Probing Molecular Interactions in Intact Antibody: Antigen Complexes, an Electrospray Time-of-Flight Mass Spectrometry Approach

Mark A. Tito,* Julie Miller,† Nicola Walker,† Kate F. Griffin,† E. Diane Williamson,† Dominique Despeyroux-Hill,† Richard W. Titball,† and Carol V. Robinson*

*Oxford Centre for Molecular Sciences, New Chemistry Laboratory, Oxford OX1 3QT, United Kingdom; and †Defence Evaluation and Research Agency Chemical and Biological Defence Sector, Salisbury, Wiltshire SP4 OJQ, United Kingdom

ABSTRACT Using a combination of nanoflow-electrospray ionization and time-of-flight mass spectrometry we have analyzed the oligomeric state of the recombinant V antigen from *Yersinia pestis*, the causative agent of plague. The mass spectrometry results show that at pH 6.8 the V antigen in solution exists predominantly as a dimer and a weakly associated tetramer. A monoclonal antibody 7.3, raised against the V antigen, gave rise to mass spectra containing a series of well-resolved charge states at m/z 6000. After addition of aliquots of solution containing V antigen in substoichiometric and molar equivalents, the spectra revealed that two molecules of the V antigen bind to the antibody. Collision-induced dissociation of the antibody-antigen complex results in the selective release of the dimer from the complex supporting the proposed 1:2 antibody:antigen stoichiometry. Control experiments with the recombinant F1 antigen, also from *Yersinia pestis*, establish that the antibody is specific for the V antigen because no complex with F1 was detected even in the presence of a 10-fold molar excess of F1 antigen. More generally this work demonstrates a rapid means of assessing antigen subunit interactions as well as the stoichiometry and specificity of binding in antibody-antigen complexes.

INTRODUCTION

Conventional electrospray (ES) mass spectrometry (MS) methods for the analysis of proteins and their complexes typically involve a combination of processes that disrupt higher order structure. These include denaturing solvents, source heating, high electric fields, and high-energy in-source collisions. To preserve noncovalent interactions, in general, solution conditions need to be at or near physiological pH often with high ionic strength and at ambient temperature. Further, the MS conditions have to be sufficiently "gentle" to desolvate the protein complexes and yet not disrupt noncovalent associations. Under these conditions the mass spectra of protein complexes carry fewer positive charges and possess significantly broader peaks for individual charge states than are generally observed for individual proteins at low pH and in the presence of denaturing solutions (Robinson et al., 1994; Light-Wahl et al., 1994; Loo, 1995). The analysis of noncovalently bound antibody-antigen complexes has not been amenable to analysis by ES from their native state primarily because their m/z values have, until recently, been outside the mass range of conventional instrumentation. For this reason MS analysis, although successfully applied to characterize antibody fragments (Krebs et al., 1995; Downard, 2000), intact antibodies from denaturing or acidified solutions (Bennett et al., 1996; Chernushevich et al. 1997), and noncovalent complexes with antibody fragments (Siuzdak et al., 1994; Chernushevich et al., 1997) have not previously been applied to intact antibodies and their antigens from solutions in which the native state is preserved. The evolution of ES into nanoflow (Wilm and Mann, 1994) together with the coupling of ES with the unlimited m/z range afforded by time-of-flight (Tof) mass analyzers (Verentchikov et al., 1994) provides the enabling technology to analyze these large protein complexes (Rostom and Robinson, 1999a; Van Berkel et al., 2000) and even whole particles (Tito et al., 2000; Rostom et al., 2000). The capability therefore exists to maintain antibody:antigen complexes and to examine their binding stoichiometry and interactions using MS.

The bacterium *Yersinia pestis* is the causal agent of bubonic plague, a zoonotic infection that is spread via the bite of an infected flea. The bubonic form of the disease may develop into the highly contagious pneumonic form, which is spread from person to person via airborne droplets. The high level of mortality and infectivity of pneumonic plague is the driving force for the development of new and more effective vaccines. As an alternative to whole cell vaccines, immunization with purified recombinant *Y. pestis* proteins, fraction 1 (F1), and V antigens has been proposed (Williamson et al., 1997; Eyles et al., 2000). In a previous MS investigation, we have examined the molecular interactions in the recombinant F1 antigen and found that it is able to form multimers with sevenfold symmetry (Tito et al., 2001). The recombinant V antigen (rV) is a 37-kDa protein secreted by *Y. pestis* implicated in a variety of different functions (Leary et al., 1995), however the oligomeric state and the molecular basis of its recognition by a monoclonal antibody (MAb) have not been determined. Here we demonstrate the application of an MS approach and examine in

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Address reprint requests to Carol V. Robinson, Oxford Centre for Molecular Sciences, New Chemistry Laboratory, South Parks Road, Oxford OX1 3QT, UK. Tel.: 44-1865-275981; Fax: 44-1865-275948; E-mail: carolr@bioch.ox.ac.uk.

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detail the binding interactions between the rV antigen from *Y. pestis* and the MAb 7.3 (Hill et al., 1997).

MATERIALS AND METHODS

Protein samples

rV and F1 antigens and MAb 7.3 were prepared as described previously (Hill et al., 1997; Williamson et al., 1997). Before analysis, all samples were buffer exchanged into 250 mM ammonium acetate (Analytical Grade, Sigma, St. Louis, MO) in Milli-Q™ water, using PD-10 columns (Amicon, Beverly, MA). Protein concentration was measured by the Bicinchoninic Acid Assay reagents (Pierce Chemical, Rockford, IL) against a bovine serum albumin standard, and solutions for analysis were diluted to 20 μM concentration. The pH of individual samples were recorded and found to be typically in the range pH 6.8 to 6.9 for the antigen solutions and pH 6.6 for the MAb solution. To investigate disulphide bond formation between monomeric rV antigen molecules, an aliquot of solution was incubated overnight in aqueous solution at 45°C. Disulphide bond reduction of the rV antigen was carried out by adding 2% (v/v) of β-mercaptoethanol to the solution containing rV antigen and incubation at 60°C for 1 h.

Polyacrylamide gel electrophoresis

rV was analyzed by reducing and nonreducing sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) using a PhastSystem (Pharmacia, Uppsala, Sweden). The antigen was heated to boiling in the presence of Laemmli sample buffer (containing 60 mM Tris, 2% (w/v) SDS, 25% (v/v) glycerol, 0.1% (w/v) bromophenol blue) with and without β-mercaptoethanol. Samples were applied to a 10 to 15% gradient gel in phosphate-buffered saline (PBS). The proteins in the gel were stained with Coomassie blue. rV was also analyzed by native PAGE by applying an untreated sample of rV antigen in PBS to an 8 to 25% gradient gel. Proteins in the gel were stained with Coomassie blue.

Size exclusion chromatography is as follows: aliquots of rV antigen (200 μl) in PBS were applied to a Superose 6 HR 10/30 column (Pharmacia) attached to an Akta Explorer (Pharmacia). The column had been previously equilibrated with PBS and the antigen was eluted with the same buffer. A range of molecular weight standards (ferritin, 440; catalase, 232; aldolase, 158; albumin, 67; and ovalbumin, 43) were applied to the column to produce a calibration curve for estimating the approximate molecular weight of the native protein.

Mass spectrometry

Spectra were recorded on two different instruments Q-Tof-1 and Q-Tof-2 as well as a liquid chromatography (LC)-Tof mass spectrometers (Micromass, UK Ltd., Manchester, UK). All the mass spectrometers are equipped with Z-Spray Nanoflow sources. Gold coated-borosilicate glass capillaries were used for sample introduction and prepared as described previously (Nettleton et al., 1998). In Q-Tof mass spectrometers ions are focused by a radio frequency lens before transmission to the quadrupole, which in these experiments was used in the RF-only mode, as a wide bandpass filter. Ions were transferred through a hexapole collision cell pressurized with dry argon. Transmission into the Tof was achieved with an acceleration voltage of 8 kV and with a pulse rate of 4 kHz for detection with a multichannel plate (MCP). A 1-GHz TDC was used for Q-Tof-1 and the LCT, whereas a 4-GHz time to digital converter was used for the Q-Tof-2. The MCP was set at 2700 V in all cases. Collisional cooling on both the Q-Tof-1 and Q-Tof-2 instruments was achieved by manually throttling the analyzer-roughing pump and applying up to 30 mBa of argon gas in the collision cell. Spectra recorded on the LC-Tof mass spectrometer were obtained with argon gas introduced into the first hexapole lens. Acceleration into the Tof was achieved with a 3-kV pulse. For all mass spectrometers, the cone voltage was typically 150 V with a needle voltage of 1.4 kV except where stated otherwise. Mass spectra were acquired without source heating. All mass spectra were calibrated against cesium iodide. Mass Lynx software version 3.1 was used, and the spectra represent the raw data with a mean smoothing algorithm applied at peak width at one-half height.

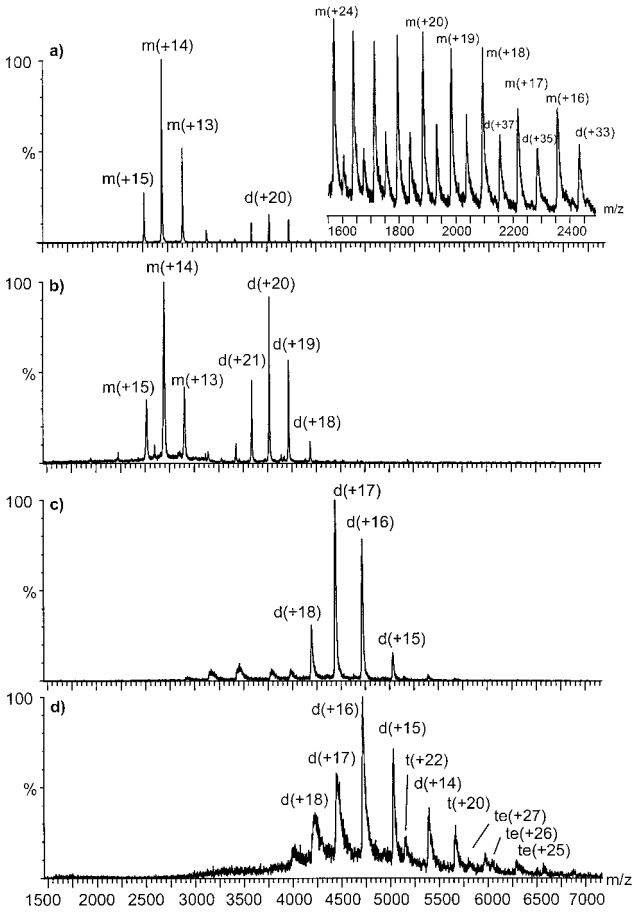

FIGURE 1 The nano-ES mass spectra of the V antigen recorded on the Q-Tof-1 at (*a*) conditions showing predominantly monomer with cone and capillary voltages 100 V and 1.5 kV, respectively, (*inset*) after incubation overnight at 45°C and addition of 50% acetonitrile. (*b*) Cone voltage of 120 V in the presence of collisional cooling (9.0^{-7} mBa) indicating the appearance of dimer at ~m/z 3500 to 4000 with an average charge of +20 (*c*) at a pressure of 1.5^{-6} mBa, in the source region of the mass spectrometer showing predominantly V antigen dimer with an average charge of +17. (*d*) Analyzer pressure of (6.0^{-6} mBa), indicating the presence of additional peaks assigned to the trimer and tetramer. The peaks labeled m, d, t, and te represent monomer, dimer, trimer, and tetramer, respectively. The charge states are given in parentheses.

RESULTS

Subunit interactions in the V antigen

Fig. 1 *a* shows the mass spectrum of the rV antigen from solution at pH 6.9 and 250 mM ammonium acetate. Assignment of the charge states enables the calculation of the mass of the neutral molecule, reported in Table 1. A second series

TABLE 1 Calculated and measured masses for the V antigen and its complexes with Mab 7.3

Protein complex	Measured mass (Da)	Calculated mass (Da)	Peak width* (Da)
V antigen monomer	37,709 ± 4	37,674	280
V antigen dimer	75,348 ± 8	75,348	180
V antigen trimer	113,307 ± 30	113,022	400
V antigen tetramer	150,802 ± 48	150,696	680
Mab 7.3	148,884 ± 8	not known	1120
Mab 7.3:V antigen	224,837 ± 20	224,242	2700
Mab 7.3:V antigen†	224,479 ± 15	224,242	1260

*Calculated for the molecular ion from peak width at half height measurements of individual charge states.
†Recorded under high collision energy conditions.

of charge states is observed at lower intensity and corresponds in mass to a dimer. It was not possible to record spectra in which there was no contribution from the dimer, even under relatively harsh MS conditions, suggesting that a proportion of the molecules (5–10% depending on the length of time in solution) are linked by disulphide bond formation. To investigate this possibility further the rV antigen was analyzed under a wide range of solution and MS conditions. The formation of disulphide-bonded dimer is demonstrated by incubation of a solution at 45°C overnight and recording the mass spectrum in the presence of organic cosolvent (Fig. 1 *a*, inset). The presence of the disulphide-bonded dimer was further confirmed by addition of 2% β mercaptoethanol (see Materials and Methods). The resulting mass spectrum revealed only the presence of rV antigen monomer (data not shown). Together these results demonstrate that the single cysteine residue in the rV antigen sequence is oxidized to form a disulphide-linked dimer upon unfolding at increased temperatures or prolonged storage in solution. To summarize the results of these experiments a freshly prepared solution of the rV antigen contains $<$5% of disulphide-bonded dimer, whereas increased proportions of the dimer were formed on storage of rV antigen in solution, particularly with heating.

Reducing the acceleration of the ions in the atmospheric pressure region of the mass spectrometer leads to a significant increase in the population of a noncovalently bound dimer (Fig. 1 *b*). The average charge states from the monomer (average +14) and dimer (average +20) do not overlap due to the reduction of charge in the associated dimer compared with the component monomers. The most likely explanation for this reduction in charge is through salt bridges that maintain the interactions between the component monomers (Vis et al., 1998). The measured mass is closer to the calculated and the peak width recorded for the dimer narrower than both these values for the monomer within the same spectrum (Table 1). These observations are surprising given the size of the protein dimer (75 kDa) and suggest that unlike the monomer, small molecules are not trapped within the protein structure or the interfacial regions of the dimer.

An increase in pressure in the analyzer region of the mass spectrometer facilitates more effective collisional cooling (Krutchinsky et al., 1998). This allows the noncovalent interactions in the dimer to be maintained without significant dissociation to the monomer, Fig. 1 *c*. The average charge state of the dimer is +17, a decrease over that observed under the conditions described above. This observation can be attributed to the appropriation of ions from the buffer during desolvation as the pressure in the mass spectrometer increases (Tito et al., 2001). This interpretation is also supported by an increase in the width of the peaks recorded with increased collisional cooling over those observed in Fig. 1 *b*. Under very low energy conditions, a series of ions at high m/z interspersed with the peaks assigned to the dimer are observed. These ions correspond in molecular weight to both trimers and tetramers. The origin of these species, either in solution or in the gas phase, is difficult to distinguish. They may be consistent with association in the protein droplet. Local protein concentrations are known to change as a result of the evaporative processes that occur during ES (Kebarle, 2000). In general, however, gas phase dimerization of protein molecules leads to significantly higher m/z ratios suggesting that the origin of this species is in solution. In summary these MS results confirm the importance of collisional cooling for studying protein-protein interactions and demonstrate that, under the solution conditions used here, the rV antigen is predominantly a dimer in equilibrium with a weakly associated tetramer.

To verify this interpretation of the mass spectra, the rV antigen was analyzed by SDS PAGE/native PAGE and size exclusion chromatography. When rV antigen was analyzed under reducing conditions, a single band was observed on the SDS gel corresponding to a molecular weight of ~37 (Fig. 2 *a*). Nonreducing conditions resulted in two bands corresponding to molecular weights of ~37 and 74. The data suggest that dimer formation is at least in part as a result of disulphide bond formation. A similar proportion of disulphide bonded dimer appeared to be present in the SDS gel, observed after incubation of rV antigen solution, and recorded the spectrum under denaturing conditions (Fig. 1 *a*, inset). These results confirm that the heating process exposes the active cysteine residue allowing intermolecular disulphide bond formation, resulting in a proportion of dimer that remains intact during SDS PAGE.

Analysis by PAGE under conditions in which native interactions are maintained resulted in two major bands of approximate molecular weight 74 and 148, consistent with the noncovalent association of dimers to form tetramers. In addition, higher molecular weight multimeric species were observed. Size exclusion chromatography of a solution of rV revealed two distinct peaks with elution volumes of 15.37 and 16.6 ml. Comparing these values with the calibration curve constructed gave an approximate molecular

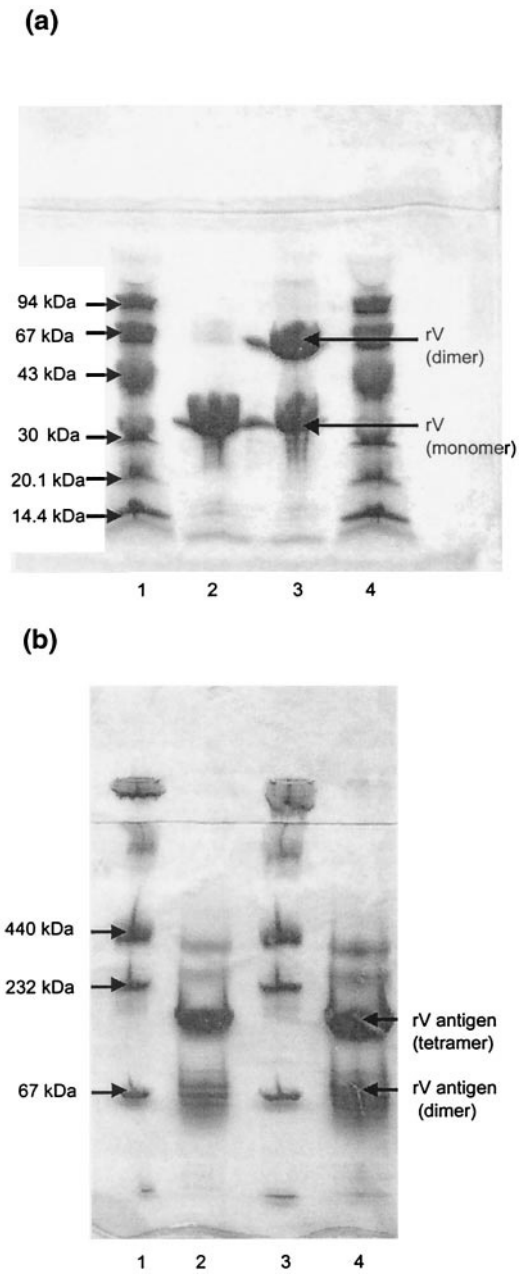

FIGURE 2 (*a*) SDS-PAGE analysis of rV antigen after heating in Laemmli sample buffer with and without β-mercaptoethanol (*lanes 2* and *3*, respectively); molecular mass markers in lanes 1 and 4. (*b*) Native PAGE analysis of rV antigen in PBS (*lanes 2* and *4*); molecular weight markers in lanes 1 and 3.

weight of 170 for peak 1 and 60 for peak 2. This method relies on the protein being analyzed having similar characteristics to the standards used and hence can be inaccurate. However the molecular weights calculated for rV antigen again confirm a dimer and a higher molecular weight species, possibly a tetramer. These solution-based data are therefore wholly consistent with the results from MS, confirming the presence of a proportion of disulphide-bonded

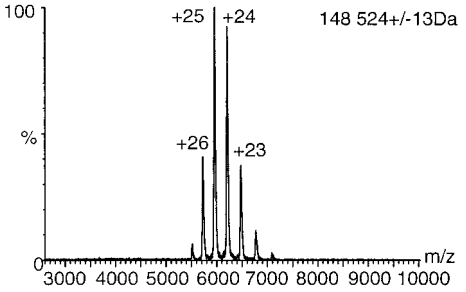

FIGURE 3 Nano-ES mass spectrum of the MAb 7.3 acquired on the Q-Tof-2 using collisional cooling at a pressure of 5.4^{-5} mBa, cone 150 V, capillary 1.6 kV, and 50 V applied to accelerating lenses in the collision cell with 30 mBa of argon gas.

dimer together with noncovalent associations to form both dimers and tetramers.

MS of the MAb

A MAb raised against the rV antigen (MAb 7.3) (Hill et al., 1997) was analyzed by nanoflow electrospray (nano-ES) MS and gave rise to the spectrum shown in Fig. 3. The antibody at pH 6.6 in 250 mM ammonium acetate gives rise to a series of charge states from m/z 5500 to 7500. The charge states were assigned as +20 to +26, and the calculated mass is reported in Table 1. The spectrum reveals a narrow distribution of charge states compared with those of previous studies where an antibody was subjected to reduction and denaturation (+38 to +50) (Bennett et al., 1996) and in the presence of 7% acetic acid (+21 to +66) (Chernushevich et al., 1997). The peak width of the molecular ion under the solution conditions used here is remarkably narrow (~700 Da). The origin of the peak width in ES mass spectra arises from the isotopic composition of the molecular ion, the chemical heterogeneity of the molecule, the resolving power of the instrument, and adduction of small molecules derived from aqueous buffer. By far the greatest contribution under the conditions used here is the heterogeneity of small molecule binding. Consequently, despite the anticipated increased ion binding and reduced desolvation efficiency under aqueous conditions, neutral pH and at ambient temperature, the charge state series recorded for the MAb is surprisingly well resolved.

Stoichiometry of antibody: antigen binding

To probe the antigen interactions with the MAb, aliquots of freshly prepared solution containing rV antigen were added stepwise to a solution containing the antibody, Fig. 4. At a 1:0.1 molar excess of antibody to antigen the most intense charge state in the mass spectrum was the +28 charge state of the unbound antibody. This increase in the charge over that observed for the free MAb (+25) is explained by the

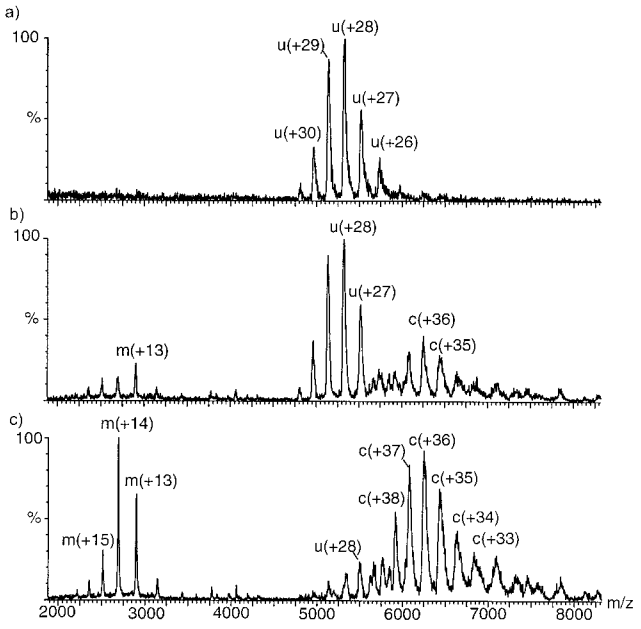

FIGURE 4 The nano-ES mass spectra recorded after addition of aliquots of the V antigen solution to MAb 7.3 (resulting pH 6.9) at (*a*) 1:0.1 molar ratio of MAb 7.3 to V antigen dimer, (*b*) 1:0.2 molar ratio, and (*c*) equimolar ratios for each protein. The letters u, c, and m represent unbound antibody, antibody in complex with V antigen, and monomeric antigen, respectively. The charge states are given in parenthesis. All spectra were recorded on the LC-Tof mass spectrometer with N_2 as a cooling gas with a cone voltage of 150 V and a capillary voltage of 1.6 kV. Instrumental conditions remained constant throughout these measurements.

different instrumental settings used to obtain these two spectra and the increased pH of the antibody antigen solution (pH 6.9) compared with that measured for the antibody alone (pH 6.6). There are no charge states indicating the presence of free monomer or dimer of the rV antigen. This presumably arises due to signal suppression of the smaller protein by the larger one (Yates et al., 2000). At a 1:0.2 molar excess of antibody to antigen, the mass spectrum reveals a series of peaks at m/z 2000 to 3000 corresponding in mass to rV antigen monomer. The absence of protein dimer in this spectrum is rationalized because a freshly prepared solution of rV antigen was added, ruling out significant contributions from the disulphide bonded dimer, and the instrument conditions are sufficient to ionize the MAb such that dissociation of noncovalent dimer is likely to occur. As well as charge states from the unbound antibody, an additional series of peaks centered at m/z 6500 and at ~40% relative intensity are observed. Assignment of their m/z values gives a series of peaks with an average charge of +36 and a molecular mass in close agreement with that calculated for a complex formed between two molecules of rV antigen and one of the antibody (Table 1). Increasing the molar ratio of rV antigen to give equimolar ratios of MAb: rV antigen increases the intensity of the MAb complex. At this concentration, the rV antigen is prominent in the spectrum, but the antigen-antibody complex charge states are the most intense. There is also a concomitant decrease in the intensity of the peaks assigned to the unbound antibody.

Increasing the rV antigen concentration in excess of that of the antibody did not show any additional antigen binding or nonspecific binding; rather excess antigen remained as a monomer in the spectrum. The observation of the monomer of the rV antigen as opposed to the dimer observed under the MS conditions used in Fig. 1 can be rationalized by the higher cone and capillary voltage that were found to increase the sensitivity for detection of the high mass complex. The increase in mass between the calculated and measured masses and the increased peak width over that measured for the unbound antibody under the same conditions (Table 1) would suggest that the intermolecular interfaces are capable of binding small molecules, either water molecules or ions derived from the buffer. Nevertheless the mass measured here for the antibody:antigen complex defines unambiguously a 1:2 stoichiometry of antibody to antigen with good accuracy.

Control experiments establish the specificity

To determine the specificity of these interactions a second antigen from the same species, *Y. pestis* F1 antigen, was examined in the presence of MAb 7.3. The rationale behind this experiment was to see if nonspecific interactions between antibodies and antigens could occur under these MS conditions. F1 antigen was added in a stepwise fashion to MAb 7.3 from substoichiometric through to a 10-fold excess of the antigen. No complex could be detected under any of the MS conditions examined. Significantly this result rules out the possibility of the rV antigen:MAb 7.3 complex existing as a result of nonspecific electrostatic interactions, often favored in the gas phase of the mass spectrometer (Robinson et al., 1996) and highlighted in the past for producing false positives in protein ligand binding experiments analyzed by MS (Aplin et al., 1994). The results of these control experiments establish therefore that the complexes observed in the mass spectra of the rV antigen and MAb 7.3 are specific.

Collision-induced dissociation of the rV antigen-antibody complex

Previous studies have shown that not only the determination of the intact molecular mass but also the dissociation behavior in the gas phase can provide structural insight into the subunit architecture of macromolecular complexes (Rostom and Robinson, 1999b; Fändrich et al., 2000). To investigate the effects of high-energy collisions on the antibody:antigen complex the voltage on the lenses that project ions into the collision cell was increased in a stepwise fashion (Fig. 5). The spectrum recorded at a collision

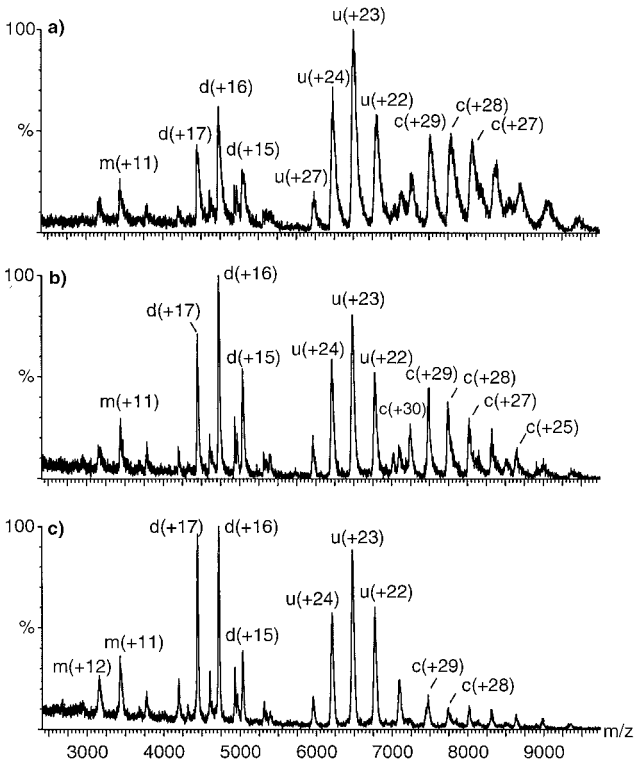

FIGURE 5 The nano-ES mass spectra of the controlled dissociation of the antibody antigen complex formed from equimolar ratios of the rV antigen dimer and MAb 7.3 at a collision energy of 25 (*a*), 50 (*b*), and 75 V (*c*). The letters u, c, d, and m represent unbound antibody, antibody in complex with V antigen, dimeric V antigen, and monomeric V antigen, respectively. The charge states are given in parenthesis. All spectra were recorded on the Q-Tof-2 mass spectrometer with 30 mBa of argon in the collision cell and in the presence of collisional cooling as described in Materials and Methods.

energy of 25 V shows that the most intense charge state is the +23 ion of the unbound antibody centered at ~m/z 6500. Also clearly visible are the antibody:antigen complex charge states ranging from +25 to +30. The rV antigen dimer centered at m/z 4500 exhibits mainly the +16 charge state ion with the +11 ion for the monomer at a relatively low intensity at m/z of 3500. Increasing the collision energy to 50 V results in a decrease in the relative intensities of the peaks assigned to the antigen:antibody complex and the unbound antibody relative to those assigned to the rV antigen dimer. Promoting higher energy collisions, by further increase of the accelerating voltages in the collision cell to 75 V, results in further disruption of the antibody:antigen complex; the unbound antibody and rV antigen dimer give rise to the most intense peaks in the spectrum. The most facile dissociation pathway for this complex is therefore loss of the rV antigen dimer from the antibody. Moreover the fact that freshly prepared rV antigen was added to the antibody, and the solution not exposed to heat, rules out the possibility of disulphide bond formation between monomers confirming that it is the noncovalent dimer that is recognized and bound by the antibody.

Consistent with other collision-induced dissociation spectra is the decrease in peak width observed for all species as the collision energy is raised (Table 1) (Rostom and Robinson, 1999a). Moreover the measured mass of the complex under the high-energy conditions used to record the spectrum in Fig. 5 is closer to the calculated mass than that measured under the lower energy regime (Table 1). This reduction in the measured mass and associated decrease in peak width is in accord with the removal of surface-bound counter ions and solvent molecules under this higher energy collision regime.

Concluding remarks

This investigation demonstrates that the binding stoichiometry and specificity within antigen and antibody complexes can be deduced solely from MS measurements. The MS and solution based measurements show that at micromolar solution concentration, the rV antigen forms a tightly associated dimer and a loosely associated tetramer. Titration experiments in which the rV antigen complex was added over a wide range of antigen concentrations reveal the stoichiometry of the antibody antigen complex. Disruption of the antibody-antigen complex, using high-energy collisions in the gas phase, shows that the major dissociation product is the rV antigen dimer. Moreover, the absence of an intermediate with 1:1 stoichiometry, even in the presence of substoichiometric amounts of the antigen, strongly suggests that the antigen recognizes the rV antigen dimer. The mass spectra do not contain any evidence for either one or four molecules of rV antigen binding to the antibody. The structure of an IgG could accommodate two molecules of rV antigen on the antigen binding site variable regions of the molecule or the antibody could bind to the dimer. Therefore, taking into account the size of the dimer molecule, it is probable that it binds between to the two variable and constant regions of each antibody arm.

From a practical point of view, even with the extended mass-to-charge ranges made available with Tof analyzers, without the use of collisional cooling, large macromolecular assemblies are not observed. This is presumably as a result of the large amount of kinetic energy associated with macromolecular assemblies with high charge states (typically +25 to +30). Minimizing these explosive forces and reducing the ions translational energy by a large number of low energy collisions maintains the integrity of the ions as they travel through the instrument to the detector. In combination, these MS developments lead to the possibility of probing protein complexes, such as the antibody-antigen complexes described here. The difficulties in defining molecular weight differences associated with the binding of antigens to intact antibodies makes the precision afforded

by this MS approach an attractive possibility for probing the interactions of this somewhat intractable class of molecules.

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