Thermodynamic Analysis of Interactions between N-Linked Sugar Chains and F-Box Protein Fbs1

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Abstract: Fbs1 is a recently discovered F-box protein that was proposed to recognize high-mannose-type asparagine-linked glycoprotein sugar chains. To reveal the specificity of Fbs1, Manα1→6Manβ1→4GlcNAc$_2$, Manα1→3Manβ1→4GlcNAc$_2$, and Manα1→3(Manα1→ 6)Manβ1→4GlcNAc$_2$ were synthesized and their affinities for Fbs1 were evaluated in comparison with previously synthesized Man$_9$GlcNAc$_2$ and Man$_8$GlcNAc$_2$. These analyses revealed that Man$_3$GlcNAc$_2$ had the strongest affinity and the chitobiose and α1→6 linked Man residue are necessary for Fbs1 to recognize a sugar.

Ubiquitination of proteins is a signal for the proteasome-mediated protein degradation pathway.[1] This process is mediated by a combination of ubiquitin (Ub) activating enzyme (E1), Ub conjugating enzyme (E2), and Ub ligase (E3). Numerous types of Ub ligase exist in the cytosol and play an important role in the selective ubiquitination of target proteins.[2] SCF complex is one of the most extensively studied families of Ub ligases.[3] It consists of Skp1, Cul1, Roc1, and an F-box protein that recognizes the target protein (Figure 1). F-box proteins comprise an F-box domain that binds to Skp1 and various C-terminal domains for target recognition.[4] Fbs1[5] and Fbs2[6] are recently discovered F-box proteins that were proposed to recognize high-mannose-type asparagine (Asn)-linked glycoprotein sugar chains (*N*-glycans).

Asn-linked sugar chains are introduced cotranslationally in the endoplasmic reticulum (ER) as a tetradecasaccharide (Glc$_3$Man$_9$GlcNAc$_2$, Figure 1).[7] Recent reports strongly suggest that misfolded glycoproteins destined for degradation are trimmed to Man$_8$GlcNAc$_2$, which in turn is recognized by the α-mannosidase I-like protein (MLP; EDEM or Htm1p).[8] The latter participates in ER-associated degradation (ERAD) as an acceptor of terminally misfolded glycoproteins, which are transported from the ER to cytosol and degradated by the Ub−proteasome system.[9] Hence, Fbs1 and Fbs2 are likely to recognize the Man$_8$GlcNAc$_2$ form of *N*-glycans. The accumulation of misfolded proteins in neurons is now considered the origin of a growing number of neurodegradative disorders.[10] The expression of Fbs1 is restricted to the adult brain and testis, implying that Fbs1 has a role in the mechanism of such disorders.

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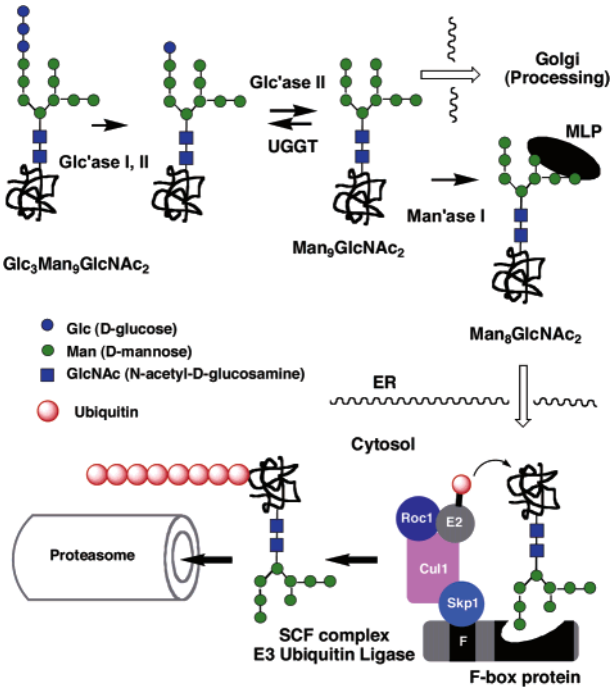

Figure 1. Glycoprotein processing and quality control in ER.

A pull-down analysis of the interaction between Fbs1 and *N*-linked glycoproteins revealed that Fbs1 recognizes *N*-glycans containing a chitobiose (GlcNAcβ1→4GlcNAc) structure with pendent mannose residues.[5] In addition, NMR-based experiments and an X-ray analysis of Fbs1 in complex with chitobiose have clarified the sugar recognition site of Fbs1.[11] However, a quantitative analysis of the binding of *N*-linked sugar chains to Fbs1 has yet to be conducted. Herein, we report the thermodynamic analysis of interactions between Fbs1 and a series of high-mannose- and complex-type oligosaccharides and their partial structures using isothermal titration calorimetry (ITC).[12] These experiments provided detailed information on the mechanism by which Fbs1 recognizes sugars.

To quantify the binding of Fbs1 to *N*-linked oligosaccharide, we prepared oligosaccharide probes (Figure 2). The synthesis of Man$_9$GlcNAc$_2$ (**1**) and Man$_8$GlcNAc$_2$ (**2**) were conducted on the basis of the reported procedure.[13,14] To reveal the specificity of Fbs1, Manα1→6Manβ1→4GlcNAc$_2$ (**5**), Manα1→3Manβ1→4GlcNAc$_2$ (**6**), and Manα1→3(Manα1→6)Manβ1→4GlcNAc$_2$ (**4**) were synthesized and their affinities for Fbs1 were evaluated in comparison with **1** and **2**. As depicted in Scheme 1, preparation of **4**−**6** was conducted in a divergent manner from triol **16**. Thus, thioglycoside **11**[15] was used as the donor, and a reaction with **10**,[16] under activation by *N*-iodosuccinimide (NIS) and triflic acid (TfOH),[17] afforded the chitobiose component **12**. **12** was deacetylated to **13**, which was subjected to β-mannnosylation via intramolecular aglycon delivery using **14** as the donor.[18] The obtained trisaccharide **15** was acetylated, deacetalized, and desilylated under high-pressure conditions[19] to afford the acceptor **16**. α-Mannosylation using the chloride **17**[20] (1.5 equiv) simultaneously provided α1→6 linked (**19**) and α1→3

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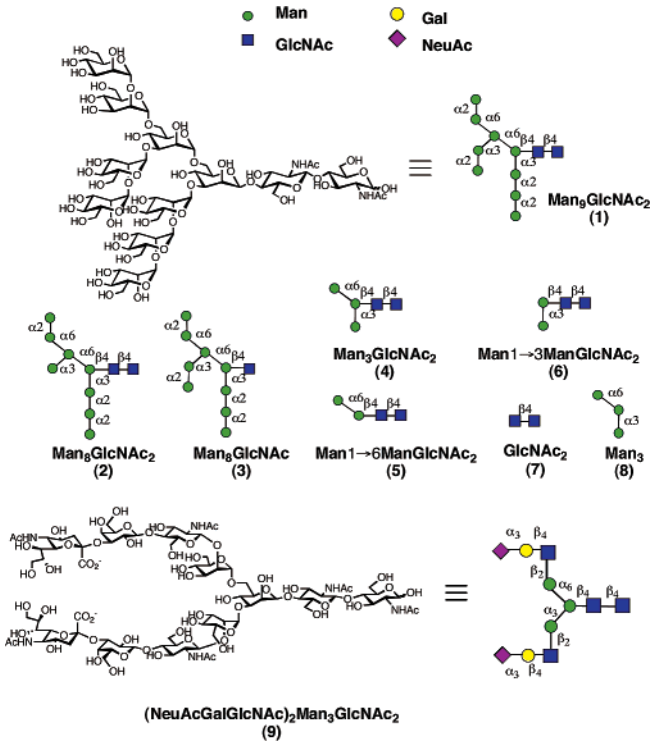
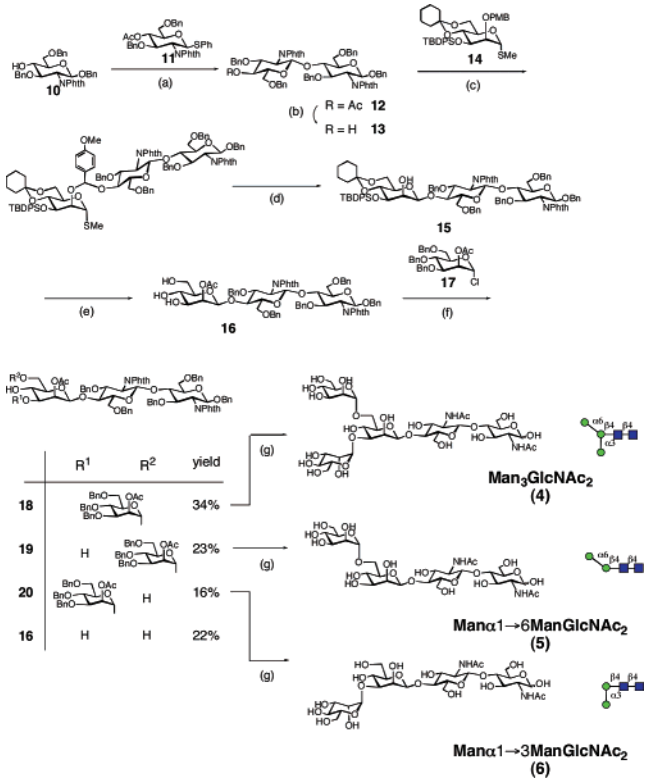


Figure 2. Oligosaccharides used in this study.

Scheme 1[a]



[a] Reagents and conditions: (a) NIS, TfOH, CH_2Cl_2, 98%; (b) 30% H_2O_2(aq), LiOH, THF, 90%; (c) DDQ, CH_2Cl_2; (d) MeOTf, DTBMP, CH_2ClCH_2Cl, 87% (two steps); (e) (i) Ac_2O, pyridine; (ii) PPTS, CH_3CN; (iii) HF/pyridine, DMF, 1 Gpa, 70% (three steps); (f) AgOTf, CH_2ClCH_2Cl; (g) (i) ethylenediamine, *n*-BuOH, 80 °C; (ii) Ac_2O, pyridine; (iii) NaOMe, MeOH; (iv) H_2, 10% Pd/C, H_2O-MeOH.

linked (**20**) tetrasacchaides and $Man_3GlcNAc_2$ (**18**) in 23%, 16%, and 34% yield, respectively, with 22% recovery of the acceptor **16**. These products were easily separated by column chromatography on silica gel and were deprotected under standard conditions. Deprotected oligosaccharides **4**–**6** were identified by comparison with previously reported NMR spectra.

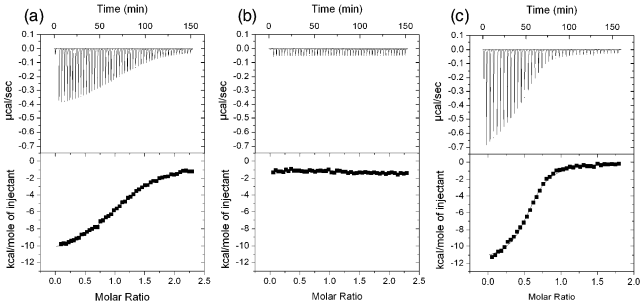


Figure 3. ITC profiles for the binding of synthetic oligosaccharides to Fbs1 at 20 °C. (a) $Man_8GlcNAc_2$, (b) $Man_8GlcNAc$, and (c) $Man_3GlcNAc_2$ were injected every 4 min into buffer solution containing Fbs1 (20 μM). Upper traces show the raw ITC data. Lower traces show the molar heat values plotted as a function of the molar ratio ([oligosaccharide]/[Fbs1]). The solid line represents the best-fit binding isotherm. The data were fitted using a single site model.

Table 1. Thermodynamic Parameters of Interactions between Fbs1 and Synthetic Oligosaccharides

entry	oligosaccharide	$K_a \times 10^{-5}$ (M^{-1})	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})
1	**1** ($Man_9GlcNAc_2$)	3.3	−7.8	−1.4
2	**2** ($Man_8GlcNAc_2$)	4.1	−5.9	5.6
3	**3** ($Man_8GlcNAc$)	ND[a]		
4	**4** ($Man_3GlcNAc_2$)	8.5	−11.7	−12.9
5	**5** (Manα1→6Man$GlcNAc_2$)	3.3	−7.2	0.7
6	**6** (Manα1→3Man$GlcNAc_2$)	0.63	−7.0	−1.8
7	**7** ($GlcNAc_2$)	ND[a]		
8	**8** (Man_3)	ND[a]		
9	**9** (Complex-type)	0.24	−10.3	−14.9

[a] No detectable binding.

We first evaluated the affinity of Fbs1 for full-length high-mannose-type glycans **1** and **2** in comparison with $Man_3GlcNAc_2$ **4**. To obtain thermodynamic parameters for the binding of these synthetic oligosaccharides to Fbs1, the binding was analyzed by ITC.[12] Titration was conducted by adding 6 μL of synthetic oligosaccharide (300 μM) every 4 min into a buffer solution (10 mM sodium phosphate, pH 7.2, and 100 mM NaCl) containing Fbs1 (20 μM). Molar heat values for the binding of **2** plotted as a function of the [**2**]/[Fbs1] molar ratio are shown in the lower panel (Figure 3a). The solid line shows the binding isotherm obtained by the fitting of the data to a binding model involving a single set of identical sites. The thermodynamic parameters obtained by the fitting are summarized in Table 1. The binding constant (K_a) of **2** (entry 2) was determined as 4.1×10^5 M^{-1}, which was similar to that of **1** (entry 1). In contrast, the binding of $Man_8GlcNAc$ (**3**)[14] was below the detection limit of ITC under these conditions (Figure 3b). This result underscores the strict requirement of the chitobiose ($GlcNAc_2$) portion for glycan recognition and corroborates well the result of a crystallographic study.[11] On the other hand, pentasaccharide **4** (entry 4) showed substantially stronger affinity ($K_a = 8.5 \times 10^5$ M^{-1}). These results suggest that Fbs1 mainly recognizes the core region of *N*-glycans, and extended outer mannose residues of full-length chains cause steric repulsion resulting in reduced affinity.

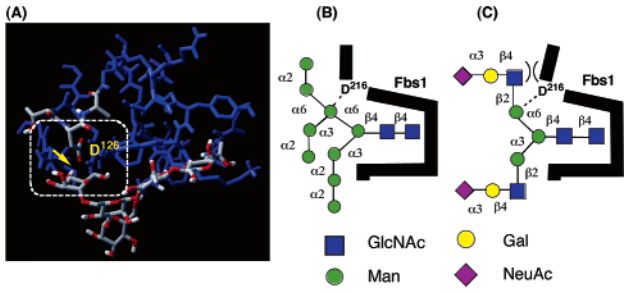


Figure 4. (A) Proposed model for the complex formed between **4** and sugar-binding domain of Fbs1. (B) Fbs mainly recoginze $Man_3GlcNAc_2$ of high-mannose-type oligosaccharide. (C) The GlcNAcβ1→2Manα1→6 arm of the complex-type oligosaccharide has reduced affinity due to steric hindrance.

Subsequently, the affinities of the partial structures of $Man_3GlcNAc_2$ were evaluated. It was found that the α1→6 linked tetrasaccharide **5** (entry 5) had an affinity marginally weaker than that of **4**, while the α1→3 linked congener **6** had a drastically reduced affinity (entry 6). The binding of smaller oligosaccharides such as **7** ($GlcNAc_2$) and **8** (Man_3) was not detectable under the same conditions. These results indicate that the α1→6 linked Man residue of the $Man_3GlcNAc_2$ core plays a significant role in the binding of Fbs1. Furthermore, a previously synthesized[18b] biantennary complex-type *N*-glycan (**9**) was subjected to a binding experiment. It had a markedly reduced affinity (entry 9), indicating that substitution at the 2-OH of the α1→6 linked Man residue caused steric congestion. It is concluded that both the chitobiose and the α1→6 linked Man residue are necessary for Fbs1 to recognize a sugar.

We calculated the structure of $Man_3GlcNAc_2$ in the complex with Fbs1 using the AMBER* force field implemented with the MacroModel program (version 8.1). A branched mannotriose was added to the crystal structure of chitobiose in the complex with Fbs1, and a conformational search was conducted with frozen Fbs1 and the chitobiose moiety using Monte Carlo multiple minimum searching. The most stable conformer for the Man_3 moiety obtained from the calculation is provided in Figure 4. 4-OH (Figure 4, yellow arrow) of the α1→6 linked Man residue is in contact with Asp216, for which a chemical shift perturbation was observed on addition of $Man_3GlcNAc_2$. This structure also agrees with our data obtained from the calorimetric analysis. In particular, the reduced affinity of the complex-type glycan can be explained by the steric hindrance caused by the presence of a bulky trisaccharide (NeuAcα2→3Galβ1→4GlcNAcβ) at the 2-position of the α1→6 linked Man residue.

Systematic analysis of the interaction between Fbs1 and synthetic oligosaccharides revealed that $Man_3GlcNAc_2$ had the strongest affinity. Whereas the major glycoform translocated into cytosol for ERAD is supposedly $Man_8GlcNAc_2$, our results indicate that *N*-glycoproteins having $Man_3GlcNAc_2$ are more efficiently recognized by the SCF^{Fbs1} complex. These facts imply that $Man_3GlcNAc_2$ or its derivatives may well be potent inhibitors of SCF^{Fbs1} complex. Further studies are in progress along this line.

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Supporting Information Available: Experimental procedures, NMR data, and ITC profiles. This material is available free of charge via the Internet at http://pubs.acs.org.

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