Introduction

Carbohydrate recognition by proteins is acknowledged to play a role in a considerable number of physiological and pathological processes such as the mechanisms of inflammation and host defense (Drickamer and Taylor, 1993). The endogenous lectins may distinguish not only between different oligosaccharide structures, but also between different presentations of oligosaccharides in the context of specific carrier proteins (Crocker and Feizi, 1996).

The serum proteins conglutinin and mannann-binding protein are soluble carbohydrate-binding receptors of the innate immune system, and are thought to promote clearance of bacteria and yeasts through their interactions with carbohydrates at the surface of the infectious agents (Holmskov et al., 1994). The two carbohydrate-binding receptors contain homologous, “C-type,” carbohydrate-recognition domains (CRDs) and show qualitatively similar specificity toward high mannose N-glycans, but only conglutinin binds to iC3b, a glycoprotein which is a proteolytically pruned form of the major serum complement glycoprotein C3, containing saccharides of this type (Childs et al., 1989; Solís et al., 1994). This interaction is mediated by recognition of the Man$_9$ or Man$_8$, N-glycan at Asn-917, as presented on iC3b but not on the parent glycprotein C3 nor on the further proteolysed glycoprotein fragment C3c on which the Man$_{5-6}$ N-glycan is preserved (Solís et al., 1994). A similar phenomenon occurs with the glycoprotein ribonuclease B (RNaseB), which contains at a single glycosylation site, Asn-34, one or other of the high-mannose N-glycans, Man$_{5-9}$, their relative molar proportions being 57, 31, 4, 7, and 1%, respectively (Fu et al., 1994). On the native glycoprotein, the oligosaccharides are not bound by conglutinin and mannan-binding protein, whereas binding occurs when the protein is reduced and denatured (Solís et al., 1994). RNaseB is a small glycoprotein (124 amino acid residues) and is an ideal model to probe in depth the structural basis of presentation of oligosaccharide for recognition by conglutinin.

The three-dimensional structure of the non-glycosylated RNase (RNaseA). Thus there are no perceptible differences between the RNase protein forms that could account for differential availability of the N-glycan for conglutinin-binding. After reduction and denaturation, the NMR spectrum became typical of a non-structured polypeptide, although the conformational preferences of the N-glycosidic linkage were unchanged, and most importantly, the Man$_9$ oligosaccharide retained the average conformational behavior of the free oligosaccharide irrespective of the carrier protein fold. This conformational freedom is clearly not translated into full availability of the oligosaccharide for the carbohydrate-recognition protein. We propose, therefore, that the differing bioactivity of the N-glycan is a reflection of the existence of different geometries of presentation of the carbohydrate determinant in relation to the protein surface within the glycan:carrier protein ensemble.

Key words: carbohydrate recognition/conformational analysis/conglutinin/oligosaccharide presentation/ribonuclease B
Here we have compared the binding of conglutinin to RNaseB-Man₈, with the Man₅₋₆ form, when the protein is in the native and in the reduced and denatured state. The N-glycan on the Man₈ form is one of the preferred ligands for conglutinin. We show that conglutinin binds strongly to the Man₈ oligosaccharide on reduced and denatured RNaseB but not on the native glycoprotein. Pursuing the molecular basis of this different oligosaccharide bioactivity, we have isolated in relatively large scale (milligram amounts) the RNase-Man₈ glycoform, for which conformational information is not available so far, and have examined, by a combination of NMR and molecular dynamics calculations, the conformation of the Man₈ oligosaccharide on the native and on the reduced and denatured RNaseB.

Results and discussion

Conglutinin binds to the Man₈ oligosaccharide on reduced and denatured RNaseB

Previous binding studies (Solís et al., 1994) with high mannose-type neoglycolipids derived from individual Man₅₋₉ N-glycans showed binding of conglutinin predominantly to the Man₇-, Man₉-, and Man₁₀ species, which are minor components of total RNaseB oligosaccharides, rather than to the major components, Man₅₋₆. To dissect the influence of carrier protein on recognition of RNaseB oligosaccharides by conglutinin, we performed microwell and nitrocellulose overlay assays (Figure 1), using equimolar amounts of the isolated RNase-Man₈ and RNase-Man₅₋₆ glycoforms before and after reduction and denaturation. With the non-reduced glycoproteins, weak binding was detected only to RNaseB-Man₈ after electrophoresis in SDS, and electrotransfer (Figure 1a, panel B). After reduction and denaturation of the proteins, however, strong binding was observed to Man₈ immobilized either on nitrocellulose (Figure 1a, panel C) or on microwells (Figure 1b) and weak binding to Man₅₋₆. This is in accord with the reactivities observed with the oligosaccharides in the absence of the carrier protein (Solís et al., 1994). Thus, the protein moiety exerts a qualitatively similar influence on the presentation of the Man₈ and Man₅₋₆ oligosaccharides for recognition by conglutinin.

Inhibition experiments of the binding of ¹²⁵I-conglutinin to reduced and denatured RNase-Man₈ using liquid-phase native RNaseB and reduced heat-denatured RNaseB as inhibitors (Table I) were in accord with the results of the binding patterns observed to the glycoproteins immobilized on plastic microwells. In the presence of dithiothreitol, RNaseB retained the three-dimensional structure of the native protein, as evidenced by NMR spectroscopy analysis (see below), and accordingly, the inhibitory activity of the glycoprotein toward conglutinin binding at the levels tested (10 mg/ml) was only 9% as compared with ~70% inhibition exerted by reduced heat-denatured RNaseB. Due to the multivalent binding ability of conglutinin, any kind of self-aggregation of the reduced heat-denatured glycoprotein could potentially result in multivalent recognition with a concomitant increase in the binding avidity. However, no evidence for such an aggregation has been observed. On gel filtration chromatography, both native and reduced heat-denatured RNaseB eluted as a major peak (about 97% of the protein) with an apparent molecular mass of 15 kDa corresponding to the monomeric glycoprotein (results not shown).

The binding of conglutinin to reduced and denatured RNase-Man₈ was inhibited by mannose and N-acetylglucosamine (Table I). Comparable levels of inhibition were observed when these monosaccharides were used as inhibitors of conglutinin binding to immobilized invertase, a highly glycosylated protein which is generally assumed to present only carbohydrate ligands to the lectin. Furthermore, no significant differences in conglutinin binding to either reduced heat-denatured RNase-Man₈ or invertase were observed under various conditions typically promoting (high ammonium

Table I. Binding of ¹²⁵I-conglutinin to reduced heat-denatured RNase-Man₈ and to invertase coated onto plastic microwells

<table>
<thead>
<tr>
<th>Additive</th>
<th>Reduced heat-denatured RNase-Man₈</th>
<th>Invertase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Binding</td>
<td>Inhibition of binding</td>
</tr>
<tr>
<td>Ethylene glycol (10%)</td>
<td>90.1</td>
<td>78.5</td>
</tr>
<tr>
<td>Tween 20 (0.5%)</td>
<td>104.6</td>
<td>85.3</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ (10 mM)⁺</td>
<td>103.8</td>
<td>100.4</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ (1 M)⁺</td>
<td>86.8</td>
<td>95.6</td>
</tr>
<tr>
<td>Man (50 mM)</td>
<td>37.3</td>
<td>62.7⁺</td>
</tr>
<tr>
<td>GlcNAc (15 mM)</td>
<td>9.6</td>
<td>90.4⁺</td>
</tr>
<tr>
<td>Dithiothreitol (12 mM)</td>
<td>58.3</td>
<td>45.4</td>
</tr>
<tr>
<td>+ RNaseB (10 mg/ml)</td>
<td>53.1</td>
<td>9.0⁺</td>
</tr>
<tr>
<td>+ Reduced heat-denatured RNaseB (10 mg/ml)</td>
<td>18.3</td>
<td>68.6⁺</td>
</tr>
</tbody>
</table>

Microwells were coated with 100 µg/ml solutions of the glycoproteins and binding of ¹²⁵I-conglutinin was determined in the absence or presence of the specified additives. For each glycoprotein coat, binding of ¹²⁵I-conglutinin in the absence of additives was taken as 100%.

¹The buffer used was 10 mM Tris–HCl, pH 7.8 (not containing NaCl).
²Percent inhibition relative to the binding in the absence of additives.
³Percent inhibition relative to the binding in the presence of 12 mM DTT.
⁴NT, Not tested.
Recognition of protein-linked Man₈ N-glycan by conglutinin

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sulfate concentrations) or weakening (low salt concentrations, water-miscible alcohols or detergents) hydrophobic interactions (Table I). Thus, the binding of conglutinin to reduced and denatured RNase-Man₈ seems to be exclusively carbohydrate-mediated and no additional hydrophobic protein–protein interactions appear to be involved.

The Man₈ oligosaccharide exhibits a similar average conformation on native RNaseB and on the reduced and denatured protein

There were no significant differences in the NOE patterns of the protein cross peaks of the isolated RNase-Man₈ (Figure 2a) and -Man₅₆ (not shown) glycoforms relative to RNaseA. Therefore, the protein moiety of RNase-Man₈ exhibits a three-dimensional structure essentially identical to that previously found for the non-glycosylated protein (Santoro et al., 1993). A similar conclusion can be drawn from these and previous studies (Joao et al., 1992) for the major RNase-Man₅₆ glycoform. Thus, no differences are apparent in the protein moiety of the individual glycoforms that could be related to the different processing of the oligosaccharide in these glycoforms. In the presence of dithiothreitol, no significant changes in the chemical shift dispersion characteristic of the native protein were observed. However, after incubation at 65°C, the NMR spectra of the resulting reduced heat-denatured RNaseB-Man₈ (Figure 2b) were characterized by limited dispersion of chemical shifts, coupling constants and smaller NOEs, close to random coil values, which is indicative of a great motion as expected for a non-structured polypeptide.

By TOCSY and NOESY NMR experiments all the spin systems of the sugar residues could be assigned and a number of sequential and remote NOE contacts between different monosaccharides deduced (Figure 3). For both the native (Figure 2c) and the reduced heat-denatured (Figure 2d) RNaseB-Man₈ glycoprotein, the protein-bound oligosaccharide exhibited ¹H chemical shifts and NOE data very similar to the free (-D₂)Man₈ oligosaccharide glycomer investigated previously (Wooten et al., 1990; Fu et al., 1994). Possible oligosaccharide conformations compatible with the NMR data were explored by molecular modeling. Full NMR data and details of the MD simulations have been described elsewhere (González et al., forthcoming). The best adjustment between modeled and experimental data was found for the simulation of
the \textit{gtgt} conformation of the two \(\alpha 1\rightarrow 6\) linkages. In particular, the observed long range NOE between ManA-H1 and GlcNAc2-methyl group indicates that the \textit{gt} conformation of the Man4′(\(\alpha 1\rightarrow 6\))Man3 linkage, where the Man4′ arm is folded back toward the trisaccharide core, is significantly populated. Thus, both on the native and on the reduced heat-denatured protein, the RNaseB-Man₈ oligosaccharide exhibits a conformational behavior similar to that described for the free oligosaccharide (Wooten \textit{et al.}, 1990), with the Man4′-ManA arm folding in and out over the rest of the glycan chain, thus approaching and moving far away from the polypeptide backbone.

NOEs to the protein were only detected for GlcNAc1, and all of them were to the Asn34 protons, as reported similarly for the \(N\)-glycan at Asn52 of the free \(\alpha\) subunit of human chorionic gonadotropin (De Beer \textit{et al.}, 1996), showing that GlcNAc1 is solvent exposed. NOE data for the \(N\)-glycosidic linkage in native RNaseB-Man₈ (Figure 3, inset) indicate that the GlcNAc1-C1H/Asn34-N\(\delta\)H and Asn34-N\(\delta\)H/C\(\gamma\)Obonds are in the trans conformation. The GlcNAc1-NAcH conformation is also trans. The same conclusions were drawn for the reduced heat-denatured glycoprotein. Only the weak NOE from Asn34-N\(\delta\)H to GlcNAc1-amide NH assigned in native RNaseB-Man₈ was not detected in the spectra of the reduced heat-denatured protein, possibly a reflection of the high degree of disorder exhibited by the protein moiety.

Overall, the three-dimensional architecture of the native RNaseB-Man₈ glycoprotein is well defined. A model generated from the protein and oligosaccharide NMR structures is shown in Figure 4. The intrinsic conformational properties of the oligosaccharide are preserved on reduced heat-denatured RNaseB-Man₈. However, the disorder exhibited by the protein moiety implies that there is a repertoire of oligosaccharide orientations with respect to the protein surface. Thus, the oligosaccharide–protein ensemble contains several very different geometries (Figure 4, inset).

On the structural basis of selective recognition by conglutinin of the RNaseB oligosaccharide

Conglutinin recognizes predominantly the terminal \(\alpha(1\rightarrow2)\)-linked mannose units (Young and Leon, 1987) which are fully exposed on the native RNaseB-Man₈. Yet, the affinity of conglutinin for the \(N\)-glycan on the native glycoprotein is not high enough for efficient binding. Mannan-binding protein behaves similarly. Crystallographic studies on recombinant mannan-binding protein CRD in complex with an asparaginyl Man₆ oligosaccharide (Weis \textit{et al.}, 1992) have shown that, in addition to the direct interaction with the terminal mannose, there are water-mediated interactions of the mannan-binding protein with internal sugars of the oligosaccharide which may play a significant role in binding. When the oligosaccharide is on a carrier protein, the ability to form these water-mediated lectin–glycan chain interactions may be crucially dependent on the precise geometry of the oligosaccharide–carrier protein ensemble. Thus, the observed differences in binding to native and to reduced heat-denatured RNaseB could arise from differences in the oligosaccharide presentations, that is, oligosaccharide orientations relative to the carrier protein surface that are available to the carbohydrate-binding proteins. The extent to which oligosaccharide presentation determines recognition by different carbohydrate-binding proteins will depend on the specific primary and secondary requirements of each recognition protein. For example, binding of calnexin and trimming by glucosidase II of monoglucosylated RNase Man₇-Man₉ glycoforms in the early stages of glycan processing have been reported to be independent of the conformation of the glycoprotein (Zapun \textit{et al.}, 1997). In contrast, the processing
enzymes in preparations of Golgi membranes from bovine pancreas can process into type chain the high-mannose chains on the reduced and alkylated RNaseB, but not on the native protein (Williams and Lennarz, 1984).

It has been proposed that recognition of specific protein-constrained oligosaccharide conformations may play a role in the control of N-linked oligosaccharide biosynthesis and carbohydrate-mediated recognition processes (Carver, 1993). The present study suggests that different carrier protein-related presentations of a conformationally free oligosaccharide may also modulate recognition.

Materials and methods

RNaseB glycoforms

Fractionation of RNaseB glycoforms has been described in detail elsewhere (González et al., forthcoming). In brief, RNaseB-Man₈ and a mixture of RNaseB-Man₅,₆ were isolated by gradient-elution affinity chromatography of RNaseB (Sigma) on concanavalin A–Sepharose and the composition of the fractions was determined by capillary electrophoresis and electrospray ionization mass spectrometry. Reduced and alkylated RNaseB-Man₅,₆ and RNase-Man₈ were prepared as described previously (Solís et al., 1994). Reduced and heat-denatured glycoproteins were prepared by incubation with 10–15 mM dithiothreitol at 65 °C for 10 min.

The aggregation state of reduced heat-denatured RNaseB was checked by gel filtration chromatography on a Superose 12 HR 10/30 column (Amersham Pharmacia Biotech) equilibrated with 10 mM Tris/HCl, pH 7.8, 0.15 M NaCl, 15 mM DTT. The flow rate was 0.5 ml/min and the elution was monitored at 280 nm. Native RNaseB as well as standard proteins for column calibration were chromatographed under similar conditions.

Conglutinin binding assays

Conglutinin isolated from heat-inactivated bovine serum (Lachmann, 1962) was radioiodinated using IODO-GEN (Pierce Eurochemie) following the manufacturer’s recommendations.

Nitrocellulose binding assays with ¹²⁵I-labelled conglutinin were carried out essentially as described (Solís et al., 1994), except that the buffer used was 10 mM Tris/HCl, pH 7.8, containing 0.15 M NaCl. Prior to overlay with labeled lectin, the amount of nonreduced and reduced glycoproteins electro-transferred onto nitrocellulose was checked by densitometric quantitation of the protein bands after Ponceau S staining, transferred onto nitrocellulose was checked by densitometric quantitation of the protein bands after Ponceau S staining, and submitted to a short (20 ps) restrained MD period, by including the distances estimated from the observed NOEs between the protons at GlcNAc₁ with those at Asn-34. The polypeptide was kept rigid during the MD. As an approach to generate a qualitative structure of the reduced and denatured glycoprotein, the disulfide bridges in the RNaseB-Man₈ model were manually removed and the resulting structure was minimized using AMBER and then subjected to a high temperature (1000 K) MD run (50 ps). Several snapshots from this simulation were taken.

Acknowledgments

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Abbreviation

RNaseB, ribonuclease B.

References
