Elucidation of the mechanism enhancing the avidity of lectin with oligosaccharides on the solid phase surface

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The mechanism underlying molecular recognition of lectins was elucidated by a novel solid phase binding assay system based on surface plasmon resonance. When the apparent affinities of interactions between chitooligosaccharides and wheat germ agglutinin were compared between lectin-immobilized and oligosaccharide-immobilized assay systems, the affinity constants (Ka) calculated for the former system were in good agreement with the previously reported values measured in solution. On the other hand, in the latter system, the calculated Ka could be more than 10,000 times higher than the values in solution at lower lectin concentrations. To elucidate the reason for this, we systematically investigated the effects of the oligosaccharide immobilized density and the lectin valence on the apparent affinity in the oligosaccharide-immobilized assay system. Both the apparent association (kass) and dissociation rate constants (kdiss) showed a tendency to decrease as the oligosaccharide density increased. This effect was most remarkable for the interaction possessing an extremely fast intrinsic kass. Oligomerization of lectin enhanced the avidity due to a significant reduction in kdiss. These phenomena could be explained by considering the nonhomogeneous conditions under which binding occurred. The reaction in a nonhomogeneous state is limited by the mass transport effect, and the effect of rebinding becomes so large that it cannot be disregarded. These findings are the first to demonstrate the importance of the mass transport effect in modulating the affinity of lectin for oligosaccharides on a solid phase surface.

Key words: avidity/clustering effect/lectin/mass transport/surface plasmon resonance

Introduction

The importance of oligosaccharide-lectin interactions in typical multicellular organism events such as development, differentiation, and morphogenesis is becoming widely recognized (Varki, 1993). Lectins have been found in a variety of species and comprise a structurally very diverse class of proteins characterized by their ability to bind carbohydrates with considerable specificity. Although the equilibrium constants between a lectin and mono- or disaccharides are generally in the range between $10^{-3}$ and $10^{-4}$ M, they are often enhanced by more than $10^3$ when the affinity is measured using a neoglycoprotein or a cell. This phenomenon is interesting because it suggests the possibility that the apparent affinity between lectin and oligosaccharide can be modulated by the densities of the lectin and the oligosaccharide. Although this has been explained by the clustering effect, multivalent binding due to the high density of the oligosaccharide and lectin subunit multivalence (Lee, 1992; Rini, 1995a,b), the precise mechanism of how lectins and oligosaccharides enhance their avidity remains mostly unknown.

Considering that oligosaccharides usually exist as glycoconjugates and most interactions with sugar-recognizing molecules occur on a solid phase surface, it will be very important to measure interactions on solid phase surfaces to clarify the enhanced avidity of lectins for cell surfaces. However, most of the existing procedures, including microdialysis, microcalorimetry, and frontal chromatography (Kobata and Endo, 1992) and frontal chromatography (Ohyama, 1985) are typical assay systems for measuring interactions on solid phase surfaces, these methods provide no information about the on- and off-rate kinetics.

We recently developed a novel method for measuring interactions between oligosaccharides and lectins using a biosensor based on surface plasmon resonance (SPR) (Shinohara et al., 1995, 1996, 1997). In this system, biotinylated oligosaccharides are immobilized on a sensor surface, and the interaction with lectin can be monitored in a micro-flow system. This method is an entirely new assay system for affinity measurements because the interaction can be kinetically monitored on the solid phase surface.

The interaction between a lectin and an oligosaccharide is quite complicated because there are many factors to be considered, e.g., the oligosaccharide density, branching in the oligosaccharide structure, lectin multivalence, and so on. Systematic evaluation of the effects of these factors on the interaction may lead to a better understanding of how oligosaccharides and lectins enhance their avidity on the cell surface. This information will also be useful for remodeling of glycoproteins, development of an anti-adhesion drug (Hodgson, 1995) and a carbohydrate-based drug delivery system (Fujita et al., 1992).

In the present study, we found that the observed avidity was quite different when the immobilized ligand was reversed in the solid phase binding assay, even though the same interaction was compared. Starting from this viewpoint, we investigated the effects of the oligosaccharide-immobilized density and the lectin's multivalence. The results obtained here provide a novel explanation for the clustering effect.
Results

Effect of immobilization of oligosaccharide or lectin on the apparent affinity

Affinity measurements between WGA and chitooligosaccharides were carried out as a model study. Since our method is monitoring the interaction on a solid phase surface, either the lectin or oligosaccharide is immobilized onto the surface of the biosensor. For the immobilization of oligosaccharides, GN$_2$, GN$_3$, and GN$_4$ were biotinylated using BPH, and the purified BPH-labeled oligosaccharides were introduced onto the streptavidin pre-immobilized surface. Note that tagging the oligosaccharide with BPH gives an adduct as its cyclic $\beta$-glycoside. Therefore, each BPH-labeled oligosaccharide involves the minimum recognition moieties. WGA was introduced onto each surface, and the interaction was analyzed. The immobilization of WGA was performed by NHS activation of the sensor surface, and free GN$_2$, GN$_3$, and GN$_4$ were each introduced onto the sensor surface at the same concentration. As shown in Figure 1, each interaction showed a relatively slow dissociation in the oligosaccharide-immobilized assay system, while the dissociation was extremely rapid in the lectin-immobilized assay system. By measuring the interaction at several concentrations of analytes in both assay systems, the $K_a$ was calculated by Scatchard plot analysis of the $[AB]_\text{eq}$ values at each concentration (Figure 2). As summarized in Table I, the $K_a$ values obtained with the lectin-immobilized assay system were in the range from $10^3$ to $10^4$ M$^{-1}$, which were in good agreement with the previously reported values, which are also shown in Table I. On the other hand, the Scatchard plot obtained with the oligosaccharide-immobilized assay system showed marked curvature (Figure 2A). Unexpectedly, the calculated $K_a$ values at low lectin concentrations (< 56.5 nM) were in the range from 1.3 to $3.0 \times 10^8$ M$^{-1}$, which were more than $10^8$ times higher than the values observed in the lectin-immobilized assay system. These results demonstrate that the apparent affinity can be significantly affected by which interactant is immobilized, even though the same interaction is being monitored.

Effect of oligosaccharide-immobilized density

To exclude the effect of oligosaccharide branching and simplify the evaluation, LacNAc, 3'-sLacNAc and 6'-sLacNAc were used as the model oligosaccharides. Following BPH labeling of each oligosaccharide, they were immobilized onto the sensor surface at four different densities. Lectins recognizing each oligosaccharide structure, i.e., RCA$_{120}$, MAM, and SSA, were then injected onto the surfaces. These three lectins are divalent. MAM is a dimer of disulfide-containing subunits (Kawaguchi et al., 1974). RCA$_{120}$ and SSA are tetrameric glycoproteins composed of two sets of a heterodimer, one of which possesses a carbohydrate-binding site (Funatsu et al., 1977, Shibuya et al., 1987, 1989). Our recent study revealed the striking structural similarity of SSA to ricin/abrin-type ribosome-inactivating proteins as well as to RCA (Kaku et al., 1996).

The apparent rate constants were calculated from plots of $k_s$ versus the lectin concentration (see Eq. 2c). The slope and the intercept of the fitted line of the plots represent the apparent $k_{\text{ass}}$ and $k_{\text{diss}}$, respectively. As shown in Figure 3, both the apparent $k_{\text{ass}}$ and $k_{\text{diss}}$ showed a tendency to decrease as the oligosaccharide density increased. However, the degree of the density effect was obviously higher for the LacNAc-RCA$_{120}$ interaction than for the 3'-sLacNAc-MAM and 6'-sLacNAc-SSA interactions. Kinetic analysis of each of three interactions revealed that the calculated $k_{\text{ass}}$ obtained for LacNAc-RCA$_{120}$ was in the order of $10^3$ M$^{-1}$ s$^{-1}$, while those obtained for the other interactions were some 10 times lower. This suggests a close relationship between $k_{\text{ass}}$ and the sensitivity of the interaction to the immobilized density.

Effect of oligomerization of lectin

To investigate the effect of the multivalence of lectin on the apparent affinity, SSA and the monomeric protomer of SSA (MSSA) were used as the model oligosaccharides. Following BPH labeling of each oligosaccharide, they were immobilized onto the sensor surface at four different densities. Lectins recognizing each oligosaccharide structure, i.e., RCA$_{120}$, MAM, and SSA, were then injected onto the surfaces. These three lectins are divalent. MAM is a dimer of disulfide-containing subunits (Kawaguchi et al., 1974). RCA$_{120}$ and SSA are tetrameric glycoproteins composed of two sets of a heterodimer, one of which possesses a carbohydrate-binding site (Funatsu et al., 1977, Shibuya et al., 1987, 1989). Our recent study revealed the striking structural similarity of SSA to ricin/abrin-type ribosome-inactivating proteins as well as to RCA (Kaku et al., 1996).

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Fig. 1. Sensorgrams showing the interaction between WGA and chitooligosaccharides. For the immobilization of oligosaccharides, the purified BPH-labeled oligosaccharides were introduced onto a streptavidin pre-immobilized surface. WGA was introduced onto each surface at a concentration of 14.5 $\mu$M. For the immobilization of lectin, WGA was introduced onto the NHS-activated surface, and GN$_2$, GN$_3$, and GN$_4$ were each introduced onto the surface at a concentration of 62.5 $\mu$M.

Fig. 2. Oligosaccharide-immobilized surface

Fig. 3. Lectin-immobilized surface

Table I

<table>
<thead>
<tr>
<th>Oligosaccharide-immobilized surface</th>
<th>Lecture-immobilized surface</th>
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</thead>
<tbody>
<tr>
<td>GN$_2$</td>
<td>SSA</td>
</tr>
<tr>
<td>GN$_3$</td>
<td>SSA</td>
</tr>
<tr>
<td>GN$_4$</td>
<td>SSA</td>
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tigate the stoichiometry (Figure 5b). The slope and the intercept of the fitted line of the plots represent $K_a$ and $K_a \times [AB]_{\text{max}}$ respectively (see Eq. 3). The calculated parameters are summarized in Table II. By forming the oligomeric state of the lectin, the apparent $k_{\text{on}}$ was slightly decreased, while the apparent $k_{\text{off}}$ was remarkably decreased, indicating that the enhanced avidity by oligomerization is mainly due to the decrease in apparent $k_{\text{off}}$. [AB]_{\text{max}} was about 4.9 times higher for SSA than for MSSA, which is in good agreement with the difference in the molecular mass of each lectin. The apparent Mr is 140 kDa for SSA and 34 kDa for MSSA (Kaku and Shibuya, 1992), indicating that both lectins showed equimolar binding to the oligosaccharide immobilized on the sensor surface. In consequence, it was strongly suggested that one of two binding sites of SSA was actually used in this binding assay. Interestingly, the dissociation phase obtained for MSSA could not be readily fitted to pseudo first-order kinetics. Although it has been reported by several authors that the dissociation phase could hardly be approximated by pseudo first-order kinetics, it is often postulated that this is due to the multivalent nature of the injected molecule (Panayotou et al., 1993; Nieba et al., 1996). However, this possibility is obviously ruled out in this case because MSSA possesses only one carbohydrate recognition site.

These results demonstrate that the interaction on the solid phase surface can be quite complicated even when the interaction can be described by a simple one-to-one interaction model. One possible explanation for the deviation from pseudo first-order kinetics even for monomeric MSSA might be rebinding of the lectin. In order to test this possibility, the effect of coinjection of an excess of 6'-sLacNAc on the dissociation rate was measured. As shown in Figure 6, increasing amounts of competing 6'-sLacNAc enhanced the dissociation rate for both lectins, although the degree of the effect was most remarkable for SSA. The dissociation rate constants obtained for MSSA and SSA without 6'-sLacNAc coinjection were 0.060 $s^{-1}$ and 0.002 $s^{-1}$, respectively. In the presence of 1.48 mM 6'-sLacNAc, the dissociation rate constants were 0.087 $s^{-1}$ for MSSA and 0.044 $s^{-1}$ for SSA, indicating that deviations from pseudo first-order kinetics are most likely due to the effect of lectin rebinding. This result also suggests that the increased number of sugar binding sites may lead to an increased chance of rebinding.

The effect of the lectin valence on the apparent affinities was

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**Table I. Comparison of WGA-chitoooligosaccharides association constants obtained using different methods**

<table>
<thead>
<tr>
<th>Type</th>
<th>Our results</th>
<th>Bains et al., 1992</th>
<th>Lotan and Sharon, 1973</th>
<th>Privat et al., 1974</th>
<th>Nagata and Burger, 1974</th>
</tr>
</thead>
<tbody>
<tr>
<td>k$A$ (M$^{-1}$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GN$_4$</td>
<td>6.3</td>
<td>5</td>
<td>13</td>
<td>4.5</td>
<td>20</td>
</tr>
<tr>
<td>GN$_3$</td>
<td>17</td>
<td>12</td>
<td>22</td>
<td>20</td>
<td>83</td>
</tr>
<tr>
<td>GN$_2$</td>
<td>17.5</td>
<td>12.1</td>
<td>36</td>
<td>23</td>
<td>—</td>
</tr>
</tbody>
</table>

*Methods used: microcalorimetry (Bains et al., 1992); fluorometry (Lotan and Sharon, 1973; Privat et al., 1974); equilibrium dialysis (Nagata and Burger, 1974).
Fig. 4. Sensorgrams showing the effect of multivalence of lectin on the interaction. MSSA and SSA were injected onto the 6'-sLacNAc-immobilized surface at concentrations of 56.8, 28.4, 14.2, and 7.1 μg/ml.

Fig. 5. Kinetic and Scatchard plot analysis of the interaction of MSSA and SSA with surface-bound 6'-sLacNAc. (a) Kinetic analysis of the $K_a$ as a function of various lectin concentrations. The slope and intercept correspond to $k_{on}$ and $k_{off}$, respectively. (b) Scatchard plot analysis of SSA/MSSA binding to surface-bound 6'-sLacNAc. The slope and intercept correspond to $-K_a$ and $K_a \times [AB]_{max}$, respectively.

Fig. 6. Effect of coinjection of excess 6'-sLacNAc in the dissociation phase on the dissociation rate for the interaction of MSSA and SSA with surface-bound 6'-sLacNAc. Each lectin was introduced onto the surface at a concentration of 10 μg/ml, and 0, 14.8, 148, or 1480 μM of 6'-sLacNAc was infused in the dissociation phase. The arrow indicates the end of lectin injection and the infusion of excess 6'-sLacNAc.

Table II. Parameters obtained for the interaction analysis between surface-bound 6'-sLacNAc and SSA/MSSA

<table>
<thead>
<tr>
<th></th>
<th>$k_{on}$ ($M^{-1}s^{-1}$)</th>
<th>$k_{off}$ (s$^{-1}$)</th>
<th>[AB]$_{max}$ (RU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSSA</td>
<td>2.90 x 10^4</td>
<td>0.0529</td>
<td>445</td>
</tr>
<tr>
<td>SSA</td>
<td>1.61 x 10^4</td>
<td>0.0024</td>
<td>2201</td>
</tr>
</tbody>
</table>

*Derived from $k_s$ vs C plot analysis.
*Derived from [AB]eq/C vs [AB]eq plot analysis.

MSSA. That oligomerization of lectin enhances the avidity due to a significant reduction in $k_{off}$ was also true when the interactions were measured using this N-linked complex type oligosaccharide. Coinjection of an excess of 6'-sLacNAc also showed a similar effect on the dissociation rate, as indicated in Figure 6 (data not shown).

Discussion

The clustering effect is a most important feature for understanding the mechanism underlying the molecular recognition of lectin. It has been explained on the basis of multivalent binding between lectin and oligosaccharide. However, the precise mechanism of how oligosaccharide and lectin enhance their avidity remains mostly unknown. In the present study, we found large differences in the apparent affinity between whether a lectin or an oligosaccharide was immobilized in the solid phase binding assay system. Since the calculated $K_a$ obtained for BPH-labeled GN$_2$ with immobilized WGA was similar to the values obtained for free GN$_2$ with immobilized WGA (data not shown), and since WGA did not show any binding to a BPH-immobilized surface, the extremely high $K_a$ values for chitooligosaccharides and WGA in the oligosaccharide-immobilized system are not due to chemical modification of MSSA.

Also evaluated using an N-linked complex type oligosaccharide: Galβ1-4GlcNAcβ1-2Manα1-6(Neu5Aca2-6Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAc1β-4GlcNAc. The obtained sensorgrams were quite different between SSA and
the oligosaccharides by BPH. We also have reported such a phenomenon for mouse macrophage lectin (mML) (Yamamoto et al., 1994). An oligosaccharide showing moderate binding during mML-immobilized affinity chromatography gave a relatively high $K_d$ ($6.2 \times 10^{-7}$ M) in a glycopeptide-immobilized SPR biosensor analysis. These findings prompted us to attempt accurate elucidation of the mechanism enhancing the avidity of lectin for oligosaccharides on a solid phase surface.

By investigating the effect of the density of the immobilized oligosaccharide, we found that both the association and dissociation rate constants tended to decrease as the oligosaccharide density increased. This tendency is the same as observed for the effect of oligosaccharide branching on the apparent affinity (Shinohara et al., 1995). The effect of oligosaccharide density on the apparent affinity was most remarkable for interactions with a fast $k_{ass}$.

For such a solid phase interaction analysis, it is important to consider an unstrained layer, because the flow rate is not constant in a laminar flow. A three-state model with a laminar flow across a surface is often used to describe this situation (Yuasa et al., 1986; O'Shanessy, 1994). In this model, an unstrained layer exists at the surface, separating the bulk flow from the surface. Such a model, depicted in Figure 7a, invokes diffusion limitations of the soluble analyte from the bulk flow into and out of the static unstrained layer. This model is kinetically far more complex than the simple Langmuir two-state model, because there are several new factors to be considered. When two molecules interact in solution, the collision frequency and the orientation of interaction sites may determine the reaction rate (Schurr and Schmitz, 1976; Northrup and Erickson, 1992).

![Diagram](image)

Fig. 7. A representation of a three-state model with laminar flow across a surface. This model invokes diffusion limitations of a soluble analyte from the bulk compartment, into and out of the static unstrained compartment (a). B is immobilized on the sensor surface, and A is injected onto the surface. $[A]_0$ and $[A]_s$ are the concentrations in the bulk compartment and in the unstrained compartment, respectively. $[B]$ is the concentration of immobilized ligand. $K_m$ and $k_m$ are mass transport rate constants. $k_{ass,int}$ and $k_{dis,int}$ are the intrinsic rate constants for the reaction between A and B, and $k_{ass,apparent}$ and $k_{dis,apparent}$ are the overall rate constants, which include both intrinsic interaction kinetics and mass transport rate constants.

However, the reaction in a nonhomogeneous state is limited by the mass transport effect. Since the larger molecule usually possesses a lower diffusion coefficient, the lectin concentration in the unstrained layer is not the same as the injected concentration (Figure 7b). It must be corrected using a mass transport coefficient. Therefore, the kinetic parameters obtained in the BIACore using a simple Langmuirian two-state model could differ from the intrinsic values. From this viewpoint, it has been reported that the obtained kinetic parameters represent the overall rate constants ($k_{ass,apparent}$ and $k_{dis,apparent}$), which include both the intrinsic interaction kinetics and mass transport rate constants (Glaser, 1993; Karlsson et al., 1994; Schuck and Minton, 1996). The apparent rate constants were proposed as

$$k_{ass,apparent} = k_{ass,int}/(1 + k_{ass,int}[B]/K_M) \quad \text{(4a)}$$

$$k_{dis,apparent} = k_{dis,int}/(1 + k_{dis,int}[B]/K_M) \quad \text{(4b)}$$

where $[B]$ is the immobilized oligosaccharide concentration, $k_{ass,int}$ and $k_{dis,int}$ are the intrinsic association and dissociation rate constants, and $K_M$ is the mass transport coefficient.

From Equations 4a and 4b, it can be concluded that $k_{ass,int}[B]/K_M$ is the limiting coefficient which can describe how the apparent kinetic constants differ from the intrinsic kinetic constants. When $k_{ass,int}[B]/K_M >> 0$, the apparent and intrinsic kinetic values will not agree. This situation occurs when the immobilized ligand density is high (high $[B]$), when a large molecule is used as an analyte and/or the flow rate is low (low $K_M$) and, most importantly, when the interaction possesses a fast $k_{ass,int}$. The same immobilized ligand density and the same flow rate were used throughout the analyses of the LacNAc-RCA120, 3'-sLacNAc-MAM and 6'-sLacNAc-SSA interactions. The molecular weights of three lectins are also quite similar to one another. When the association rate kinetics were calculated for LacNAc-RCA120, 3'-sLacNAc-MAM and 6'-sLacNAc-SSA, the calculated values obtained for LacNAc-RCA120 were in the order of $10^5$ M$^{-1}$s$^{-1}$, while those obtained for the other interactions were some 10 times lower. Therefore, it was strongly suggested that the extremely fast $k_{ass,int}$ nature of the LacNAc-RCA120 interaction was the reason that the degree of the density effect was obviously higher for the LacNAc-RCA120 interaction than for the other interactions. These results suggest that the apparent affinity of lectin possessing a fast $k_{ass,int}$ could be modulated by controlling the oligosaccharide expressed density. Since the carbohydrate–protein interaction plays an important role in the upstream of the cell adhesion cascade, these interactions are believed to be quite fast (Lawrence and Springer, 1991). An extremely fast $k_{ass}(>10^7$ M$^{-1}$s$^{-1}$) has actually been reported for P-selectin (Alon et al., 1995). The dependence of $K_a$ on the amount of immobilized oligosaccharide may explain the marked curvature in the Scatchard plot observed for WGA with surface-bound chitooligosaccharides. Curvilinearity in a Scatchard plot is often interpreted as being indicative of the existence of negative cooperativity between the binding sites, or the presence of several binding sites with intrinsically different affinities (Kabat, 1976). However, since such curvature in the Scatchard plot was not observed when the interaction between WGA2 (WGA islectin 2) and GN$_2$ was analyzed by equilibrium dialysis (Nagahora et al., 1995), it was most likely due to the effect of immobilization of the oligosaccharide. As WGA bound to surface-immobilized chitooligosaccharides, the concentration of the free oligosaccharide decreased. This may be the cause of the reduction in apparent $K_a$. 

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Regarding the contribution of the lectin valence to the apparent affinity, we found that the apparent affinity was enhanced by oligomerization of lectin due to remarkable reduction in the dissociation rate. A similar observation was reported by MacKenzie et al. (1996). They studied the effect of the valence of the single-chain antibody variable domain (scFv) specific for Salmonella serogroup B O-poly saccharide, and observed approximately a 20-fold decrease in $k_{\text{diss}}$ due to dimerization of scFv. They concluded that this was due to bivalent binding because the dissociation phase for the dimer of scFv shows better fitting with the biexponential decay function describing the dissociation of two components from the immobilized antigen rather than to the single exponential expression. In the present study, however, there was no evidence of bivalent binding for SSA because the calculated $[\text{AB}]_{\text{max}}$ was about 4.9 times higher for SSA than for MSSA, which corresponds well to the difference in the molecular mass of each lectin. Furthermore, the interaction of the monovalent form of SSA with the surface-bound 6'-sLacNAc also showed a deviation from pseudo first-order kinetics. Although MacKenzie et al. reported that the dissociation phase of the monomeric form of scFv showed good curve fitting to the monoexponential decay model and, therefore, the binding is monovalent, they used only a limited range (1.5 sec) for the dissociation phase. The results of the present series of studies indicate that a bivalent form of lectin can show enhanced avidity without simultaneous bivalent binding, and that even monovalent lectin can show quite a complicated interaction if the interaction occurs on a solid phase surface. Coinjection of a hapten sugar also had a dramatic effect on the $k_{\text{diss}}$, especially for SSA. This suggests that the delayed apparent $k_{\text{diss}}$ by oligomerization was due to enhanced rebinding. Rebinding of lectin could also be explained by the mass transport effect. Even after the lectin solution was replaced by the buffer, there might be some lag time for complete removal of the lectin from the sensor surface. When removing lectin from the surface, the free immobilized oligosaccharide concentration increases gradually, which may enhance the possibility of lectin rebinding. Coinjection of an excess of hapten sugar also had a dramatic effect on the delayed $k_{\text{diss}}$ caused by oligosaccharide branching (Shinohara et al., 1997).

If we assume that the observed dissociation phase is the sum of the intrinsic dissociation and rebinding of lectin, the dissociation phase can be described as

$$[\text{AB}]_t = [\text{AB}]_0 \exp(-k_{\text{diss, int}} t) + \text{(rebinding)},$$  \[5\]

where $[\text{AB}]_0$ is the lectin–oligosaccharide complex concentration when dissociation starts. Although the association is usually expressed as Equations 2a–c, $[A]_0$ is not constant, but a function of time in the dissociation phase. $[A]_0$ can be described as

$$[A]_t = [A]_{I1} \exp (-k_{\text{m}} t),$$  \[6\]

where $[A]_{I1}$ is the initial lectin concentration, which can be approximated by the injected lectin concentration, and $k_{\text{m}}$ is the mass transport rate constant from the sensor surface to the bulk flow.

Using these equations, the dissociation phase with rebinding can be expressed as

$$[\text{AB}]_t = [\text{AB}]_0 \exp(-k_{\text{diss, int}} t) + k_{\text{ass, apparent}}([A]_{I1} \exp(-k_{\text{m}} t))[\text{AB}]_{\text{max}}\exp(-k_{\text{ass, apparent}}([A]_{I1} \exp (-k_{\text{m}} t)) + k_{\text{diss, apparent}}([A]_{I1} \exp (-k_{\text{m}} t) + k_{\text{diss, apparent}}).$$  \[7\]

Although this equation is too complicated to be directly fitted, we confirmed that the experimental data showed a good overall fit even when $k_{\text{diss, in}}$ was set at 0.1 s$^{-1}$, which is close to the value observed in the presence of 1.48 mM 6'-sLacNAc during the dissociation phase (Figure 8). The calculated $k_{\text{m}}$ value under this condition was about 2.5 times higher for MSSA than for SSA. This observation may indicate that the enhanced avidity caused by oligomerization of lectin is due to the reduced mass transport rate from the sensor surface to the bulk flow.

Multivalent binding would be important both to produce high-affinity binding and to define specificities for the branching pattern of a single N-linked oligosaccharide, as previously demonstrated with the endocytic receptors for glycoproteins (Lee et al., 1989; Rice et al., 1990; Lodish, 1991). However, to date, almost no attention has been paid to the importance of the nonhomogeneous conditions under which binding occurs. The apparent affinity between lectin and oligosaccharide was most remarkably enhanced when the oligosaccharide was immobilized at a high density and soluble multivalent lectin was immo-

![Fig. 8. Nonlinear least-square fitting of the dissociation phases obtained for MSSA and SSA interaction with surface-bound 6'-sLacNAc using Equation 7. $[\text{AB}]_0$ was used as the observed value, the injected lectin concentration was used as the value of $[A]_{I1}$, $[\text{AB}]_{\text{max}}$ was used as calculated in Table II, and $k_{\text{diss, in}}$ was set at 0.1 s$^{-1}$](image-url)
ject ed as an analyte. As mentioned above, the apparent affinity of the interaction possessing a faster intrinsic $k_{\text{on}}$ is essentially affected by the mass transport effect under nonhomogeneous conditions. Immobilizing a molecule possessing a higher diffusion coefficient (oligosaccharide in this case) at high densities and introducing a multivalent molecule possessing a lower diffusion coefficient would be quite effective in making the reaction conditions nonhomogeneous. By positively utilizing the mass transport effect, the apparent affinity between lectin and oligosaccharide can be readily modulated using the same molecules at different densities. An important feature of the interaction of lectin with oligosaccharide on a solid phase predicted in the present study is that affinity modulation is regulated mainly by altering the $k_{\text{on}}$. This may be closely related to the regulation of the half-life of the lectin concentration. Mass transport should be considered not only on the sensor surface but in several biological states in which the ligand is immobilized. These results strongly suggest the importance of affinity measurements between lectins and oligosaccharides on a solid phase surface.

**Materials and methods**

**Instrumentation and materials**

BIACore and BIAcore 2000 (BIACORE AB, Uppsala, Sweden), which are based on SPR, were used to measure the biomolecular interactions. *Sambucus sieboldiana* lectin (SSA), *Maackia amurensis* lectin (MAM), *Ricinus communis* agglutinin-120 (RCA120), wheat germ agglutinin (WGA), di-N-acetylchitobiose (GN$_2$), tri-N-acetylchitotriose (GN$_3$), and tetra-N-acetylchitotetraose (GN$_4$) were purchased from Homon Seiyu (Tokyo), N-Acetylatedasosamine (LacNAc) was obtained from Toronto Research Chemicals (Downview, Canada) while 3'- and 6'-sialyl LacNAc (3'- and 6'-LacNAc) and GaIβ1-4GlcNAcβ1-2Manα1-6Galα1-4GlcNAcβ1-2Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc were purchased from Oxford GlycoSystems (Abingdon, UK). The BIACore sensor chip SA-5, surfactant P20 and chemical activation reagents were obtained from BIACORE AB: 100 mM N-hydroxysuccinimide (NHS) in water, 400 mM N-ethyl-N'-(-dimethylamino propyl) carbodiimide hydrochloride in water, and 1 M ethanolamine hydrochloride adjusted to pH 8.5 using NaOH. The HBS buffer comprised 10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM CaCl$_2$ and 0.05% BIAcore surfactant P20 in distilled water. Lectins were purified by Superdex 200 (Pharmacia Biotech, Uppsala, Sweden) using HBS buffer as a solvent. The lectin concentrations were determined using a micro BCA protein assay kit (Pierce, Rockford, 4-(Biotinamido)phenylacetylhydrazide (BPH) was synthesized as previously reported (Shinohara et al., 1996).

**Preparation of stable SSA subunit**

The stable SSA subunit (MSSA) was prepared by selective reduction and alkylation of SSA according to a previously reported method (Kaku and Shibuya, 1992). Briefly, SSA (5 mg/ml) in 0.4 M N-ethylmorpholine/acetate buffer (pH 8.3 containing 1% DTT and 0.25 M lactose) was incubated at 20°C for 15 min under N$_2$. The monomeric S-B-(4-pyridylethyl) cysteine-SSA subunit (MSSA) was purified by affinity chromatography on immobilized fetuin-Sepharose 4B and gel filtration on Superdex 200. Formation of MSSA was confirmed by gel filtration on Superdex 200 and also by SDS-PAGE in the absence of β-mercaptoethanol.

**BPH tagging of the oligosaccharide**

BPH tagging of the oligosaccharide was performed under the previously reported conditions (Shinohara et al., 1996). Briefly, the oligosaccharide in water was incubated with a 4-fold molar excess of BPH in 30% acetonitrile at 90°C for 1 h. After the reaction, 50 mM formate buffer (pH 3.5) was added and stored at 4°C for 12 h to promote the tautomerization from the acyclic Schiff-base type hydrazone to stable B-glycosides. The reaction mixture was injected directly into reversed-phase HPLC to purify the BPH-adducts.

**Affinity analysis using SPR**

Affinity measurements between lectins and oligosaccharides were carried out using both lectin-immobilized and oligosaccharide-immobilized assay sys-
References


