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Binding of selected carbohydrates to apo-concanavalin A studied by electrospray ionization mass spectrometry

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Electrospray ionization mass spectrometry was used to investigate the binding of selected carbohydrates to different multimeric forms of apo-concanavalin A (apo-Con A). Information on the stoichiometry of the different quaternary Con A complexes, bound to high-affinity carbohydrate ligands, could be derived from the mass spectra. It was observed that specific carbohydrate binding takes place with metal-free apo-Con A. The data revealed that the apo-dimer and -tetramer (which are both present in solution) have similar affinities for the studied carbohydrates.

Introduction

Non-covalent interactions are crucial parameters in protein–ligand recognition, and these interactions play a vital role in the formation of tertiary and quaternary protein complexes. As biological activity is highly dependent on molecular conformations and intermolecular interactions, the application of mass spectrometry to these areas is of great interest. Over the last few years, several studies have appeared in which electrospray ionization mass spectrometry (ESI-MS) was used to investigate enzyme substrate and/or inhibitor[1–3] interactions to 'screen' combinatorial peptide libraries[2] or to investigate specific molecular interactions.[3–6] The use of mass spectrometry in characterizing non-covalent complexes is far from established, as these investigations are still in their early days. A few review articles have appeared,[7–10] highlighting some early successes, but also some experimental uncertainties. Method development, using relatively well characterized model systems, is still required before these new, exciting approaches in biological mass spectrometry can be used generally to obtain reliable and relevant information on protein complexes. One of the strengths of mass spectrometry in the analysis of protein interactions lies in the fact that complex stochiometries may be revealed. Because mass spectrometry is able to distinguish species of different molecular masses, it has the potential to analyze macromolecular complexes with multiple components in equilibrium. Many classes of protein systems may be multimeric under native conditions. This is especially true for lectins, *i.e.*, carbohydrate binding proteins.[11] It has been established that binding of carbohydrate molecules to oligomeric lectins may be dependent not only on the secondary and tertiary structure of the protein, but also on the quaternary structure of the protein complex.[12]

Other methods in structural biology such as X-ray diffraction mostly yield information only on a single (crystallized) quaternary complex, and a variety of multimeric systems are generally too complex to be studied by X-ray diffraction and/or NMR spectroscopy. At present microcalorimetry is probably one of the best methods to determine the thermodynamics of binding of ligands to proteins. However, calorimetry measures overall enthalpies of binding and does not directly distinguish between the binding to potentially different quaternary structures. In this study, we investigated the binding of the multimeric lectin concanavalin A (Con A) to a small library of carbohydrate molecules, with the aim of establishing whether we may extract qualitative and quantitative information on carbohydrate binding to the different multimeric forms of Con A.

Concanavalin A

Con A is a lectin isolated from jack bean (*Canavalia ensiformis*) seeds and has numerous biological functions related to its carbohydrate binding specificity. The tertiary and quaternary structures of Con A and its carbohydrate binding characteristics have been extensively studied over the years.[11,13–15] The Con A monomer consists of 237 amino acids ($M_r \approx 26\,500$ Da) and is arranged in two anti-parallel β-sheets. Con A has a high affinity to glucose/mannose carbohydrates, and is especially specific for carbohydrates with a 3,6-di-*O*-(α-D-mannopyranosyl)-D-mannose core. The carbohydrate binding site is situated on a solvent exposed cap of the monomer unit. Close to the carbohydrate binding site are two metal binding sites; a transition metal ion site (S1) (typically Mn^{2+}) and a Ca^{2+} site (S2).[16,17] It has been reported that metal-free Con A undergoes significant, but slow, conformational changes when fully metallized, and it has also been suggested that metal binding is essential for carbohydrate binding capacity and biological activity.[15]

In solution Con A exists in a dimer–tetramer equilibrium, with the dimer predominating below pH 5.6 and at low temperatures and the tetramer above pH 7.0 and at room temperature.[18] Below pH 6.5 the Con A tetramer dissociates into its canonical dimers. The monomer–dimer–tetramer equilibrium is thought to be highly dependent on pH and metal ion binding, whereby the number of interdimer hydrogen bonds appears to be significantly reduced in the metal-free Con A.[15,19] It is also believed that the dimer–tetramer equilibrium may have an impact on the carbohydrate binding, since certain N-linked oligomannose glycopeptides were shown to be divalent and bind more specifically with the Con A tetramer, but not with dimeric Con A. In contrast, however, Mandal *et al.*[20] reported that dimeric and tetrameric Con A bind similarly to a variety of carbohydrates (as expressed by their K_D measured by micro-

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calorimetry). Extensive X-ray crystallographic studies have been reported on metal-free Con A, metallized Con A and Con A co-crystallized with specific binding carbohydrates.[14] For instance, the X-ray crystal structure of the complex between tetrameric Con A and methyl 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside has been reported. [21] The carbohydrate binding ability of Con A has been widely used to investigate the properties of normal and transformed cells and to isolate carbohydrates, glycoconjugates and cells on Con A affinity matrices.[13,22] Binding affinities of Con A for a wide range of mannose containing carbohydrates have been determined recently by titration microcalorimetry.[20,23] As mentioned above, it should be noted that normally calorimetry measures overall enthalpies of binding and does not directly distinguish between the binding to potentially different quaternary structures and is, therefore, *a priori* inadequate to differentiate carbohydrate binding affinities of different multimeric forms of Con A.

In one of the earliest experiments in the area of non-covalent interactions studied by ESI-MS Light-Wahl *et al.* investigated Con A.[24,25] It was observed that in the mass spectra Con A could be detected as both the non-covalent dimer and the tetramer, depending on the experimental conditions. The occurrence and stability of monomeric, dimeric and tetrameric forms of Con A were investigated as a function of pH, and up-front source collision-induced dissociation (CID) collision energy. These early mass spectrometric results for the apo- (metal-free) Con A corresponded fairly well with literature data, confirming that at pH 7 Con A is predominantly a tetramer. In this work, we investigated the formation of complexes of selected carbohydrates (listed in Table 1) with different multimeric forms of Con A using electrospray time-of-flight (TOF) mass spectrometry with the aim of studying whether solution carbohydrate binding properties may be reflected by mass spectrometry.

Experimental

ESI mass spectra of ConA were recorded using a Micromass Q-TOF quadrupole time-of-flight mass spectrometer (Micromass UK, Manchester, UK) equipped with a nanospray source. Samples were introduced using a 'Z-spray' nanoflow electrospray source, using in-house pulled electrospray needles. The needles were made of borosilicate glass capillaries (Kwik-Fil, World Precision Instruments, Sarasota, FL, USA) using a P-97 puller (Sutter Instruments, Novato, CA, USA). They were gold-coated using an Edwards Scancoat six Pirani 501. Electrospray conditions were optimized as described in the Results and discussion section. The standard mass range scanned was 700–6000 Th. Con A was obtained from Sigma (St. Louis, MO, USA) and the mannose containing carbohydrates **a**–**e** (see Table 1) were obtained from Dextra Laboratories (Reading, UK). These carbohydrate molecules were selected as their binding properties to Con A had been investigated previously by microcalorimetry.[20,23] All chemicals were used without further purification. Stock standard solutions of Con A and/or the carbohydrates were prepared in pure water at a concentration of 1 mg mL^{-1}. Samples were further diluted and mixed in 20 mM ammonium acetate buffer to obtain the appropriate concentrations.

Calculations and analysis of data on carbohydrate binding

The raw mass spectra were processed (averaged and smoothed) using MassLynx software (version 3.1). Deconvolution of the spectra was accomplished using MaxEnt. For the quantitative analysis of carbohydrate binding, the abundance of the relevant species, *i.e.*, Con A multimers bound to none, one or more of the carbohydrate molecules **a**–**e** (see Table 1), was determined by integrating the signal intensities of each individual species. The extent of carbohydrate binding was calculated by defining the ligand occupancy percentage. The ligand occupancy percentage was determined by calculating the number of bound carbohydrate ligand molecules, *n*, divided by the potential number of ligand bound, *N*. It was assumed that the potential occupancy meant binding of two and four carbohydrate molecules to the Con A dimer and tetramer, respectively, *i.e.*, one binding site per monomer. *n*/*N* was calculated as a function of the carbohydrate concentration, at constant pH and Con A concentration.

Results and discussion

Experimental factors affecting the mass spectra of Con A non-covalent complexes

pH dependence. Con A oligomerization is known to depend on the pH of the solution.[15,20,24,25] Therefore, as a first test to investigate whether our recorded ESI spectra would reflect solution phase properties, Con A mass spectra were recorded as a function of the pH of the spray solution. These experiments were carried out with a Con A monomer concentration of 10 μM in 20 mM ammonium acetate buffer. The pH of this solution was 6.8. The pH of the buffer was varied by adding small amounts of formic acid or ammonia. Spectra were acquired at pH 5.1, 6.8, 7.8 and 9.8, after an incubation time of

Table 1 Structures of carbohydrates used in binding studies with Con A

Code	Structure	Abbreviation[a]	Mass/Da	K_D/L mol $^{-1}$[b]
a	α-D-Man*p*-OCH_3	Man-OMe	194.1	8.2×10^3
b	α-D-Glc*p*-(1→4)-D-Glc*p*	Glc_2	342.2	1.3×10^3
c	α-D-Man*p*-(1→6)-D-Man*p* ↑3 1 α-D-Man*p*	Man_3	504.4	3.4×10^5
d	α-D-Man*p*-(1→6)-α-D-Man*p*-(1→6)-D-Man*p* ↑3 ↑3 1 1 α-D-Man*p* α-D-Man*p*	Man_5	828.7	3.5×10^5
e	β-D-Glc*p*NAc-(1→2)-β--D-Man*p*-(1→6)-D-Man*p* ↑3 1 β-D-Glc*p*NAc-(1→2)-α-D-Man*p*	$GlcNAc_2Man_3$	910.8	1.4×10^6

[a] Man-OMe = methyl-α-D-mannopyranoside; Glc_2 = α-D-glucopyranosyl-(1→4)-D-glucopyranose; Man_3 = α-D-mannopyranosyl-(1→3)-[α-D-mannopyranosyl-(1→6)-D-mannopyranose; Man_5 = α-D-mannopyranosyl-(1→3)-[α-D-mannopyranosyl-(1→6)]-D-mannopyranosyl-(1→6))-[α-D-mannopyranosyl-(1→3)]-D-mannopyranose; and $GlcNAc_2Man_3$ = 3,6-di-*O*-[β-*N*-acetyl-D-glucosaminyl-(1→2)]-β-D-mannopyranosyl-(1→6)-D-mannopyranose. [b] K_D values determined by microcalorimetry.[20,23]

approximately 15 min. It was observed that at pH 5.1 Con A was mainly present in the dimer form, although some signals originating from Con A monomers were also observed. The spectra of Con A recorded at pH 6.8 and 7.8 mainly revealed Con A in the dimer and tetramer form. At pH 9.8 less than 20% of the total ion current could be attributed to signals of Con A multimers and 80% appeared to originate from the monomeric form. These results are in close agreement with previous mass spectrometric data by Light-Wahl *et al.*[24,25] and in agreement with the properties of Con A in solution.[18] Attempts were made to see whether the carbohydrate binding influenced the dimer–tetramer equilibrium. No real significant shifts were observed in the relative abundance of the tetramer and dimer ions upon addition of a binding carbohydrate. If anything, the equilibrium shifted slightly towards higher tetramer abundance relative to dimer abundance upon addition of the carbohydrate.

Source parameters. Electrospray is a relatively soft ionization technique which usually induces no or only limited fragmentation, allowing the detection of even weakly bound intact non-covalent molecular complexes such as the Con A protein oligomers. However, several experimental parameters have to be carefully adjusted in order to optimize the detection of such non-covalent complexes. To optimize the experimental conditions for the detection of the lectin Con A subunits bound to the carbohydrate ligands, a study was undertaken varying a few crucial experimental parameters on the Q-TOF electrospray ionization source, *i.e.*, the voltage applied on the nanoflow needle and the voltage on the cone. It appeared to be essential to optimize the needle spraying voltage. The amount of gold coating, the internal diameter of the needle and the distance between the needle and the sampling cone were all found to be important parameters affecting the optimum needle voltage. Therefore, for each new nanospray needle the spraying voltage needed to be optimized. Changes in the needle voltage led to significant changes in the resolution of the observed signals, but less so to the total ion signal intensities. In Fig. 1 mass spectra are shown recorded as a function of the needle voltage for a 20 mM ammonium acetate solution of pH 6.8 containing Con A and the trisaccharide Man_3, **c** in Table 1 (both at 10 μM concentration). Fig. 1 shows the ion signals for dimeric Con A, unbound and bound to one or more carbohydrate molecules. For instance, the signal at *m*/*z* 3680 corresponds to the $[2\text{Con A} + 14\text{H}]^{14+}$ ions. We introduce the following notation for the observed ion signals in the recorded ESI-MS spectra. The multiple charge state peaks are annotated with X_Y^{Z+}, in which X represents the aggregation state of Con A ($X = 2$ and 4 for the dimer and tetramer, respectively), Z^+ is the number of protons attached to the non-covalent complex and Y is the number of carbohydrate molecules attached to the Con A multimer. From top to bottom the spectra were recorded at spraying voltages of 2250, 2000, 1800, 1700 and 1500 V, using a constant cone voltage of 75 V. At the lowest applied spraying voltages the ion signals broadened significantly, and even more so at high spraying potentials. This peak broadening may be caused by ineffective desolvation or electrochemical reactions at the needle end. For all the needles used the voltage was varied between 1000 and 2500 V. It was observed that the optimum needle spray voltage for Con A can range from 1000 to 2000 V depending on the needle and its position. However, most needles performed best at a spraying voltage between 1600 and 1800 V.

Additionally, it was found to be essential to optimize the potential across the high-pressure region between the atmospheric pressure ESI source and the mass spectrometer, usually termed the cone voltage. Acceleration of ions in this region probably results in 'harder' collisions with the residual gas. These collisions at relatively low energies (10–150 eV) cause the ions to be focused and further decluster the initial ion droplets, resulting in improved, resolved mass spectra. However, at higher cone voltages unwanted disruption of non-covalent interactions and/or ion fragmentation may occur. Consequently, for studying non-covalent interactions the cone voltage has to be optimized with respect to maximum resolution without disrupting interactions between the Con A subunits and their binding to the carbohydrate molecules. Fig. 2 shows part of the ESI spectra recorded for a 20 mM ammonium acetate solution of pH 6.8 containing Con A and the trisaccharide Man_3 (both at 10 μM concentration) at cone voltages of, from top to bottom, 25, 50, 75, 100 and 125 V at a constant spraying voltage

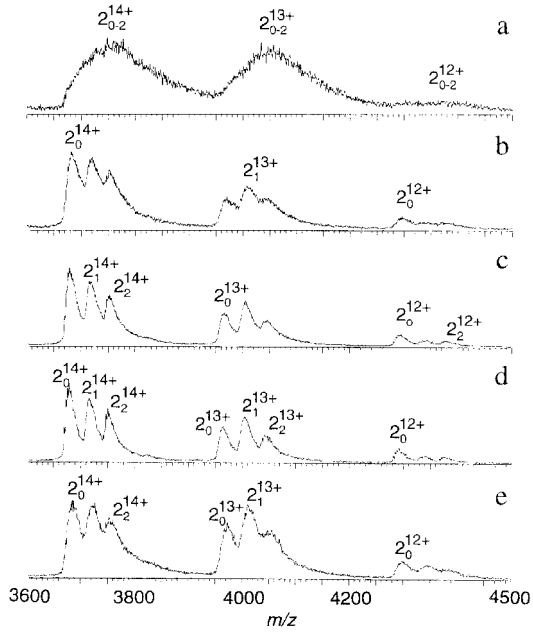

Fig. 1 ESI-TOF mass spectra as a function of a varying needle spraying voltage for 20 mM ammonium acetate solution of pH 6.8 containing Con A and the trisaccharide Man_3 (both at 10 μM concentration). From top to bottom the spectra were recorded at spraying voltages of 2250, 2000, 1800, 1700 and 1500 V, using a constant cone voltage of 75 V. Peak annotations are described in the text.

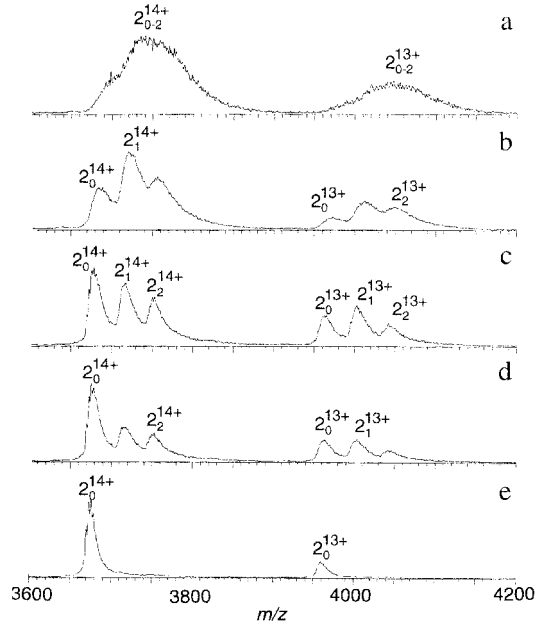

Fig. 2 ESI-TOF mass spectra as a function of a varying cone voltage for 20 mM ammonium acetate solution of pH 6.8 containing Con A and the trisaccharide Man_3 (both at 10 μM concentration). From top to bottom the spectra were recorded at cone voltages of 25, 50, 75, 100 and 125 V at a constant spraying voltage of 1700 V. Peak annotations are described in the text.

of 1700 V. It is evident that too low cone voltages lead to insufficient declustering, causing the ion signals to broaden. At cone voltages above 85 V it was observed that the saccharide ligands dissociated from the Con A dimer ions.

In summary, it was determined that typically the optimum cone voltage had to be around 75 V, whereas the spraying voltage should be, for Con A and the electrospray needles used, around 1750 V. The data shown in Fig. 1 and 2 do, however, reveal clearly that several experimental parameters have to be carefully adjusted for each new application when studying non-covalent interactions by mass spectrometry.

Binding and titration of carbohydrates to Con A

Binding studies were performed in 20 mM ammonium acetate solution of pH 6.8. It should be noted that we therefore investigated carbohydrate binding to metal-free Con A at relatively low salt concentrations, very different from the conditions used in microcalorimetric studies.[20,23] These conditions, *i.e.*, using a volatile buffer and low salt concentration, were selected as they are more compatible with nanoflow ESI-MS studies. Moreover, when 1 mM $CaCl_2$ and 1 mM $MnCl_2$ were added to the spray solutions, the observed ion signals were found to be substantially broadened, owing to the competitive binding of the metals and protons. Solutions of Con A were mixed and titrated in subsequent individual experiments with the carbohydrates **a**–**e** listed in Table 1. The Con A concentration was always 10 μM in 20 mM ammonium acetate buffer (pH 6.8). In experiments using Man-OMe (**a**), for which a K_D value of 8.2×10^3 L mol^{-1} has been reported,[20,23] no significant non-covalent Con A–carbohydrate complexes could be detected even when 10 μM Con A was mixed with 160 μM of the carbohydrate **a**. With the disaccharide Glc_2 (**b**), with a reported lower K_D value of 1.3×10^3 L mol^{-1},[20,23] only some very weak binding to Con A could be observed. At a Glc_2 concentration of 160 μM, approximately 10% of the available binding sites of both the dimeric and the tetrameric form of Con A were occupied. Both of these carbohydrate molecules are known to have a relatively low affinity for Con A.

In sharp contrast, highly abundant non-covalent lectin complexes were observed in experiments with the carbohydrate molecules Man_3 (**c**), Man_5 (**d**) and the biantennary $GlcNAc_2$-Man_3 **e** (see Table 1). This is may not be that surprising as these carbohydrate molecules **c**–**e** are known to have in solution at least a 100-fold higher affinity than **a** and **b** for Con A.[20,23] However, the data would then also indicate that specific carbohydrate binding occurs already with metal-free apo-Con A. The carbohydrates **c**–**e** have reported K_D values of 3.4×10^5 3.5×10^5 and 1.4×10^6 L mol^{-1}, respectively, albeit measured in 0.1 M HEPES buffer (pH 7.2) containing 0.9 M NaCl, 1 mM $MnCl_2$ and 1 mM $CaCl_2$.[20,23] As an example, Fig. 3 shows the electrospray mass spectrum of a solution containing 10 μM Con A and 50 μM of the carbohydrate Man_5 (**d**). In the mass spectrum shown in Fig. 3, dimeric and tetrameric forms of Con A were observed with a fine structure superimposed on them which originates from binding to different numbers of carbohydrate molecules **d** (Δmass ≈ 830 Da, the molecular weight of **d**). It is gratifying to see that the small mass differences between the unbound and bound tetrameric forms of Con A (~830 Da on a total mass of ~106 000 Da) can still be resolved, which allows us to 'count' the individual numbers of bound carbohydrate ligand molecules. It can be deduced from the spectrum shown in Fig. 3 that the majority of the expected Con A binding sites were occupied by the carbohydrate **d**, since 2_2^{Z+} and 4_4^{Z+} are the most abundant ions in the spectrum. The spectrum shows also some non-specific carbohydrate binding (*e.g.*, the peaks marked with 2_3^{Z+} and 4_5^{Z+}), which was, however, exclusively observed when the protein was saturated with a large excess concentration of carbohydrate. From the data in Fig. 3 it may be concluded that the binding to **d** is specific, since even at a substrate excess of 5 only marginal non-specific binding was observed.

Spectra showing the results of the titration experiments on 20 μM Con A with the carbohydrate **c** are shown in Fig. 4 and 5. Fig. 4 shows parts of the ESI mass spectra of dimeric Con A and Fig. 5 those of the tetrameric species. As may be deduced from these spectra, even at a carbohydrate **c** concentration of 5 μM already significant signals are detected for Con A–carbohydrate complexes [Fig. 4(b) and 5(b)]. At a carbohydrate concentration of 20 μM nearly all the expected ligand binding sites are

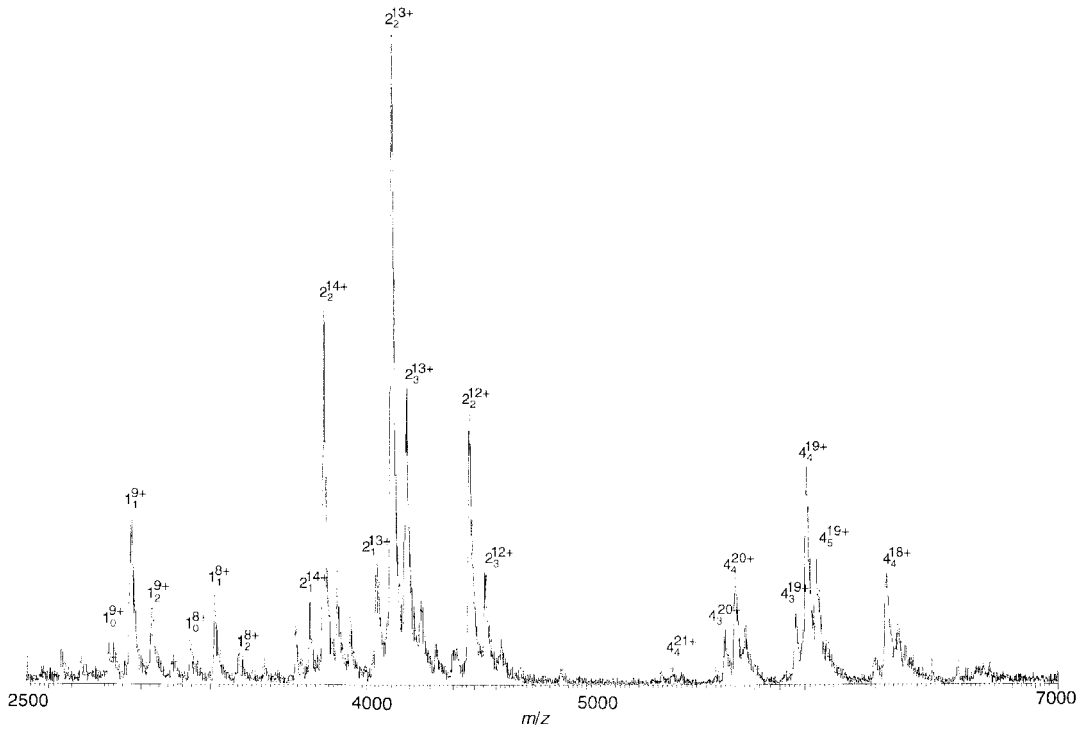

Fig. 3 ESI-TOF mass spectrum of 10 μM Con A and 50 μM carbohydrate Man_5 (**d**) in 20 mM aqueous ammonium acetate buffer. Peak annotations are described in the text.

occupied for both oligomers [Fig. 4(d) and 5(d)]. In principle, it should be possible to obtain association constants directly from these mass spectrometric data.[4,6,26] As the experimental factors affecting the appearance of the spectra were found to be very critical for this particular system, we only wish to conclude from these titration experiments that the association constants for **c**–**e** are indeed in the micromolar range, as the spectra clearly change in appearance in this range of carbohydrate concentrations. Instead, we compared the carbohydrate occupancy of the dimer and tetramer forms of Con A with the different carbohydrates in order to reveal whether the dimer and tetramer have distinct affinities. In Fig. 6 the results are shown for carbohydrate **c**, where the ligand occupancy was determined and plotted *versus* the carbohydrate concentration. As described in the experimental section, it was assumed that 100% ligand occupancy meant two for dimeric Con A and four for tetrameric

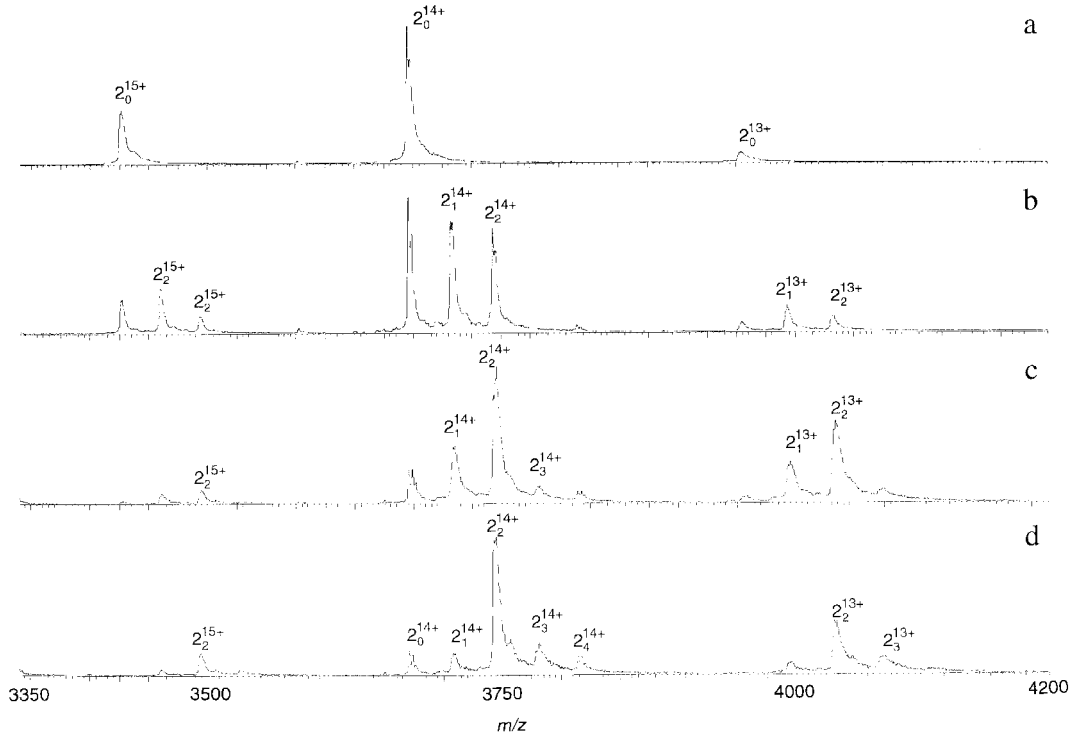

Fig. 4 Titration of Con A with the trisaccharide Man_3, (**c**).The ESI-MS spectra shown display the *m*/*z* region of dimeric Con A: (a) 10 μM Con A; (b) 10 μM Con A + 5 μM **c**;(c) 10 μM Con A + 10 μM **c**; (d) 10 μM Con A + 20 μM **c**. Peak annotations are described in the text.

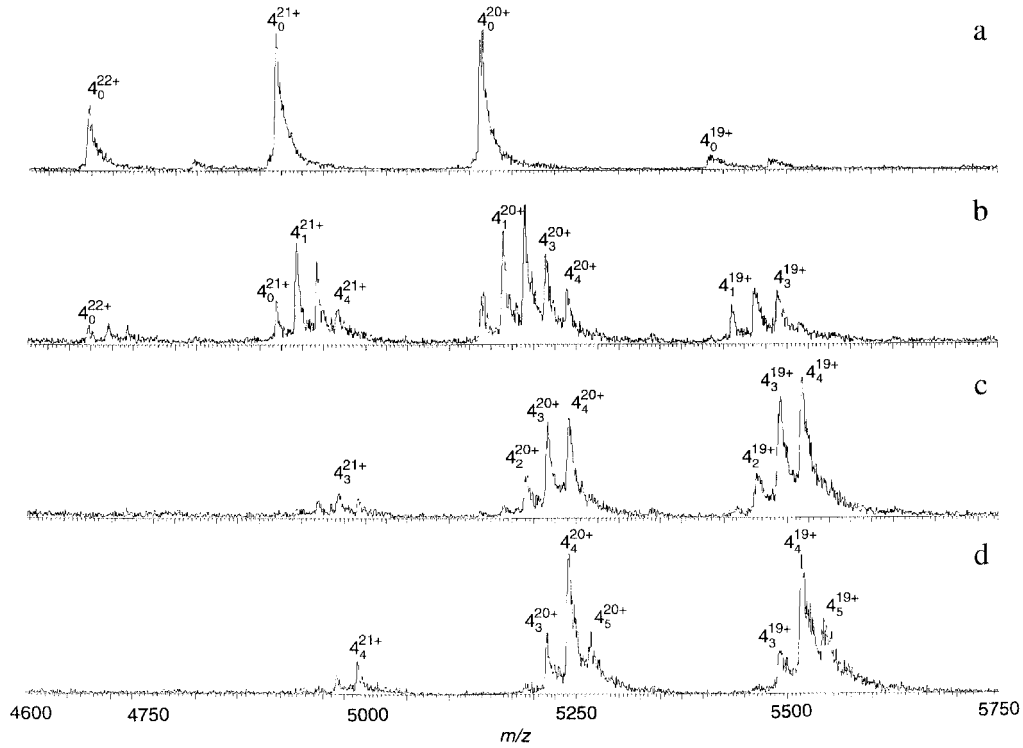

Fig. 5 Titration of Con A with the trisaccharide Man_3, (**c**). The ESI-MS spectra shown display the *m*/*z* region of tetrameric Con A: (a) 10 μM Con A; (b) 10 μM Con A + 5 μM **c**; (c) 10 μM Con A + 10 μM **c**; (d) 10 μM Con A + 20 μM **c**. Peak annotations are described in the text.

Con A. In Fig. 6 it can be seen that the percentage ligand occupancies [($n/N \times 100\%$] for the dimer (crosses) and tetramer (diamonds) follow exactly the same trend. Similar results were obtained in the titration experiments with carbohydrates **d** and **e** (Fig. 7 and 8, respectively). From these results it may be concluded that dimer and tetramer Con A have similar ligand occupancy profiles, at least for the studied carbohydrate systems **c**–**e**. Fig. 8 seems to indicate that carbohydrate **e** might have a lower affinity than **c** and **d** to Con A as revealed by the less steep slope observed in the titration curve in Fig. 8, but in contrast with reported binding constants measured at high salt concentrations and in the presence of Ca^{2+} and Mn^{2+} in the metal binding sites. Whether this reduced binding is significant and/or maybe influenced by the lack of metal binding is currently under investigation in competitive binding experiments.

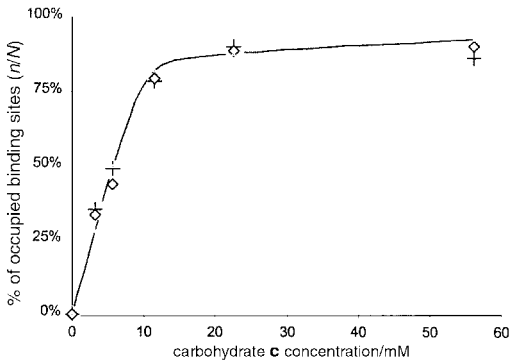

Fig. 6 Ligand occupancy profiles for the dimeric and tetrameric forms of Con A titrated with the carbohydrate **c**. The data points marked with a cross reflect the ligand occupancy to the dimer of Con A and those marked with a diamond show the ligand occupancy to the tetramer of Con A. For the calculation of the ligand occupancy, see text.

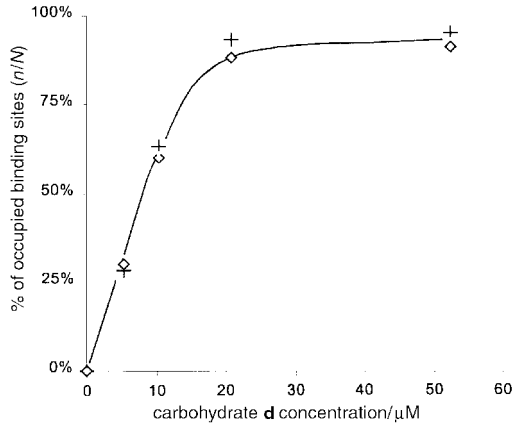

Fig. 7 Ligand occupancy profiles for the dimeric and tetrameric form of Con A titrated with the carbohydrate Man_5 (**d**). The data points marked with a cross reflect the ligand occupancy to the dimer of Con A and those marked with a diamond show the ligand occupancy to the tetramer of Con A. For the calculation of the ligand occupancy, see text.

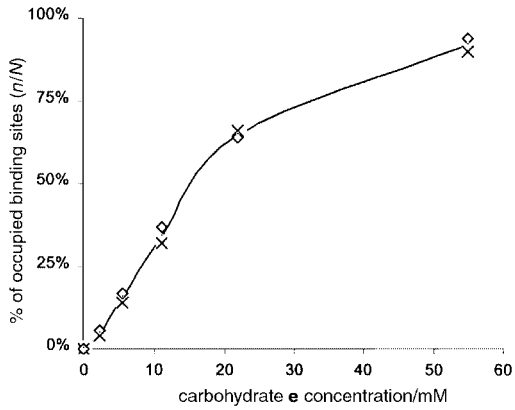

Fig. 8 Ligand occupancy profiles for the dimeric and tetrameric form of Con A titrated with the carbohydrate $GlcNAc_2Man_3$ (**e**). The data points marked with a cross reflect the ligand occupancy to the dimer of Con A and those marked with a diamond show the ligand occupancy to the tetramer of Con A. For the calculation of the ligand occupancy, see text.

Conclusion

We used ESI-MS to study the binding of selected carbohydrates to different multimeric forms of apo-Con A. Our results indicate that the detection of such non-covalent complexes may be influenced significantly by the experimental parameters. However, using carefully adjusted ESI source parameters, mass spectrometry can be a very useful tool to study the binding between multimeric protein structures and carbohydrate ligands. Information on the stoichiometry of the different quaternary complexes and ligands can be directly derived from the mass spectra, in addition to some semi-quantitative information on carbohydrate binding. ESI-MS experiments performed on dimeric and tetrameric Con A revealed that significant binding to carbohydrates, with a 3,6-di-*O*-(α-D-mannopyranosyl)-D-mannose core, takes place already for apo-Con A, without metal binding into the two potential metal binding sites S1 (Mn^{2+}) and S2 (Ca^{2+}). Our results indicate that metal binding is therefore not essential for ligand binding, although it has been suggested that it is required to promote the biological activity of Con A.[15]

From the mass spectrometric data, it could be deduced that the ligand occupancies of dimeric and tetrameric apo-Con A for carbohydrates with a 3,6-di-*O*-(α-D-mannopyranosyl)-D-mannose core were virtually identical, indicating that the dimer and tetramer have similar affinities for these carbohydrates. These latter findings are in compliance with the microcalorimetric data of Mandal *et al.*,[20] who found that for several carbohydrates, the affinities of the dimeric forms of acetyl-Con A and the dimeric forms of succinyl-Con A were virtually identical with that observed for tetrameric Con A.[20]

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