asn-87, while two mannose molecules at the other sites were phosphorylated. The total mass of carbohydrate is around 10 kDa, 20% of which was attached to Asn-87.1,2

Carboxypeptidase Y has three distinct forms, p1CPY, p2CPY, and mCPY. p1CPY, found in endoplasmic reticulum, has a mass of 67 kDa and is modestly glycosylated. p1CPY is transported into the Golgi compartment and converted to p2CPY, which has a mass of 69 kDa and is highly glycosylated. p2CPY is further moved to the vacuolar compartment where the final maturation process occurs to produce mCPY.2,4 Although the carbohydrate moiety was suspected of being involved in the protein maturation process, i.e., sorting, folding and stability,2,3,5,6 the role of carbohydrate in the maturation process has not been fully defined.

Understanding of the role of carbohydrate moiety in glycoprotein has been a subject of interest for those concerned with protein signaling inside living cells. De- or unglycosylation has been used to clarify the role of carbohydrate moieties in glycoproteins. Generally, carbohydrate is deleted from proteins in three ways, enzymatic removal, cell growth in the presence of tunicamycin, and expression of mutant enzymes by site-directed mutagenesis.

When tunicamycin was added to the culture medium to prevent protein glycosylation, carboxypeptidase Y was correctly transported into the vacuole.6 Similarly, normal transport of carboxypeptidase Y was observed in vivo among the mutant enzymes, the carbohydrate attaching sites of which were altered. The only difference among them was the transport rate; the rate for the Asn-87 mutant was much slower than those for the three other mutants.7 Therefore, the role of carbohydrate in the maturation process of carboxypeptidase Y should be re-examined.

Enzymatically deglycosylated carboxypeptidase Y was prepared by using Endo F or Endo H.9,10 Although many properties of the deglycosylated enzyme resemble those of the native enzyme, reduced stability toward proteolysis as well as organic solvent was demonstrated.9 Endo F-treated enzyme gave crystals from which a high resolution X-ray structure was obtained.10 The crystal structure identifies three N-acetyl-d-glucosamine residues remaining at Asn-87, 168, and 368 after End F digestion. The native structure of carboxypeptidase Y, with the complete carbohydrate unit, has not been crystallized yet.

In this study, completely unglycosylated carboxypeptidase Y was prepared by site-directed mutagenesis and the structural properties were characterized for the first time.

Materials and Methods

Materials. Plasmid pTSY3, containing the PRC1 gene coding for carboxypeptidase Y was kindly provided by Dr. Klaus Breddam of Carlsberg Laboratory, Denmark. Bacteriophage M13mp18 and the Sculptor in vitro mutagenesis system were purchased from Amersham (Buckinghamshire, England), synthetic oligonucleotides from

† To whom correspondence should be addressed. Fax: +81-75-753-6128

Abbreviations: ATDE, α-N-acetyl-l-tyrosine ethyl ester; BTPNA, α,N-benzoyl-l-tyrosine p-nitroanilide; CD, circular dichroism; CPY, carboxypeptidase Y; Endo F, Flavobacterium sp. endo-β-N-acetylglucosaminidase; Endo H, Streptomyces plicatus endo-β-N-acetylglucosaminidase H; Z, benzoyloxycarbonyl
Japan Bio Services (Saitama, Japan), restriction endonucleases and T4 polynucleotide kinase from Toyobo Ltd (Osaka, Japan), a Transformer site-directed mutagenesis kit from Clontech (Palo Alto, USA), a DNA ligation kit from Takara (Shiga, Japan), and an ABI PRISM dye terminator cycle sequencing ready reaction kit from Perkin Elmer (Foster City, USA).

An Applied Biosystems 373A DNA sequencer and Perkin Elmer Cetus model 480 were used for DNA sequencing and PCR, respectively. An Applied Biosystems model 476A protein sequencer was used for N-terminal amino acid sequencing. An ultrafiltration apparatus with a PM 30 membrane was from Amicon, Inc (Danvers, USA). For solvent evaporation, Savant Vapornet AES 1000 Speedvac concentrator was used. UV-VIS spectra were measured on a Shimadzu UV-160A. For CD and fluorescence measurements, a JASCO J-720W spectropolarimeter and Shimadzu RF-5300PC spectrophotometer was used, respectively. For SDS-PAGE and isoelectric focusing, Hoefer Mighty Small single slab units and an Atto flat bed isoelectric focusing apparatus (Tokyo, Japan) were used, respectively. For the pressurization, a Hikari Kouatsu high pressure pump (Hirosima, Japan) was used and a detailed protocol was written elsewhere.10

A wild-type carboxypeptidase Y was obtained from Oriental Yeast Co. (Lot OY 73-11). DEAE-Sephadex A-50 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), BTPNA from Nacalai Tesque (Kyoto, Japan), Z-Phe-Leu-OH from Sigma Chemicals (St. Louis, USA), ATEE from Protein Research Foundation (Osaka, Japan), Z-Phe-CH2Cl from Fox Chemical Co. (Los Angeles, USA), and urea Ultra Pure from ICN Biomedicals, Inc. (Ohio, USA). Porcine pancreas elastase was purchased from Boeringer Mannheim (Tokyo, Japan), bovine pancreas α-chymotrypsin, trypsin, bakers yeast proteinase A and hog stomach mucosa papain were from Sigma Chemicals. Bacillus thermoproteolyticus thermolysin, Bacillus amyloliquofaciens subtilisin BPN', and Tritirachium album proteinase K were from Nacalai Tesque, Staphyloccocus aureus proteinase V5 from Seikagaku Co. (Tokyo, Japan), and penicilloproteinase LF from Funakoshi Co. (Tokyo, Japan). All other chemicals and solvents were of analytical grade and obtained locally. Endo F was a gift from Dr. Kenji Yamamoto of Kyoto University.


\[
\text{5'} \rightarrow \text{ACTACAAAAGAGCTCACCCTCGCGCGTG} < 3'.
\]

After the construction of a subclone of sequences containing the 2.6-kb SaI-SacI fragment of pTSY3 (PRC1) inserted into the SaI and SacI sites of M13mp18, the mutagenesis (N13, 87, 168, 368A) was done with a Sculptor in vitro mutagenesis system.10 The sequences of the oligonucleotides, mutant residues underlined, were as follows:

\[
\begin{align*}
5' & \rightarrow \text{GGCATTGACCCA-} \\
& \text{GCCGTCAACAGATAC} < 3' \quad (\text{Asn13} \rightarrow \text{Ala}) \quad 5' & \rightarrow \text{TCTTGGAAACGCGCCGCCGT-} \\
& \text{GATC} < 3' \quad (\text{Asn87} \rightarrow \text{Ala}) \quad 5' & \rightarrow \text{GACAGAAAATTCTGCTTAACCTCCGT} C' < 3' \\
& \text{C} \quad (\text{Asn168} \rightarrow \text{Ala}) \quad 5' & \rightarrow \text{CAAAAAGTACGTGCCTGGACTTCT} < 3' \\
& \text{A} \quad (\text{Asn368} \rightarrow \text{Ala}) \end{align*}
\]

The mutant PRC1 gene containing four mutated alanine residues was reintroduced into the original PRC1 gene context of the plasmid pTSY3 (PvuII→SacI). This plasmid encoding carboxypeptidase Y lacking four glycosylation sites was named pTSY3-degCPY. All DNA subcloning steps were done with a DNA ligation kit. Transformation of the yeast strain SEY2202 was done by the lithium acetate method. 15 E. coli TG1 was transformed with the standard transformation buffer.16

\[
\text{CPY purification. Wild-type carboxypeptidase Y was prepared as described previously,17,18 and further purified by hydroxylapatite chromatography. Unglycosylated carboxypeptidase Y (N13, 87, 168, 368A) was purified from the yeast strain SEY2202 transformed by pTSY3-degCPY. Extracts were prepared by breaking yeast cells with glass beads as follows. The cells were centrifuged at 7,000 rpm for 15 min at 4°C, and its 50 g was resuspended in 150 ml of 50 mM Tris-HCl buffer at pH 7.5, containing 10 mM magnesium sulfate, 10 mM potassium acetate, 1 mM EDTA, and 1 mM DTT. To the cell suspension was added 200 ml of glass beads, and the suspension was vortexed vigorously in a 350-ml cell for 1 min with intermittent cooling on ice for 2 min. This step was repeated 3 times. The glass beads were allowed to settle and the supernatant was transferred to a centrifugation bottle and centrifuged at 10,000 rpm for 60 min at 4°C. Unglycosylated carboxypeptidase Y was purified from this supernatant through fractionation by ammonium sulfate, dialyzing against 10 mM potassium phosphate buffer, pH 7.0, with 0.1 M potassium chloride, and chromatographed in two steps on DEAE-Sephadex A-50. Elution of the second chromatography on DEAE-Sephadex A-50 was done with a linear increase in potassium chloride concentration from 0 to 0.5 M. The eluent was desalted and concentrated to a protein concentration of 1 mg/ml by ultrafiltration. Purity and molecular mass were monitored by SDS-PAGE.}
\]
Characterization of wild-type and unglycosylated CPY. The N-terminal amino acids were sequenced with a protein sequencer. CD measurements were done at room temperature, where 6 repeated measurements were accumulated at the concentration of 0.2 mg/ml of wild-type or unglycosylated carboxypeptidase Y. For fluorescence measurements, 0.1 mg/ml of enzyme solution was excited at 280 nm at room temperature. Activities were monitored at 410 nm for the hydrolysis of 0.3 mM BTP-NA at 37°C, at 570 nm for the increase in ninhydrin color on the hydrolysis of 0.2 mM Z-Phe-Leu-OH at 25°C, or at 230 nm for the hydrolysis of 5 mM ATEE at 25°C. Inhibition by Z-Phe-CH3Cl was done with a 300-fold molar excess of Z-Phe-CH3Cl over protein concentration. After the incubation, residual activities were tested with 0.3 mM BTPNA at pH 7.0, 37°C.

Heat inactivation of enzymes was done by incubation at 20–60°C for 30 min in 0.05 mM bis-Tris-1 mM EDTA buffer, pH 6.5, in the protein concentration of approximately 25 μg/ml. Inactivation of both enzymes by urea was done at 25°C for 40–180 min in 2–8 M urea using the protein concentration of approximately 40 μg/ml. Inactivations of both enzymes by proteases were done at 25°C for 24 hr in 0.1 M CH3COOH–CH3COONa buffer of pH 4.5 for proteinase A and in 0.1 M Tris-HCl buffer of pH 8.0 for other proteases. The concentration of the enzyme in the incubation mixture was approximately 50 μg/ml and the ratio of protease and carboxypeptidase Y was 1:10 (w/w).

Inactivation of enzymes by pressure was done at 25°C for 30 min under 200 or 400 MPa in 0.05 mM bis-Tris-1 mM EDTA buffer, pH 6.5. The concentration of carboxypeptidase Y in the incubation mixture was approximately 25 μg/ml. After each incubation, residual enzyme activities were measured toward 0.3 mM BTPNA in 0.1 M phosphate buffer, pH 7.0, at 37°C.

Enzymatic removal of carbohydrate moiety. Carboxypeptidase Y (100 μg) was incubated with Endo H (0.4 units) for 24 h at 37°C in 200 mM sodium acetate buffer, pH 6.0. Samples of the reaction mixture were analyzed by SDS-PAGE. Carbohydrate content was measured by a colorimetric method.20

Results

Expression of mutant CPY

A degPRC1 gene containing N13A, N87A, N168A, and N368A mutations was prepared on M13mp18 system. Initial attempts to prepare the expression vector from the SalI–SacI fragment (2632 bp) were unsuccessful, probably due to the fragment being too large. Therefore, degPRC1 was digested with BgII to give two fragments, SalI–BgII and BgII–SacI, which were then cloned into the pTSY3 vector. DNA sequence analysis confirmed the codon changes. A yeast strain, SEY2202, was then transformed to express unglycosylated carboxypeptidase Y.

Purification of mutant CPY

Large scale cultivation (65 l) of mutant carboxypeptidase Y was done to give 2,040 g of wet cell paste. As summarized in Table 1, 15.6 mg per 1,000 g of wet cell paste of the purified unglycosylated carboxypeptidase Y was obtained to give an yield of 13.5%.

In ion exchange chromatography, unglycosylated carboxypeptidase Y was eluted at a higher salt concentration (0.45 M KCl) than the wild-type enzyme (0.35 M KCl) (Fig. 1).

Properties of enzymatically deglycosylated CPY

One major and two minor deglycosylated bands were obtained on SDS-PAGE after the 24 h incubation with Endo H (Fig. 2). The apparent mass of the major band was estimated to be 53.7 kDa. The content of carbohydrate in the 53.7 kDa fraction was about 44% of that in the untreated wild-type enzyme.

Structure of mutant CPY

The apparent molecular mass of unglycosylated carboxypeptidase Y was 51 kDa as shown in SDS-PAGE (Fig. 2), which was considerably smaller than that of the Endo H treated native carboxypeptidase Y. The isoelectric focusing point of the unglycosylated carboxypeptidase Y was 4.2 while that of the wild-type was 3.5. Pro-

Table 1. Summary of Unglycosylated Carboxypeptidase Y Purification Steps

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total volume (ml)</th>
<th>Total protein (μg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/μg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dialysis</td>
<td>2,500</td>
<td>30,600</td>
<td>263</td>
<td>0.0086</td>
<td>100</td>
</tr>
<tr>
<td>DEAE-Sepharose stepwise</td>
<td>126</td>
<td>266</td>
<td>62.4</td>
<td>0.235</td>
<td>23.8</td>
</tr>
<tr>
<td>Chromatography</td>
<td>14</td>
<td>15.6</td>
<td>35.5</td>
<td>2.24</td>
<td>13.5</td>
</tr>
</tbody>
</table>

Fig. 1. Second DEAE-Sepharose A-50 Chromatography of Unglycosylated Carboxypeptidase Y (N13, 87, 168, 368A).

The effluent of the first DEAE-Sepharose A-50 chromatography having BTPNA hydrolysis activity was put on the column (5 cm diameter × 6 cm height) and eluted with a linear salt gradient (5 ml/fraction). Solid line indicates an absorbance monitor at 280 nm and open circle indicates an activity. Arrow shows the elution position for the wild type carboxypeptidase Y.
tein sequence analysis for the first 5 cycles of the mutant enzyme confirmed that the N-terminal sequence was the same as the wild-type enzyme (NH$_2$-Lys-Ile-Lys-Asp-Pro). Circular dichroism (CD) of the mutant enzyme in the far UV region was measured. Almost identical CD spectra for the wild-type and mutant enzymes indicate the similarity of their secondary structures. Intrinsic tryptophan fluorescence spectrum showed little changes in the fluorescence intensity as well as the band position. These results suggest that the removal of all four carbohydrate moieties has little effect on the structure of carboxypeptidase Y.

**Enzymatic properties of mutant CPY**

Peptidase, esterase, and anilidase activities of unglycosylated enzyme toward Z-Phe-Leu-OH, ATEE, and BTpNA, respectively, are summarized in Table 2. The mutant enzyme showed slightly lower (5–8%) peptidase and esterase activities than the wild-type enzyme while anilidase activity of mutant enzyme was a little higher. Inhibition profiles of the wild-type and mutant carboxypeptidase Y with Z-Phe-CH$_2$Cl are shown in Fig. 3. Both enzymes were inhibited in an identical manner. Results suggest that wild-type and mutant enzymes are catalytically compatible.

**Stability of mutant CPY**

Stability of the unglycosylated enzyme against typical denaturants, i.e. urea, heat, and pressure, was examined. When mutant enzyme was incubated with urea, the activity loss increased as incubation time and urea concentration increased (Fig. 4); the profile was nearly identical to that of the wild-type enzyme. After 30 min of heat treatment at various temperatures, the obtained heat denaturation curves for the mutant and wild-type enzymes indicated the denaturation temperature to be 53°C. The pressure treatment on the wild-type enzyme at 200 and 400 MPa reduced the activity to 90 and 78% of the untreated enzyme, respectively. The mutant enzyme had 8% less activity than the wild-type enzyme throughout the pressure range examined (Fig. 5).

**Table 2.** Relative Specific Activity of Wild-type and Mutant Carboxypeptidase Y toward Different Substrates

<table>
<thead>
<tr>
<th></th>
<th>CPY</th>
<th>Z-Phe-Leu-OH</th>
<th>ATEE</th>
<th>BTpNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>(100)$^b$</td>
<td>(100)$^b$</td>
<td>(100)$^b$</td>
<td></td>
</tr>
<tr>
<td>Mutant$^c$</td>
<td>95</td>
<td>92</td>
<td>110</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Assay conditions: 0.2 mM Z-Phe-Leu-OH at pH 6.5, 25°C; 5 mM ATEE at pH 7.5, 25°C; 0.3 mM BTpNA at pH 7.0, 37°C.

$^b$ Wild-type activities for each substrate were set as 100% active.

$^c$ Mutant represents unglycosylated carboxypeptidase Y (N13, 87, 168, 368A).
This suggests that the carbohydrate moiety is not required for the catalytic function of carboxypeptidase Y. Our results are consistent with the results obtained with an analogous mutant, T15A+N87I+N168Q+N368Q, except that this latter mutant had 20% lower activity, which might be due to the difference in the substituted amino acids and their locations.

A carbohydrate moiety contributes to the heat stability of some glycoproteins. For example, yeast invertase, the carbohydrate contents of which, being near 50% of its mass of 270 kDa, showed a significantly lowered $T_m$ when the carbohydrate moiety was removed with Endo H. On the other hand, the heat stability of other glycoproteins is not affected by the carbohydrate moiety. Unlike invertase, the removal of the carbohydrate moiety in carboxypeptidase Y either by site-directed mutagenesis of this study or Endo H caused no changes in the heat stability.

The outcome of the tests with different structural perturbants suggests the denaturation by pressure takes a significantly different path from that by heat and urea. Since activity was also monitored as a structural probe, we have examined how far carbohydrates were from the active site of the enzyme by molecular modeling. The use of Quanta on a IRIS computer has shown that the locations of the four carbohydrate attaching sites are clearly away from both catalytic center and substrate binding pocket. Therefore, it is unlikely that the carbohydrate moiety directly affects the catalytic function of carboxypeptidase Y. We can assume that pressure-induced denaturation must originate from the area where carbohydrate moieties are nearby. Under such circumstances, it is reasonable to assume that the presence of the carbohydrate moiety protects the enzyme from pressure-induced denaturation.

Glycoproteins expressed in the presence of tunicamycin become susceptible to proteolysis in vivo. This result has implied that the carbohydrate moiety covers the potential proteolytic site(s) in proteins; thus, unglycosylated proteins become the targets of in vitro proteolysis. Wild-type carboxypeptidase Y is highly resistant toward proteolytic cleavage, probably due to its high carbohydrate contents. Our experiment here demonstrated that unglycosylated carboxypeptidase Y lost up to 23% of its activity. This suggests that unglycosylated carboxypeptidase Y becomes susceptible to in vitro proteolysis. The most likely explanation is that the carbohydrate moiety covers up the potential cleavage sites, thus preventing proteolytic attack. This view is supported by our preliminary data, which indicates protease resistance is diminished once the protein structure is partially denatured (Watani, M., et al., manuscript in preparation). Since the unglycosylation lowered the protease resistance only by 7–23%, the mutant enzyme is still considered to be proteolysis resistant. The clue to the nature of the proteolysis resistance may be found in the unique structural features built in the enzyme in addition to the carbohydrate moiety covering the cleavage sites. Further structural studies may clarify the elements which make carboxypeptidase Y highly resistant toward proteolysis.

### Proteolytic susceptibility of mutant CPY

Incubation with the proteases for 24 h reduced the activity of both the wild-type and mutant enzymes. However, the loss was 7 to 23% greater in the mutant enzyme than the wild-type enzyme (Table 3).

### Discussion

Since the role of the carbohydrate moiety has been the point in question in the enzyme maturation process in vivo, we have designed and examined the efficiency of the expression of the mutant enzyme, unglycosylated carboxypeptidase Y (N13, 87, 168, 368A). Yield of the mutant enzyme was within the range of the wild-type and mutant enzyme preparations, which suggests the role of carbohydrate moiety is insignificant in the biosynthesis of this enzyme. In other words, the carbohydrate moiety has only a little role in protein folding and protein sorting. The mutant enzyme had enzymatic activity fully compatible with the wild-type carboxypeptidase Y.

![Fig. 5. Effects of Pressure on the Wild-type and Unglycosylated Carboxypeptidase Y.](image)

**Table 3. Effects of Proteases on Wild-type and Mutant Carboxypeptidase Y**

<table>
<thead>
<tr>
<th>Protease</th>
<th>Activity remained (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
</tr>
<tr>
<td>Penicilliproteinase LF</td>
<td>93</td>
</tr>
<tr>
<td>Proteinase V8</td>
<td>97</td>
</tr>
<tr>
<td>Papain</td>
<td>93</td>
</tr>
<tr>
<td>Thermolysin</td>
<td>87</td>
</tr>
<tr>
<td>Trypsin</td>
<td>90</td>
</tr>
<tr>
<td>Elastase</td>
<td>93</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>84</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>84</td>
</tr>
<tr>
<td>Proteinase A</td>
<td>85</td>
</tr>
<tr>
<td>Subtilisin</td>
<td>72</td>
</tr>
</tbody>
</table>

* $^a$ Incubated at 25°C for 24 h under the protease/CPY ratio of 1:10 (w/w).
* $^b$ $d$ designates difference in activity between the wild-type and mutant enzymes.
Acknowledgments

We wish to thank Kenji Yamamoto for kindly providing Endo F, Klaus Breddam for the gift of the carboxypeptidase Y expression vector, and Michihiko Kataoka and Sakayu Shimizu for the use of their large scale cultivation facility.

References


