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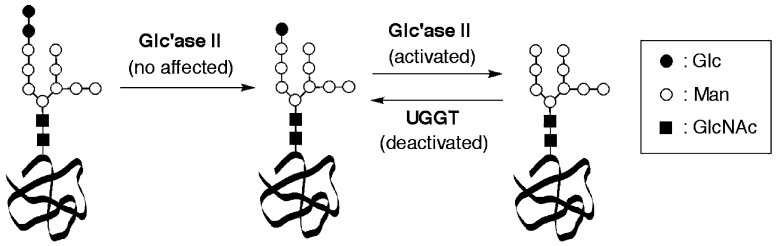

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Effects of Macromolecular Crowding on Glycoprotein Processing Enzymes

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Abstract: Intracellular environments are highly crowded due to the presence of various biomacromolecules. In this study, we estimated the property of the endoplasmic reticulum glucosidase II (G-II) under macromolecular crowding conditions. A crowded milieu that contains bovine serum albumin greatly enhanced the second trimming step (cleavage 2), which deglucosylates $Glc_1Man_9GlcNAc_2$, but not the first trimming step (cleavage 1), which removes the terminal glucose residue from $Glc_2Man_9GlcNAc_2$. A similar effect was obtained with ribonuclease A and high molecular weight polyethylene glycol 20 000. An analysis of CD spectra suggested that G-II enhanced its cleavage 2 activity through conformational change. We also investigated the effects of molecular crowding on other *N*-linked glycan-processing enzymes, UDP-Glc: glycoprotein glucosyltransferase and 1,2-α-mannosidase. Our results indicate that the kinetics of glycan processing under crowded conditions may be quite different from those measured in dilute buffers.

Introduction

The intracellular environment is highly crowded because of the presence of various biomacromolecules such as nucleic acids, proteins, and polysaccharides.[1−4] The total concentration of these macromolecules is extremely high, reaching several hundred grams per liter.[5,6] Such an environment may be substantially different from that commonly encountered in biochemical experiments, in which the typical concentration of biomacromolecules is less than 1 g/L. Under such crowded conditions, the biochemical equilibrium is shifted to a state in which the excluded volume is minimized. Under these circumstances, compact conformation of macromolecules is favored, and their association would be enhanced.[7] As a result, elevation of enzyme activities has been observed in certain cases.[8−12] Such phenomena are referred to as volume exclusion effect (positive macromolecular crowding effects).[13] However, a crowded environment decelerates the diffusion of molecules,[14] and the rate of diffusion-controlled reactions may decrease (negative macromolecular crowding effect).[15] Consequently, the balance of these counteracting factors determines the velocity of reactions under crowded conditions. Effects of crowding agents on the stability of DNA structures were also reported.[16−18] In the latter case, the effects of molecular crowding were ascribed to the changes of water activity and DNA hydration.

Glucosidase II (G-II)[19] plays a key role in glycoprotein processing in the endoplasmic reticulum (ER). This enzyme trims two α-1,3-linked glucose residues, Glcα1 → 3Glc (cleavage 1) and Glcα1 → 3Man (cleavage 2) from high-mannose type $Glc_2Man_9GlcNAc_2$ (G2M9)-proteins (Figure 1).[20] Upon cleavage 1, G2M9 is converted to $Glc_1Man_9GlcNAc_2$ (G1M9), which is retained in the ER by virtue of its binding with lectin-like chaperones calnexin (CNX) and calreticulin (CRT).[21,22] The other activity (cleavage 2) converts G1M9 to non-glucosylated glycoform $Man_9GlcNAc_2$ (M9), thereby liberating glycoproteins from CNX/CRT. However, M9 of incompletely folded glycoproteins are reglucosylated by UDP-glucose: glycoprotein glucosyltransferase (UGGT),[23] the folding-sensor enzyme in the ER. This reglucosylation promotes the

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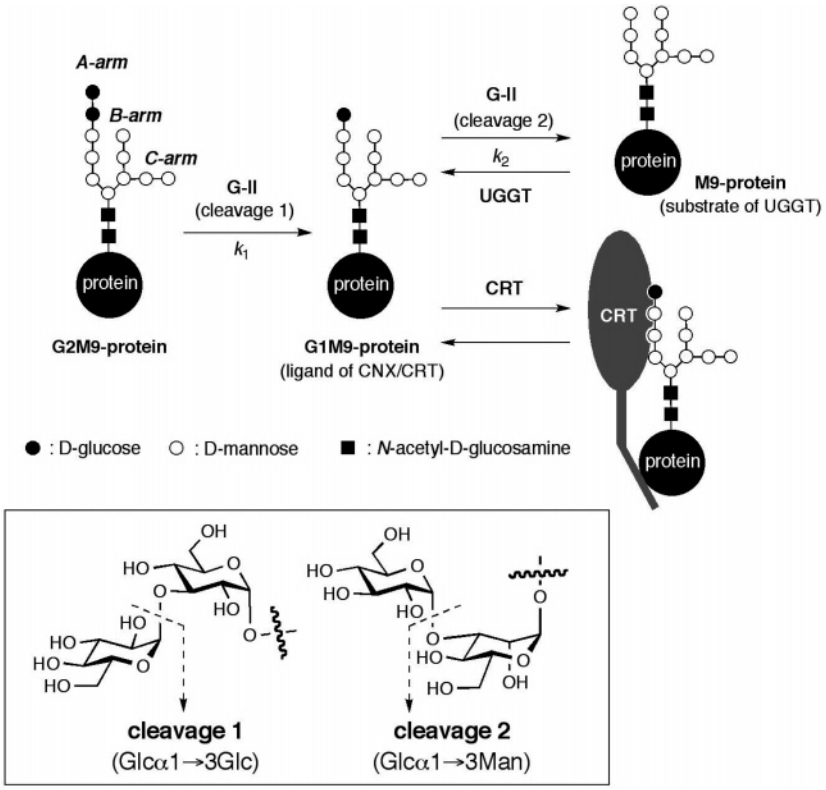

Figure 1. Dual activity of G-II.

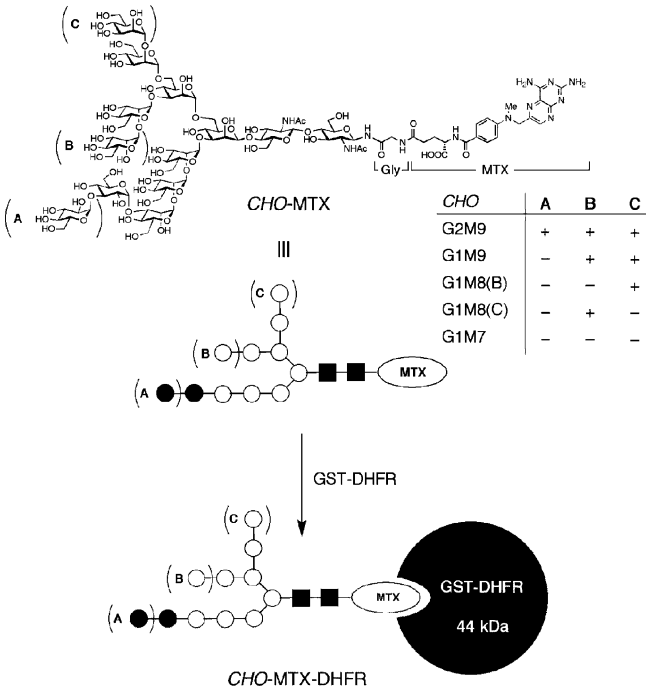

Figure 2. Synthetic oligosaccharide substrates used in this study. GST, glutathione-*S*-transferase; DHFR, dihydrofolate reductase.

association of un(mis)folded glycoproteins with CNX/CRT to maximize their folding. Our previous studies focused on quantitative analyses of the specificities of UGGT[24] and G-II[25] using synthetic substrates.

In order to conduct quantitative analyses of G-II and UGGT, we have employed high-mannose-type glycan-methotrexate (MTX) derivatives (*CHO*-MTXs) as substrates (Figure 2)[24,26] for the following reasons. First of all, a strategy for systematic synthesis of various *CHO*-MTXs has been established.[27] Second, MTX exhibits specific absorption at 304 nm, which enables quantitative monitoring of enzymatic reactions by HPLC.[24,25] Third, MTX-derivatized undecasaccharide (M9-MTX) was discovered as a non-peptidic substrate of UGGT.[24] Fourth, since MTX is a tight-binding (K_D < nM) inhibitor of dihydrofolate reductase (DHFR),[28] *CHO*-MTX can be easily converted to glycan-protein conjugate (*CHO*-MTX-DHFR), which could be used as glycoprotein mimetics (Figure 2).[26] In this study, an artificially crowded environment was produced by adding macromolecules, such as bovine serum albumin (BSA, 68 kDa), ribonuclease A (RNase A, 14 kDa), and polyethylene glycol (PEG, 0.2∼20 kDa), all of which were expected to be inoffensive to the reactions of interest.

Materials and Methods

Materials. *p*NP-Glc and glutathione *S*-transferase (GST) were obtained from Sigma-Aldrich Japan K.K. (Tokyo, Japan). GST-CRT was expressed in *E. coli* as previously described.[29] G2M9-, G1M9-, $Glc_1Man_8(B)GlcNAc_2$ (G1M8B)-, $Glc_1Man_8(C)GlcNAc_2$ (G1M8C)-, $Glc_1Man_7GlcNAc_2$ (G1M7)-, and M9-MTX (Figure 2) were prepared based on a previously reported convergent approach.[26,30] All samples

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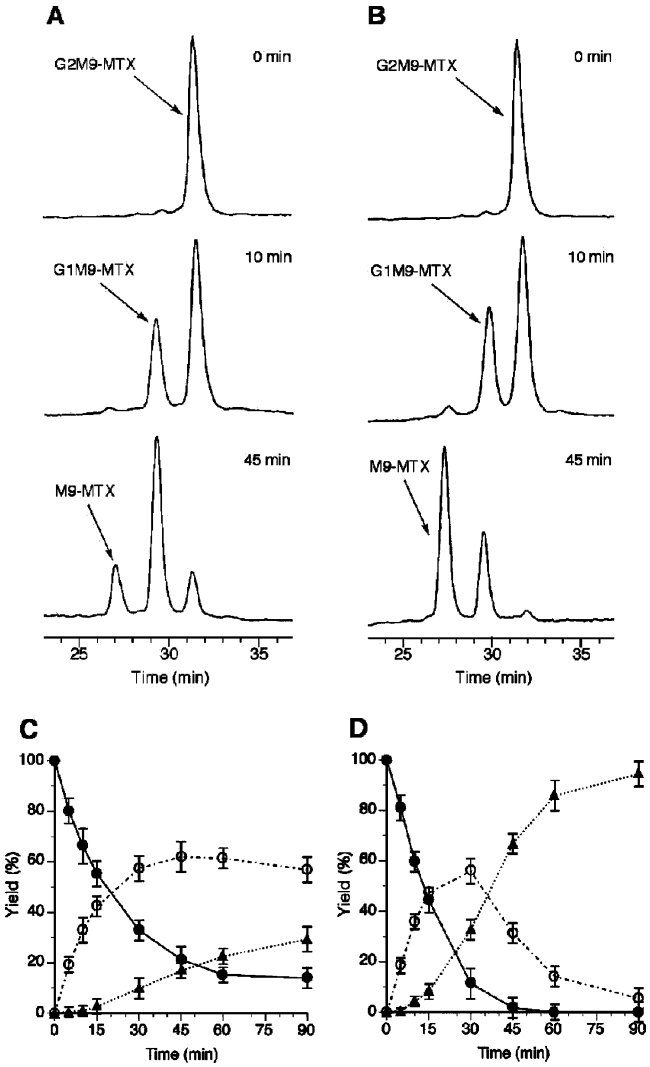

Figure 3. G-II-mediated total glucose-trimming profiles of G2M9-MTX under standard or crowded conditions. (A) HPLC profiles of the trimming of Glc from G2M9-MTX under standard conditions. (B) HPLC profiles of the trimming of G2M9-MTX under crowded (10 wt % BSA) conditions. Conditions for HPLC: TSK-GEL Amide-80 column (4.6 mmϕ × 25 cm), mobile phase CH_3CN/3% AcOH−Et_3N, pH 7.3, linear gradient from 65:35 to 50:50 in 50 min, flow rate 1.0 mL/min at 40 °C. (C) Time course of the trimming of Glc from G2M9-MTX under standard conditions. (D) Time course of the Glc trimming of G2M9-MTX under crowded (10 wt % BSA) conditions. ●, G2M9-MTX; ○, G1M9-MTX; ▲, M9-MTX. All enzyme assays were carried out in a 100-μL reaction mixture containing 50 μM of substrate, 1.2 μg of G-II, 1 mM deoxymannojirimycin, 10 mg BSA (10 wt %) [for panel (B) and (D)], 0.05% Triton X-100, and 10 mM Hepes (pH 7.4), at 37 °C. After the reactions were stopped by heating at 100 °C for 1 min, reaction mixtures were analyzed by HPLC under the conditions described above.

were fully characterized by ^{1}H NMR and matrix-assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF−MS). G1M9-MTX-DHFR was prepared by mixing G1M9-MTX with recombinant GST-DHFR as reported.[26] G-II was purified from rat liver as previously reported.[31] SDS-PAGE of purified G-II showed that the protein contains α- and β-subunits.[25] UGGT was purified from rat liver as reported.[32] 1,2-α-mannosidase was purchased from Seikagaku Co. (Tokyo, Japan). BSA and RNase A were purchased from Sigma-Aldrich Co. PEG200 and PEG2000 were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). Deoxynojirimycin and deoxymannojirimaycin were purchased from Seikagaku Co. and PEG20000 was purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). HPLC was conducted with Waters 2695 (separation module) and Waters 996 (photodiode array detector).

Glucosidase II Assay with *CHO*-MTX. Reaction mixtures contained, in a total volume of 100 μL, 50 μM of *CHO*-MTX, 1.2 μg of soluble rat liver G-II (0.23 milliunits; activity was measured using *p*NP-Glc as a substrate,[31] with one unit defined as the amount that released 1 μmol/min of *p*-nitrophenol at 37 °C), 1 mM deoxymannojirimycin, 0.05% Triton X-100, 0−40 wt % of crowding agent (BSA, RNase A, PEG200, PEG2000, or PEG20000) and 10 mM Hepes (pH 7.4). In some cases, the glucose trimming reactions were performed in the presence of CRT (75 μM). After 5−90 min at 37 °C, 10-μL portions of the mixtures were removed by a micropipette and diluted with 90 μL of H_2O and 100 μL of CH_3CN, then heated at 100 °C for 1 min to inactivate the enzyme. The percentage of glucose trimming in each reaction was analyzed by HPLC [TSK-GEL Amide-80 (4.6 mmϕ × 25 cm) column with 3% AcOH−Et_3N (pH 7.3)/CH_3CN mixed solvent (35:65 to 50:50, linear gradient for 50 min) at 40 °C, 1 mL/min]. *CHO*-MTXs were detected by measuring absorption at 304 nm.

Glucosidase II Assay with *p*-Nitrophenyl α-D-Glucopyranoside (*p*NP-Glc). Reaction mixtures contained, in a total volume of 250 μL, 10 mM of *p*NP-Glc, 3.8 μg of soluble rat liver G-II, 0.05% Triton X-100, 0−40 wt % of BSA, and 10 mM Hepes (pH 7.4). After 5−25 min at 37 °C, 50 μL of the mixtures were removed by a micropipette and diluted with 50 μL of 2 M Tris to terminate the reactions. The reaction rates were calculated from the absorption of released *p*-nitrophenol at 405 nm.

UGGT Assay. Reaction mixtures contained, in a total volume of 100 μL, 100 μM of M9-MTX, 10 mM UDP-Glc, 0.28 μg of soluble rat liver UGGT, 1 mM deoxynojirimycin, 1 mM deoxymannojirimycin, 10 mM $CaCl_2$, 0.6% Triton X-100, 0−40 wt % BSA, and 4 mM Tris·HCl (pH 8.0). After 1−8 h at 37 °C, 10 μL of the mixture was removed by a micropipette and diluted with 90 μL of H_2O and 100 μL of CH_3CN, then heated at 100 °C for 1 min to terminate the reactions. The percentage of glucose transfer in each reaction was analyzed by HPLC (same conditions as described in *Glucosidase II Assay*).

1,2-α-Mannosidase Assay. Reaction mixtures contained, in a total volume of 100 μL, 100 μM of G1M8B-MTX, 0.25 μg of recombinant 1,2-α-mannosidase, 0−40 wt % BSA and 100 mM AcOH−Et_3N (pH 5.5). After 5−60 min at 37 °C, 20 μL of the mixtures were removed by a micropipette and diluted with 80 μL of H_2O and 100 μL of CH_3CN, then heated at 100 °C for 1 min to terminate the reactions. The percentage of mannose trimming in each reaction was analyzed by HPLC (conditions are described in the section on *Glucosidase II Assay*).

CD Measurements. The structural changes of G-II in a crowded environment were assessed by CD spectroscopy. The CD spectra were acquired in the range 200−275 nm with a 1 mm path length at 25 °C on a JASCO J-720 spectrometer. The scan speed was set to 50 nm/min, and the response time, at 0.25 s. For sample preparation, 250 μL of the protein solutions in 10 mM Hepes (pH 7.4) (0.12 mg/ mL) were mixed with various concentrations of PEG20000 to adjust their final concentration to 0, 10, or 20 wt %.

Results

We first compared the glucose-trimming efficiency of G-II (rat liver) with or without a crowding agent. G-II is a heterodimeric enzyme, which consists of an α-subunit (104 kDa) and a β-subunit (58 kDa).[31] The αβ-subunit-complex appears to have a highly extended structure, which is markedly sensitive to protease digestion, giving a globular fragment of 70 kDa comprising two-thirds of the α-subunit.[33] Interestingly, the fragment was fully active against *p*NP-Glc, implying that the α-subunit is the catalytic component, which is conformationally

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rigid.[34] However, the function of the β-subunit has been obscure.[31] Although the role of the HDEL domain as the ER retention signal[33,35,36] and hypothetical lectin activity toward high-mannose-type glycans due to the presence of the mannose 6-phosphate receptor-like (M6PR) domain[37] have been reported, clear evidence to support these notions has not been provided.

As a crowding agent, we selected BSA, for its high molecular weight (68 kDa), excellent solubility, and structural robustness. These properties are particularly important, because larger molecule creates greater excluded volume, giving rise to an enhanced crowding effect,[13] and intracellular concentrations of macromolecules are extremely high (300−400 g/L).[6] In previous works, BSA has often been used as a crowding agent to create pseudo-cellular environments.[3] The excluded volume effect of typical cellular fluid was estimated to be comparable to 35% BSA.[6] In addition, BSA is a nonglycosylated protein, with no carbohydrate-binding activity.

To begin with, G-II mediated trimming of G2M9-MTX was conducted under crowded (10 wt % BSA) or standard (0 wt % BSA) conditions. The generation of G1M9-MTX and M9-MTX was monitored by HPLC (Figure 3A vs 3B), and time courses are provided in Figure 3, parts C and D. As shown in Figure 3, parts A and B, their HPLC profiles were drastically different after 45 min incubation. While the yield of M9-MTX was 19% under standard conditions, the presence of BSA gave rise to 67% of M9-MTX. Under standard conditions, the slope for the generation of G1M9-MTX was much steeper than that for M9-MTX (Figure 3C) and an accumulation of G1M9-MTX was observed. This observation is consistent with the perception that the cleavage 1 is faster than the cleavage 2 ($k_1 \gg k_2$).[38] However, in the presence of BSA, the amount of M9-MTX increased markedly, indicating that G1M9-MTX formed by the cleavage 1 was rapidly converted to M9-MTX (Figure 3D). These results suggest that, under crowded conditions, the rates of cleavage 1 and cleavage 2 may be more similar than widely believed.

In order to evaluate cleavage 1 and cleavage 2 activities separately, we exploited the lectin activity of CRT, which specifically captures G1M9 and strongly retards the cleavage 2.[25] Namely, the cleavage 1 was measured with G2M9-MTX in the presence of excess CRT (1.5 equiv), while the measurement of the cleavage 2 was conducted with G1M9-MTX in the absence of CRT. As anticipated, the cleavage activity was barely affected by the presence of BSA, event at 40 wt % concentration (Figure 4A). However, a remarkable acceleration of cleavage 2 was observed in the presence of BSA in a concentration dependent manner (Figure 4B). The activity rose more than 4-fold with the addition of 40 wt % BSA. Protein-conjugated substrate (G1M9-MTX-DHFR) also achieved acceleration to an extent marginally higher than G1M9-MTX (Figure 4C). By contrast, the addition of BSA did not cause any acceleration of the hydrolysis of *p*NP-Glc, which has been used for in vitro assays of G-II (Figure 4D).[31] These results indicates that the reaction

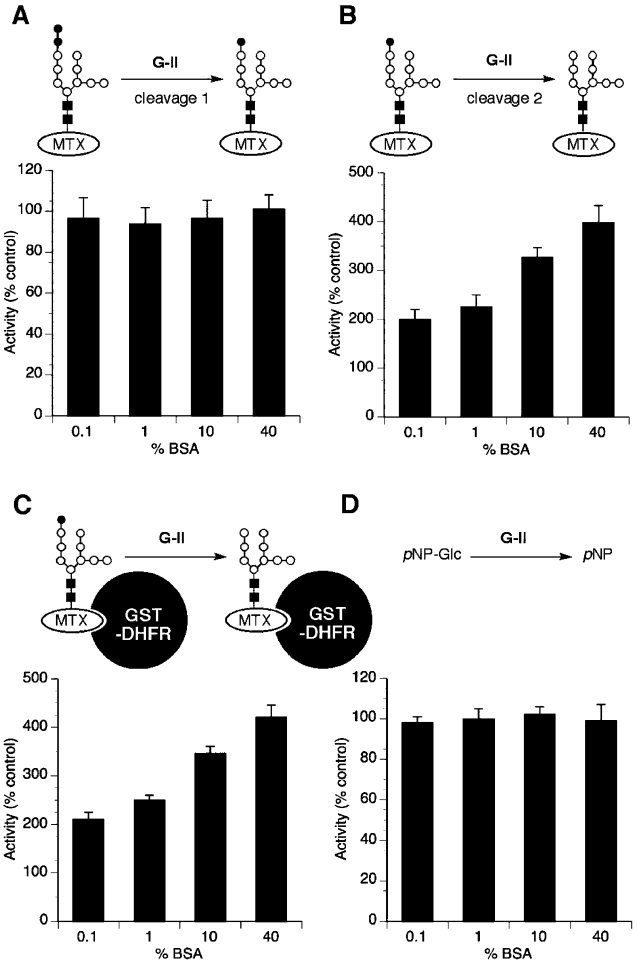

Figure 4. Effect of BSA induced crowding on G-II activity toward various substrates. (A) The trimming of Glc from G2M9-MTX (cleavage 1) in the presence of BSA (0−40%). (B) The trimming of G1M9-MTX (cleavage 2) in the presence of BSA (0−40%). (C) The trimming of G1M9-MTX-DHFR (cleavage 1) in the presence of BSA (0−40%). (D) The hydrolysis of *p*NP-glucose in the presence of BSA (0−40%). All of the enzyme assays were carried out in a 100-μL mixture containing 50 μM of substrate, 1.2 μg of G-II, 1 mM deoxymannojirimycin, 75 μM CRT [for panel (A)], 0−40 mg BSA (0−40 wt %), 0.05% Triton X-100, 1 mM $CaCl_2$ [for panel (A)], and 10 mM Hepes (pH 7.4) at 37 °C. After 10 min of incubation, the reactions were stopped by heating at 100 °C for 1 min. Then the mixtures were analyzed by HPLC under the conditions as follows: TSK-GEL Amide-80 column (4.6 mmϕ × 25 cm), mobile phase CH_3CN/3% AcOH−Et_3N, pH 7.3, linear gradient from 65:35 to 50:50 in 50 min, flow rate 1.0 mL/min at 40 °C. Activities of G-II were calculated from the value for Glc-trimming at 10 min in each reaction.

modes of cleavage 1 and cleavage 2 are different, and the hydrolysis of *p*NP-Glc proceeds in a similar manner as cleavage 1.

In addition to G1M9, several monoglucosylated glycoforms such as G1M8B, G1M8C, and G1M7 may exist in the ER. They are formed either by the trimming of G1M9 (by mannosidases) or by the glucosylation of Man_8(B)$GlcNAc_2$ (M8B), Man_8(C)-$GlcNAc_2$ (M8C) and $Man_7GlcNAc_2$ (M7) (by UGGT).[21,22] We examined whether a crowded environment has any impact on the substrate specificity of G-II (Figure 5A). Our previous study under standard assay conditions revealed that the reactivity order was G1M9-MTX [100] ≥ G1M8B-MTX [95] > G1M8C-MTX [52] > G1M7-MTX [42].[25] When the same assays were conducted in 10 wt % BSA, negligible changes in relative reactivity was observed; reactivity values being G1M9-MTX [100], G1M8B-MTX [90], G1M8C-MTX [47], and G1M7-

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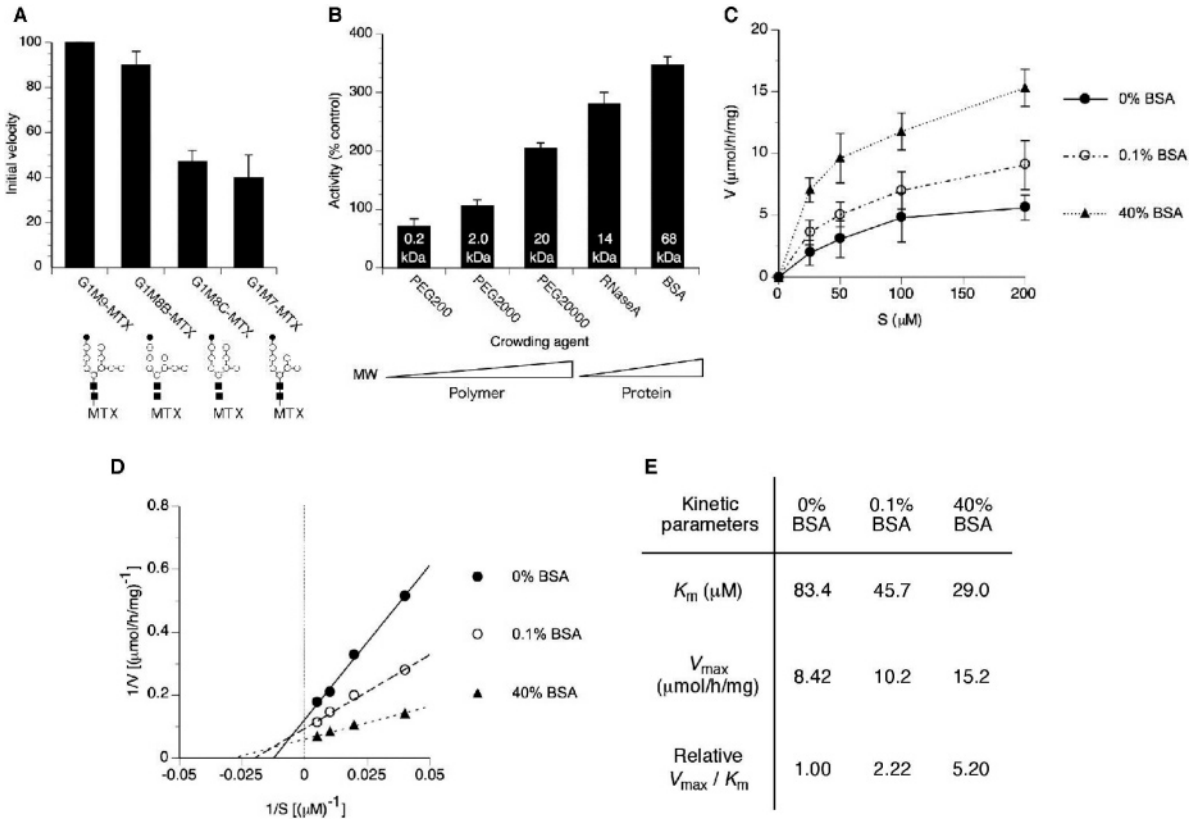

Kinetic parameters	0% BSA	0.1% BSA	40% BSA
K_m (μM)	83.4	45.7	29.0
V_{max} (μmol/h/mg)	8.42	10.2	15.2
Relative V_{max} / K_m	1.00	2.22	5.20

Figure 5. Effects of crowding on G-II mediated Glc trimming. (A) Initial velocity for G1M9-MTX, G1M8B-MTX, G1M8C-MTX, and G1M7-MTX. Initial velocity was calculated from the value for trimming at 10 min in each reaction. (B) The effect of crowding agents at 10% concentration upon the trimming of Glc from G1M9-MTX. Activities of G-II were calculated from the value for Glc-trimming at 10 min in each reaction. The enzyme assays of panels (A) and (B) were carried out in a 100-μL mixture containing 50 μM of substrate, 1.2 μg of G-II, 1 mM deoxymannojirimycin, crowding agent [40 mg of BSA for panel (A); 10 mg of PEG200, PEG2000, PEG20000, RNaseA, or BSA for panel (B)], 0.05% Triton X-100, and 10 mM Hepes (pH 7.4) at 37 °C. After appropriate incubation, the reactions were stopped by heating at 100 °C for 1 min. Then the mixtures were analyzed by HPLC under the conditions as follows: TSK-GEL Amide-80 column (4.6 mmϕ × 25 cm), mobile phase CH_3CN/3% AcOH−Et_3N, pH 7.3, linear gradient from 65:35 to 50:50 in 50 min, flow rate 1.0 mL/min at 40 °C. (C) Correlation of substrate concentration with trimming rate of Glc from G1M9-MTX (0−40% BSA). (D) Lineweaver−Burk plot of G1M9-MTX under crowded conditions (0−40% BSA). (E) K_m, V_{max}, and relative K_m/V_{max} values of G1M9-MTX under crowded conditions (0−40% BSA). The enzyme assays of panel (C) were carried out in a 20-μL mixture containing 25−200 μM of substrate, 0.24 μg of G-II, 1 mM deoxymannojirimycin, 0.2−8 mg BSA (0−40 wt %), 0.05% Triton X-100, and 10 mM Hepes (pH 7.4) at 37 °C. After 5 min incubation, the reactions were stopped by heating at 100 °C for 1 min. Then the mixtures were analyzed by HPLC under the conditions as follows: TSK-GEL Amide-80 column (4.6 mmϕ × 25 cm), mobile phase CH_3CN/3% AcOH−Et_3N, pH 7.3, linear gradient from 65:35 to 50:50 in 50 min, flow rate 1.0 mL/min at 40 °C.

MTX [40]. These results indicate that crowding accelerated the trimming of these glycoforms to the same extent.

Subsequently, we explored the effects of other crowding agents, such as RNase A and PEG. RNase A is a non-glycosylated globular protein, which is much smaller (14 kDa) than BSA. It is stable and soluble enough to be employed as a crowding agent. PEG was chosen as a non-proteinic polymer, various lengths of which are available. Our experiments revealed that the effect of PEG on the cleavage 2 was strongly dependent upon its molecular weight (Figure 5B). When PEG200 (10 wt %) was included, G-II was less active than under standard conditions, possibly because PEG raised the viscosity of the solution, and diminished the diffusion rate of the substrates. Low molecular weight PEG was revealed to interact with proteins through van der Waals interaction,[39] being less suitable as a crowding agent.[3] However, PEG20000 exhibited a substantial degree of acceleration, indicating that this polymer created a crowded environment. PEG of intermediate size (PEG2000) had no effect, possibly because the association-enhancing (positive) and diffusion-suppressing (negative) effects of the macromolecular crowding cancelled each other out. In spite of its smaller molecular weight (14 kDa), RNase A was more effective than PEG20000 in accelerating the deglucosylation of G1M9-MTX. This observation may reflect the lower viscosity of the solution containing RNase A.[12] Alternatively, RNase A might be more effective in producing a larger exclusion volume due to its globular shape. In any event, RNaseA and PEG were less effective than BSA, reflecting their smaller size.

Kinetic parameters of G-II (cleavage 2) under crowded conditions were obtained. The relationship between substrate concentration and glucose trimming velocity was plotted (Figure 5C) and then expanded into a Lineweaver−Burk plot (Figure 5D). K_m, V_{max}, and V_{max}/K_m values were obtained from the approximate straight line in a standard manner (Figure 5E). These values revealed that a crowded environment gave rise to the reduction of K_m and increase of V_{max}. At 40 wt % BSA, V_{max}/K_m was 5× greater than that seen at 0 wt % BSA. Although the decrease of K_m would be ascribed to the excluded volume effect of BSA, the reason for the simultaneous increase of V_{max} seemed less clear.

We hypothesized that the crowded environment induced a conformational change of the enzyme leading to its more active

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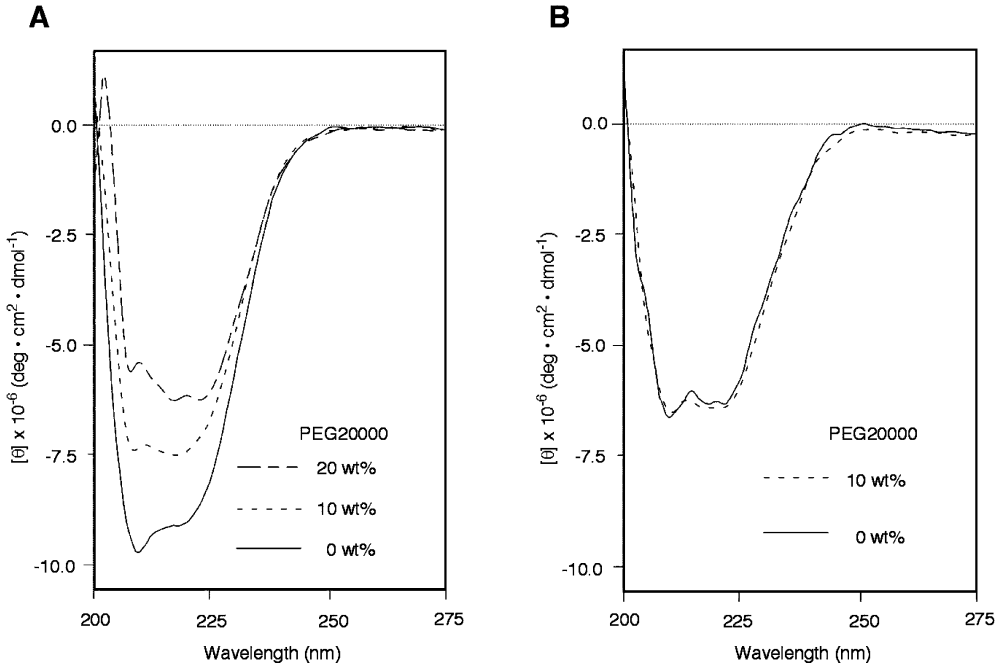

Figure 6. Conformational change of G-II under crowded conditions. (A) CD spectra of G-II (0.12 mg/mL) in the absence or presence (10 and 20 wt %) of PEG20000. (B) CD spectra of glutathione-*S*-transferase (0.12 mg/mL) in the presence of PEG20000 (0 wt %, 10 wt %).

form. To examine this possibility, CD experiments were carried out. For this purpose, we employed PEG20000 as the crowding agent, because the use of BSA was precluded due to its intrinsic absorbance. Thus, CD spectra of G-II in a buffer that contained 0, 10, or 20 wt % PEG20000 were acquired as presented in Figure 6A. As a reference, CD spectra of homo-dimeric GST is provided in Figure 6B. Interestingly, the addition of PEG20000 dose-dependently caused a change in the CD spectrum of G-II, whereas GST did not show any change under the same conditions. These results imply that the crowded environment induced conformational change of G-II.

In our experiments, crowding did not affect the cleavage 1 of G-II but greatly enhanced the rate of the cleavage 2. How does macromolecular crowding impact on other intracellular glycan-processing enzymes? We analyzed properties of the ER glucosyltransferase UGGT[32] and Golgi 1,2-α-mannosidase[40] under crowded conditions. UGGT is an extremely important protein in the ER glycoprotein quality control system, which functions as a "folding sensor". It conducts the reaction opposite to G-II, glucosylating M9 attached to unfolded proteins to give G1M9.[23] We compared its glucose transfer activity under crowded and non-crowded conditions. Assays were carried out using UGGT obtained from rat liver with UDP-glucose as a donor substrate, in the presence of various concentrations of BSA (0, 0.1, 1, 10, and 40 wt %) (Figure 7A). These results showed that the change in the activity of UGGT under crowded conditions was far less significant, with only a marginal reduction in activity as the concentration of BSA increased.

Since we are interested in analyzing the effects of macromolecular crowding on various glycoprotein processing enzymes, our attention was turned to 1,2-α-mannosidase, a Golgi enzyme that removes α-1,2-linked mannose residue(s) from *N*-linked glycoprotein oligosaccharides.[40] To avoid the cleavage

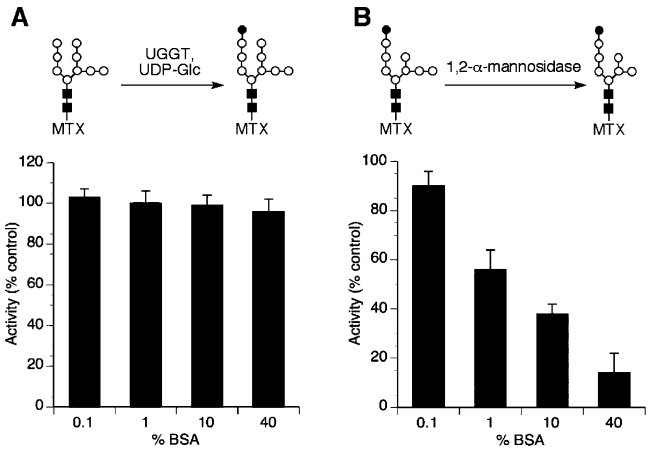

Figure 7. Effect of BSA-induced crowding UGGT and 1,2-α-mannosidase. (A) UGGT-mediated transfer of Glc to M9-MTX in the presence of BSA (0% to 40%). All enzyme assays were carried out in a 100-μL mixture containing 100 μM of substrate, 0.4 μg of UGGT, 0.6 mM deoxymannojirimycin, 0.6 mM deoxynojirimycin, 10 mM $CaCl_2$, 0−40 mg BSA (0−40 wt %), 0.6% Triton X-100, and 4 mM Tris-HCl (pH 8.0) at 37 °C. After 1 h incubation, the reactions were stopped by heating at 100 °C for 1 min. Then the mixtures were analyzed by HPLC [TSK-GEL Amide-80 column (4.6 mmϕ × 25 cm), mobile phase CH_3CN/3% AcOH−Et_3N, pH 7.3, linear gradient from 65:35 to 50:50 in 50 min, flow rate 1.0 mL/min at 40 °C]. Activities of UGGT were calculated from the value for the transfer of Glc at 1 h in each reaction. (B) The 1,2-α-mannosidase mediated Man trimming of G1M8B-MTX in the presence of BSA (0% to 40%). All enzyme assays were carried out in a 100-μL mixture containing 100 μM of substrate, 0.25 μg of 1,2-α-mannosidase, 0−40 mg BSA (0−40 wt %), and 0.1 M AcOH/Et_3N (pH 5.5) at 37 °C. After 10 min incubation, the reactions were stopped by heating at 100 °C for 1 min. Then, mixtures were analyzed by HPLC under the conditions described above. In each reaction, activity of 1,2-α-mannosidase was calculated as the extent of mannose trimming after 10 min.

of multiple mannose residues, we chose G1M8B-MTX as a substrate, which, by treatment with the enzyme, should afford G1M7-MTX as the sole product. In stark contrast with G-II, this reaction was strongly retarded when BSA was included in the assay mixture. (Figure 7B). The fact that the BSA-associated

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reduction in the enzyme's activity was dose-dependent indicates that this reaction is a diffusion-controlled process and retarded in a crowded medium.

Discussion

In the present study, enzymatic properties of ER G-II in a crowded milieu as a pseudo intracellular environment were investigated using chemically synthesized substrates, *CHO*-MTX. Our analyses showed that the crowding did not affect the first trimming of glucose from G2M9, but greatly accelerated the second trimming, converting G1M9 to M9. These results support the proposed notion of a mechanistic difference between the two activities of G-II.[40,41] Assays in dilute buffer have shown that the cleavage 1 proceeds much faster than the cleavage 2.[25,38] However, analyses under crowded conditions revealed a dramatic enhancement of the cleavage 2, while the rate of the cleavage 1 remained unchanged. As a result, the rates of these reactions are more similar than in dilute buffers.

Inspection of the CD spectrum revealed a conformational change of G-II in the presence of high-molecular weight PEG. This implies that the conformational change associated with macromolecular crowding enhances the activity for the cleavage 2. G-II is a heterodimeric protein consisting of α- and β-subunits, and the β-subunit may be essential for the cleavage 2. Our results seem to support the proposed role of the β-subunit as the regulatory fragment. Data given in Figure 4, parts A and D, indicate that the cleavage 1 of G2M9-MTX is not affected by molecular crowding, nor is the hydrolysis of *p*NP-Glc. These results are intriguing in light of previous reports, which showed that the β-subunit was not required for the hydrolysis of *p*NP-Glc,[34] and the cleavage 1 proceeded even when the β-subunit was disrupted.[41] Given that molecular crowding induces the conformational change of G-II, which promotes the cleavage 2 activity, why does it not affect the activity for the cleavage 1 or the hydrolysis of *p*NP-Glc?

Trombetta et al. showed that trypsin-digestion leaves two-thirds of the α-subunit, which contains the catalytic core and adopts a stable globular shape.[33] Therefore, the conformation of the α-subunit is likely to be robust, being barely perturbed by environmental changes such as molecular crowding. Their work also revealed the highly asymmetric extended structure of G-II.[34] As the α-subunit has a nearly globular shape, the β-subunit would likely to be non-globular and conformationally flexible. This notion is supported by its susceptibility to trypsin treatment.[34] It is conceivable, therefore, that the β-subunit adopts a more compact conformation in crowded medium, which renders G-II active for the cleavage 2. A recent study by Wilkinson et al. proposed the participation of the β-subunit in cleavage 2, based on the observation that a β-subunit-disrupted yeast G-II showed no cleavage 2 activity for high-mannose-type glycans in the ER.[41] Our results strongly support this and suggest that the β-subunit regulates the dual activity of G-II in association with its conformational change. Deprez et al. has proposed a model in which the M6PR-like domain of the β-subunit acts as a lectin that recognizes the 6′-pentamannosyl branch of high-mannose-type glycans.[42] The precise role of the β-subunit in substrate recognition would be revealed by an analysis of its lectin activity or by the glucose-trimming activity of the M6PR-mutant of G-II.

Conclusions

Although the macromolecular crowding perturbs activities of enzymes, the effects are diverse. Under conditions that mimic the crowdedness of intracellular environments (40% BSA), the cleavage 2 activity of G-II was 5-fold higher than under dilute conditions, whereas that of 1,2-α-mannosidase was suppressed (<20%). However, activities of the cleavage 1 of G-II and UGGT were barely affected. Our study suggests the crowdedness of environments is an important factor that should be taken into consideration in analyses of intracellular events such as glycoprotein processing. In order to prove biological relevance of these results, it is necessary to compare them with the effects of a real intracellular environment. This would be an important issue, which should be addressed in future studies.

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