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# RESEARCH ARTICLES

## Dissection of Multi-Protein Complexes Using Mass Spectrometry: Subunit Interactions in Transthyretin and Retinol-Binding Protein Complexes

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***ABSTRACT*** **Complexes formed between transthyretin and retinol-binding protein prevent loss of retinol from the body through glomerular filtration. The interactions between these proteins have been examined by electrospray ionization combined with time-of-flight mass analysis. Conditions were found whereby complexes of these proteins, containing from four to six protein molecules with up to two ligands, are preserved in the gas phase. Analysis of the mass spectra of these multimeric species gives the overall stoichiometry of the protein subunits and provides estimates for solution dissociation constants of 1.9 ± 1.0 × $10^{-7}$ M for the first and 3.5 ± 1.0 × $10^{-5}$ M for the second retinol-binding protein molecule bound to a transthyretin tetramer. Dissociation of these protein assemblies within the gas phase of the mass spectrometer shows that each retinol-binding protein molecule interacts with three transthyretin molecules. Mass spectral analysis illustrates not only a correlation with solution behavior and crystallographic data of a closely related protein complex but also exemplifies a general method for analysis of multi-protein assemblies. Proteins Suppl. 2:3–11, 1998.** 

**Key words: mass spectrometry; time-of-flight; nanoflow electrospray; transthyretin; retinol binding protein**

### INTRODUCTION

Assembly of molecules into complexes is a key aspect of control and regulation of biological processes. Many aspects of the formation and composition of multi-protein complexes are, however, difficult to define under physiological conditions. In plasma, retinol-binding protein (RBP) is the specific carrier of retinol (vitamin A) transporting it from storage sites in the liver to receptors on the surface of its target cells.[1] A second serum protein, transthyretin (TTR) involved in the plasma transport of thyroid hormones, forms a complex with RBP.[1,2] Complex formation by RBP and transthyretin is thought to prevent filtration in the glomerulus of both the RBP and bound retinol.[3] TTR:RBP complexes have been identified in a variety of species including human[1,2] and chicken.[4] The retinol-binding proteins and transthyretins of these species have molecular masses of 21kDa and 55kDa respectively; the transthyretins being composed of four identical subunits. The binding affinities of TTR:RBP complexes (typically 1.1 × $10^{-7}$ to 1.5 × $10^{-7}$ M) are essentially the same within species and across species in chimeric complexes.[5] Early studies of the protein assemblies, however, presented conflicting results regarding the stoichiometry, showing both one[1,2,3,6] and four[7] molecules of RBP in complex with the transthyretin tetramer. Subsequent studies involving gel filtration, electrophoresis, and circular dichroism reported that one mole of human transthyretin binds up to a maximum of 1.35 molar equivalents of RBP using protein concentrations up to 20mg per ml and at pH 7.0.[8] Fluorescence polarization studies, under different experimental conditions, indicated that less than two molecules of human RBP and up to four molecules of chicken RBP were bound to human and chicken transthyretin respectively.[5] These results are intriguing given that transthyretin is one of the most strongly-conserved plasma proteins with trans-

*Abbreviations:* RBP, retinol binding protein; TTR, transthyretin tetramer; ttr, transthyretin monomer; m/z, mass to charge ratio

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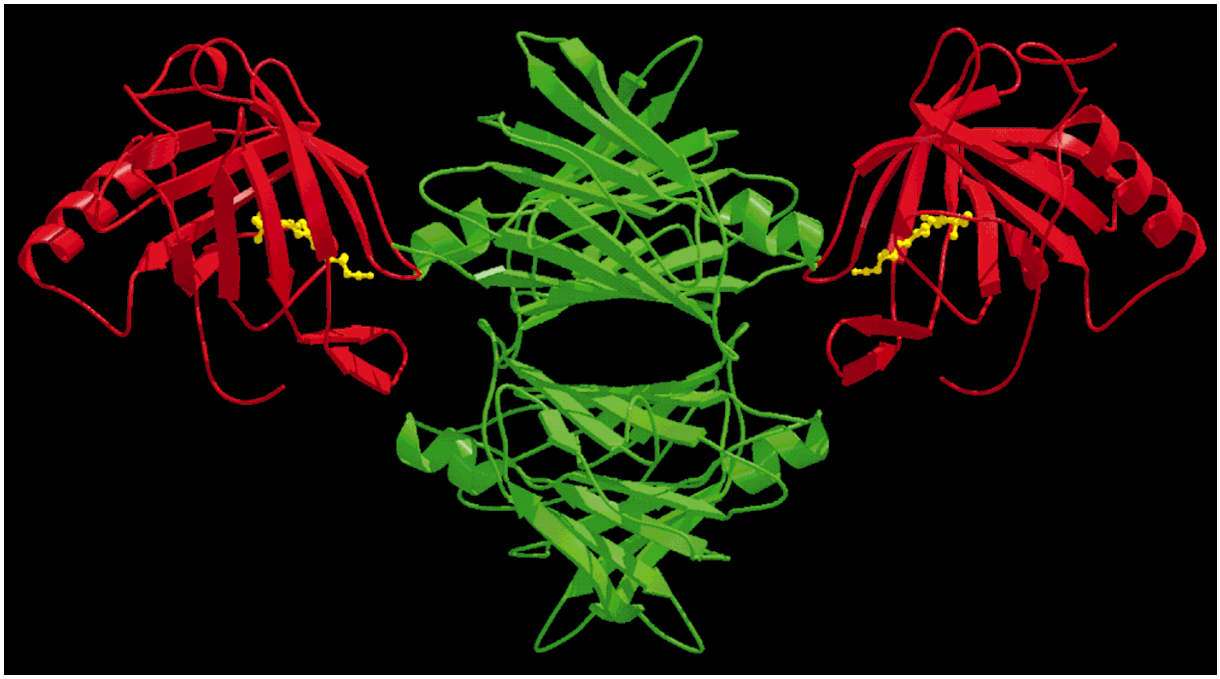

Fig. 1. A view of the human transthyretin:chicken RBP complex along the axis of the thyroxine binding channel in transthyretin. The transthyretin tetramer is shown in green, the RBP in red and the retinol ligand in a ball-and-stick representation in yellow. The co-ordinates are from the Brookhaven Data Bank, ID code 1RLB, and the figure produced with the programme MOLSCRIPT[35] adapted by R Esnouf[36] and Raster 3D[37].

thyretin from chicken showing 75% sequence identity with transthyretin from human sources.[9,10]

The X-ray structure of transthyretin reveals a largely β topology.[11] The protein is tetrameric, and involves two transthyretin dimers which associate through 4 loops to form a central channel in which thyroxine binds. The X-ray structure of RBP shows that the molecule has eight strands of antiparallel β-sheet and a short α-helical segment.[12,13] As well as the studies of the isolated molecules, the X-ray structure of a complex formed between human transthyretin and chicken RBP has been reported, Figure 1.[14] In the crystal structure of this chimeric complex, two retinol-binding protein molecules establish molecular interactions with the same transthyretin dimer and each also makes contact with one of the other monomers from the opposite dimer. This X-ray analysis reveals two equivalent binding sites for RBP on the transthyretin tetramer. These crystals were, however, grown with the proteins in a ratio of two molecules of RBP to one transthyretin tetramer. The normal concentration of RBP in plasma is about 2mM, and that of transthyretin tetramer about 4.5mM, the predominant complex *in vivo* is believed to consist of one molecule of RBP combined with one transthyretin tetramer.[7]

Here we show that recent developments in mass spectrometry can provide key information about large multi-protein complexes such as those formed between transthyretin and RBP. Despite the fact that mass spectrometry measures species in the gas phase, it is clear that, under the appropriate electrospray conditions, non-covalent features of protein structure relevant to the solution state are retained.[15] The survival in the gas phase of non-covalent complexes formed in solution has improved dramatically with the introduction of nanoflow electrospray ionization techniques.[16] During the conventional electrospray process solutions are infused at a flow rate of 10 μL/min, requiring high needle voltages and large volumes of gas to produce a stable spray and to effect the evaporation of droplets to produce ions. The low flow rates (typically 2–10 nL/min) and needle voltages associated with nanoflow sample introduction remove the need for large volumes of gas, therefore reducing the frequency of high-energy collisions with the gas phase protein assemblies and enhancing their survival. Large proteins analyzed from aqueous solutions at neutral pH, however, are often not sufficiently charged to lie within the mass-to-charge range of most commercial mass spectrometers, typically up to 3,000 or 4,000 m/z. This limitation has been overcome with the introduction of time-of-flight mass analyzers coupled with electrospray ionization.[17] Time-of-flight mass analyzers, although not a new technology, are more readily coupled with matrix-assisted laser desorption ionization since it is possible to synchronize the timing of the laser pulse with the time of flight.[18] The coupling of a continuous flow ionization method,

such as electrospray, is more difficult but is achieved using an orthogonal time-of-flight mass analyzer with gated ion flow.[19] In this arrangement, accelerator electrodes are pulsed to extract ions orthogonally from the continuous ion beam. In theory the mass range of time-of-flight analyzers is unlimited; in practice mass-to-charge ratios up to 20,000 have been reported.[20] Thus electrospray ionization coupled with orthogonal extraction time of flight mass analysis enables much larger proteins to be studied. In this study we show that the physiologically important complexes formed between chicken transthyretin and RBP can be maintained in the gas phase during mass spectrometry analysis providing new insight into the stoichiometry of the interacting proteins and the nature of the interactions between the component proteins.

## METHODS

### Preparation of the TTR:RBP Complex From Chicken Serum

Chicken *Gallus gallus* whole blood (8 l) was collected and allowed to clot at room temperature for 4 h, then left overnight at 4°C. Serum (4 l) was decanted, centrifuged at 16,000 g for 1 h and then dialyzed against 0.04 M Tris-HCl, pH 7.4 containing 0.5 M NaCl at 4°C. The serum was then loaded onto a column of Sepharose 4B coupled to human RBP. Human RBP had been purified from human urine as described previously.[26] The column was washed with the above buffer until all unbound protein had been eluted. Glass-distilled water was then used to elute the bound protein. The bound protein consisted of chicken transthyretin and also chicken transthyretin-RBP complex. These eluted from the column in the same fractions, and were dialyzed against 0.5 M Tris-HCl, pH 6.8, then separated in a non-denaturing preparative polyacrylamide gel using 0.375 M Tris-HCl, pH 8.9. The transthyretin fraction eluted before the transthyretin-RBP. The transthyretin-RBP complex was concentrated using Diaflo with a PM10 membrane (Amicon, Beverly, Massachusetts) at 40 psi $N_2$, then with a Centricon-10 ultrafiltration device (Amicon). The protein complex was stored at −70°C, then transported as 75% ammonium sulphate slurry. Immediately prior to analysis by electrospray mass spectrometry the sample was dialyzed against buffer and washed extensively, Centricon-10 (Amicon) at pH 7.0 in 10mM ammonium acetate buffer. The total protein concentration was determined using the Bicinchoninic acid Assay reagents (Pierce Chemical Company, Rockford, Illinois).

### Mass Spectrometry

Mass spectra were collected on a prototype ESI TOF spectrometer assembled at Micromass (Floats Road, Wythenshawe, Manchester). The spectrometer was equipped with a nanoflow electrospray ionization source and protein sample (1–2 μL) was introduced at a concentration of 35 μM in 10 mM ammonium acetate buffer, pH 7.0, from a gold-coated borosilicate needle with an internal diameter of 1–10 μm prepared in-house. Ions produced by the electrospray source are transferred to an orthogonal time-of-flight analyzer via two independently pumped hexapole lenses. On entry to the time-of-flight analyzer, the ions are accelerated to 5,300 V by a two-stage orthogonal acceleration pusher. A single stage reflectron then reflects the ions back to a dual microchannel plate detector. The effective instrument path length is 1.2 m, although the actual analyzer length is 0.7 m. An ion counting detection system incorporates a 1 GHz time-to-digital converter. Individual spectra are summed in a histogram memory, and the histogram spectrum is transferred to the host computer. The mass spectrometer was calibrated using equine myoglobin. The measured masses quoted represent the average masses of all the charge states in the spectrum. The standard deviations do not represent the broad nature of the high m/z peaks but arise from differences between the molecular masses calculated from individual charge states. Instrument control and data acquisition were carried out using the MassLynx (Micromass) data system. The charge states of the protein complexes were simulated using Gaussian distributions based upon the known isotopic content and measured peak width at half height. The simulations and integration of the electrospray peaks were performed in Sigma Plot (Jandell Scientific, Erkrath, Germany).

## RESULTS AND DISCUSSION

### Multi-Protein Complexes Are Maintained in the Gas Phase

A solution containing chicken transthyretin and RBP, at pH 7.0 in ammonium acetate, was analyzed by nanoflow electrospray time-of-flight mass spectrometry and the resulting spectrum is shown in Figure 2. The most intense series of peaks, between m/z 2,000–3,000, can be assigned directly on the basis of their mass-to-charge ratios, as unbound RBP with seven, eight and nine positive charges. Lower intensity charge states, attributable to the transthyretin monomer, with seven, eight and nine positive charges are observed below m/z 2,000. In addition to these peaks, a remarkable series of broad m/z peaks is clearly evident above m/z 3,000. These peaks must be due to non-covalent complexes formed from the component proteins which have survived the electrospray time-of-flight analysis. The broad nature of these peaks has been suggested to arise from a distribution of water molecules or other small molecules trapped within the protein assemblies.[21,22] These high mass species are, however, difficult to assign because uncertainty in their exact mass leads to an ambiguity in the identification of the individual charge states. To overcome this assignment problem,

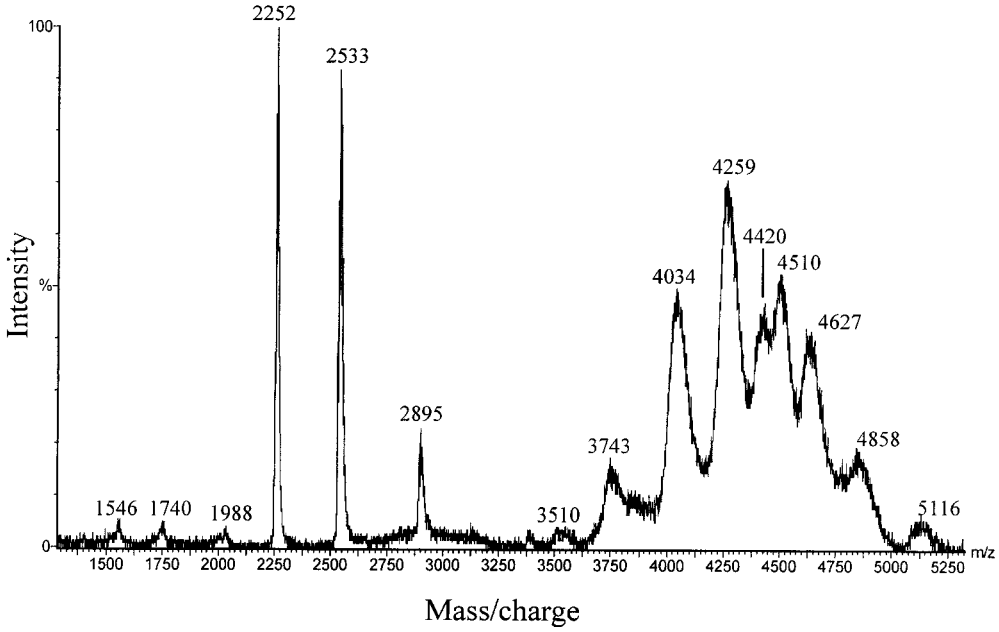


Fig. 2. Positive ion nanoflow electrospray mass spectrum of complexes purified from chicken plasma consisting of transthyretin and retinol-binding protein. The charge states series below m/z 3000 are assigned to *apo* retinol-binding protein and to transthyretin monomer. The spectrum was obtained with a cone voltage of 100V.

the isotopic composition of all possible combinations of transthyretin with up to four molecules of RBP and a distribution of water molecules from 0–100 was calculated. This simulation ruled out any contribution from TTR:3RBP:3retinol and TTR:4RBP:4retinol to the observed spectrum. The charge states simulated for the transthyretin tetramer, TTR:RBP:retinol and TTR:2RBP:2retinol, however, coincided with the diffuse peaks in the spectrum. Their relative proportions were adjusted until good agreement was found between simulation and experimental data, Figure 3. The number of water molecules used in the simulation was adjusted to coincide with the width of the peak. The distribution of water molecules defined by the simulation is 10 to 90, implying that under these mass spectrometry conditions a range of water molecules are present, presumably in the central channel. Examination of the crystal structure of transthyretin from chicken shows a large central channel which is 50 Å long and 80 Å in diameter.[23] Assuming an average volume for a water molecule (22.9 Å)[3,24] a cylindrical channel of this size can accommodate ~100 water molecules. The maximum of the distribution, however, is centered at 27 ± 3 water molecules suggesting that the channel is occupied to about one third of its total capacity. This analysis has been supported by a mass spectrometry study of human transthyretin where ligand binding experiments have shown that thyroxine binding in the central channel displaces 30 water molecules leading to narrower peaks and a reduction in the mass increase observed for the tetrameric species.[22]

The simulation of the various charge states for the complexes together with the existence of unbound RBP in the mass spectrum allows an estimate of the proportions of unbound and complexed proteins that were present in the solution. The proportion of protein present in a complexed state observed in the gas phase is, however, dependent upon a combination of experimental variables, including electrospray needle voltage, cone, and skimmer voltages. These variables affect the internal energy of the protein assemblies and hence will have a profound affect on the relative proportions of different species recorded in the mass spectrum. Careful examination of the experimental conditions affecting the integrity of the human transthyretin tetramer in the gas phase, however, has suggested that excellent agreement with known ultra-centrifugation data can be obtained by careful control of the mass spectrometric conditions.[22,25] Spectra were obtained under these conditions and the proportions of the various species were estimated by integration of the areas under the simulated charge states. From the known protein concentrations, and assuming that the equilibrium in solution is not disturbed by the nanoflow process, the solution dissociation constants of the complexes can be estimated. This procedure results in a Kd of $1.9 \pm 1 \times 10^{-7}$ M for TTR:RBP, in close agreement with the solution Kd reported for chicken TTR:RBP:retinol complex, $1.1–1.6 \times 10^{-7}$ M,[5] and from bovine sources $0.34 \pm 0.02 \times 10^{-6}$ M.[13] A value of the Kd for transthyretin:2RBP:2retinol has not been reported but is estimated here to be $3.51 \times 10^{-5}$ M. This value

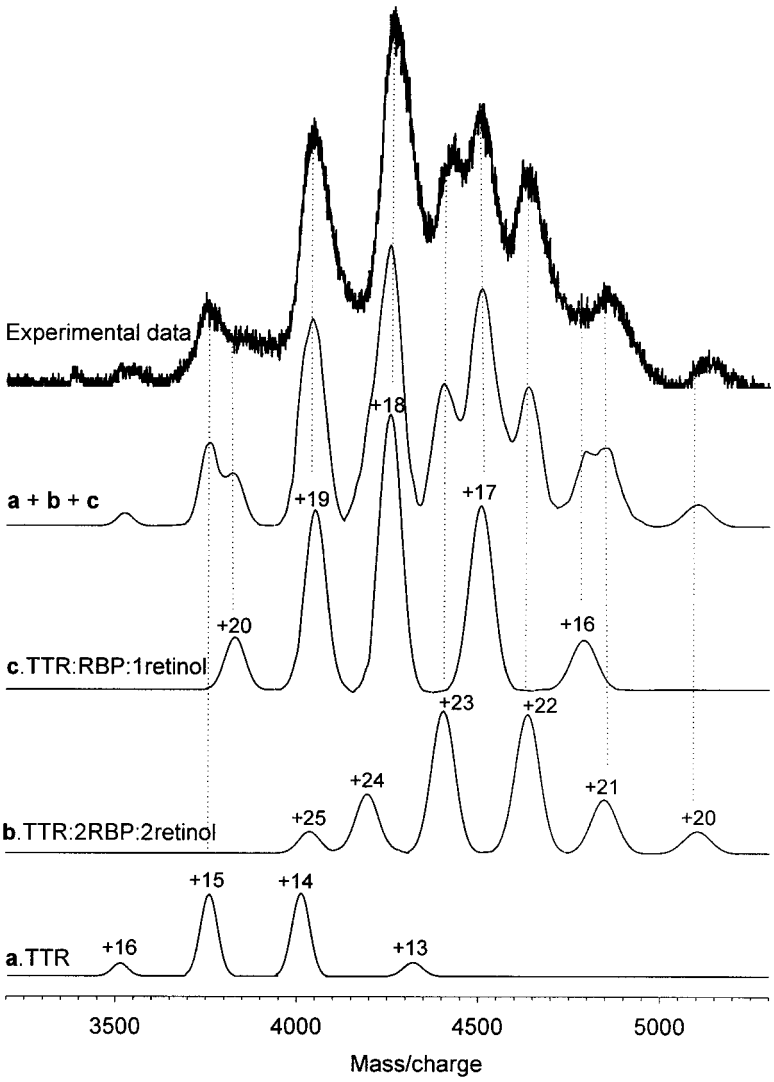


Fig. 3. Comparison of the experimental data with simulation of the charge states for the component protein complexes. Individual charge stage simulations for transthyretin tetramer **(a)**, TTR:2RBP with two retinol molecules **(b)** and TTR:RBP with single retinol **(c)** were summed to give a simulation, similar to that observed in the experimental data. Minor discrepancies arise since the simulation employs a Gaussian distribution centered around the average isotopic composition while the experimental data are more accurately described by a binomial distribution[38]. Furthermore no attempt has been made to model the asymmetry of the peak arising from salt adducts. The simulations were carried out in Sigma Plot (Jandell Scientific). Good agreement was found between simulation and experiment assuming an average of 27 water molecules in the transthyretin tetramer and in complexes with RBP.

supports the proposal that binding of the first RBP molecule induces negative co-operative effects, decreasing the affinity towards the second RBP in both human and chicken systems.[5] Further support for the correlation between solution behavior and gas phase measurements in these complexes comes from the ratio of the proportion of RBP molecules to transthyretin tetramer, defined by the simulation, as 1.28:1 ± 0.1, in close agreement with the finding from solution studies of the human complex where the molar ratio of RBP to transthyretin tetramer was found to be 1.35:1 under similar conditions.[26]

### The Nature of the Interactions

The relationship between the observed charge state of a protein in the electrospray mass spectrum and the amino acid composition of the protein depends on the interplay of many different factors. These include the pH and ionic strength of the solution from which the protein is analyzed,[27] the protein structure,[28] the counter ions present[29] and the cone and needle voltages set in the electrospray interface. The amino acid composition of chicken transthyretin gives a total of 17 acidic residues including the C-terminus, and 18 basic groups including the N-terminus per monomer. Thus at neutral pH the overall charge on the protein will be +1. Given the observed charges on the monomeric form of transthyretin (+7, +8, and +9), the charge state of the protein is affected by the electrospray conditions. Under positive ion electrospray conditions a voltage (typically +1 kV) is applied to the electrospray needle containing the protein solution, causing positively-charged solvent ions $H_3O^+$ and $NH_4^+$ to flow out of the tip of the needle together with more highly-charged protein molecules as a result of proton attachment.[30] Since both transthyretin monomer and tetramer are observed within the same spectrum, and thus under identical solution and instrument settings, charge states ranging from +28 to

**TABLE I. Measured and Calculated Masses and Charge States for Monomeric and Complexed Proteins†**

| Protein | Cone voltage (V) | Measured mass (Da) | Calculated mass (Da) | Predicted charge states | Observed charge states |
|---|---|---|---|---|---|
| Transthyretin monomer | 100 | 13910 ± 2 | 13904 | +1 | +7, +8, +9 |
| Transthyretin tetramer | 100 | 56105 ± 20 | 56102 | +28, +36 | +15, +16 |
| apo Retinol binding protein | 100 | 20260 ± 2 | 20253 | +2 | +7, +8, +9 |
| TTR:RBP:retinol | 100 | 76650 ± 23 | 76641 | +22, +23, +24, +25 | +17, +18, +19 |
| TTR:2RBP:2retinol | 100 | 97200 ± 30 | 97180 | +24, +25, +26, +27, +28 | +19, +20, +21, +22 |
| TTR:RBP:retinol | 150 | 76667 ± 26 | 76641 | | +15, +16, +17, +18, +19 |
| 3ttr:RBP:retinol | 150 | 62504 ± 7 | 62505 | | +9, +10, +11, +12, +13 |
| 2ttr:RBP:retinol | 150 | 48607 ± 12 | 48603 | | +8, +9, +10, +11, +12 |

†Predicted charge states were calculated from the number of basic and acidic amino acid residues in monomeric transthyretin[10] and RBP.[32] The predicted charge states of the protein complexes were calculated from the observed charge states for the appropriate monomeric protein and their complexes. The calculated masses of the protein complexes were calculated from the isotopic composition of the component proteins with 27 water molecules. The masses calculated for the 3ttr:RBP:retinol and 2ttr:RBP:retinol, however, were in agreement with the isotopic composition of the component proteins with an average of 14 water molecules. This is consistent with the loss of 13 water molecules, presumably due to disruption of the central channel under the high-energy conditions used to obtain this spectrum.

+36 might be predicted by simple multiplication of the +7, +8, and +9 charge states observed for the monomer. However the predominant charge states for the transthyretin tetramer are +15 and +16 suggesting that 13–20 charges are lost from the component monomers when the tetramer is formed. In the transthyretin tetramer the BC loops are composed almost entirely of charged side chains (10 charged residues from each monomer) located in a long narrow array that forms a two-dimensional network of balancing charge.[11] Since the basic and acidic residues are presumably protonated in the monomer under the electrospray conditions, ionic interactions in the tetramer lead to a change in the overall charge. Thus a maximum of 20 basic sites could be involved in ion pairing interactions in the tetramer. This may provide an explanation of the observed loss of 13–20 positive charges from the component monomers.

The changes in charge states that occur upon binding one molecule of RBP to the transthyretin tetramer were examined in a similar manner, Table I. The observed charge states of the 1:1 complex suggest that five or six positively-charged residues are involved in ion pairing interactions between the RBP and transthyretin tetramer. Since binding of retinol to RBP involves hydrophobic burial of the uncharged retinol molecule within RBP, and as the small conformational change associated with binding is unlikely to result in any changes in the burial of charged residues,[31] the observed changes must arise from interactions between RBP and transthyretin. By analogy with the chimeric complex and from the known X-ray structure of transthyretin[23] and amino acid sequence of chicken RBP,[32] it is predicted that the number and nature of interactions are essentially the same as those defined in the chimeric complex.[33] Five basic residues are implicated in the proposed model and the changes in the charge states observed suggest that an average of five ionic interactions are involved in the TTR:RBP complex. Further support for these ionic interactions comes from the change in charge states upon binding the second RBP to TTR:RBP. The observed charges on the complex suggest that five or six ionic interactions are formed between transthyretin and the second RBP molecule. Because the observed charges on the individual proteins do not correlate with the predicted charges, the changes in charge states associated with assembly provide insight into the nature and number of interactions. Binding of one and two RBP molecules to transthyretin, for example, involves the same number of charged residues, the five and six predicted from this analysis are in close agreement with those predicted from interactions in the chimeric complex.[14]

### Mapping Subunits Within the Multi-Protein Complex

The fact that these multi-protein complexes are maintained in the gas phase of a mass spectrometer provides an opportunity to explore their intersubunit contacts. Increasing the energy of collisions with residual gas molecules in the electrospray interface gives rise to a markedly different electrospray mass spectrum from that obtained under conditions chosen to mimic solution phenomena, Figure 4. The low m/z charge state series now dominate while the intensities of the broad peaks at higher m/z are significantly reduced. The peaks below m/z 3,500 correspond to *apo* RBP and monomeric transthyretin suggesting, in common with previous experiments, that high energy collisions are sufficient to disrupt the transthyretin tetramer [22] and to increase the proportion of unbound RBP in the gas phase of the mass spectrometer. A series of much weaker high m/z species (m/z 4,000–7,000) is also generated, extending beyond the m/z of the protein complexes (m/z 3,500–5,500). These must arise from species carrying fewer charges than the

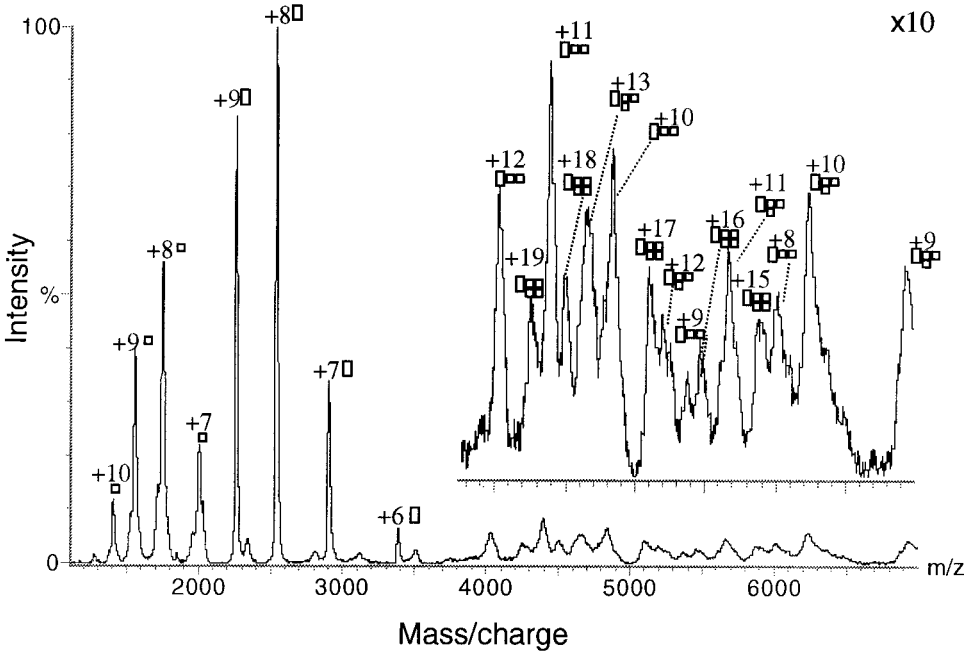


Fig. 4. Electrospray mass spectrum of the chicken plasma protein complexes under high energy conditions. The monomeric species occur below m/z 3000. Ions occurring above m/z 3000 have been assigned to subunits of the protein complexes and reveal the important interactions that stabilize the protein complexes. The identify of the high m/z ions are RBP:3ttr, RBP:2ttr, RBP:TTR, RBP and ttr. The mesured masses of these ions, table 1, are consistent with loss of 14 water molecules or $NH_4^+$ ions from both RBP:2ttr and RBP:3ttr. The mass of RBP:TTR is closely similar to that determined for the intact complex under the low energy conditions, figure 3. The standard deviations arise from mass measurements of all the charge states shown in the figure. The spectrum was obtained with a cone voltage of 150V.

protein complexes or from larger protein assemblies. Since it is unlikely that high-energy collisions would lead to the formation of larger assemblies, these charge states must arise from species carrying fewer charges. Analysis of the spacing between the peaks confirms that they correspond in mass to varying numbers of protein subunits, and reveals that the most intense series of peaks correspond to 3ttr:1RBP:1retinol. The second highest intensity peaks correspond to 2ttr:1RBP:1retinol and a lower-intensity series correspond in mass to TTR:1RBP:1retinol. The change in the charge states observed for TTR:1RBP:1retinol under the conditions chosen to mimic solution and under high energy collisions suggests that high-energy collisions not only disrupt the multi-protein complexes but also extend the charge state series to include lower-charge states, see Table I. This suggests, therefore, that the protein complexes which have undergone high-energy collisions with residual gas molecules have lost charge, presumably by proton transfer to water molecules present in the nitrogen collision gas. This phenomenon has been reported in the study of multiply-charged protein ions undergoing collisions with basic gas molecules.[34]

Analysis of the protein subunit complexes which survive the high-energy collisions reveals a number of important features of these assemblies. Firstly each RBP molecule is in contact with up to three transthyretin monomers and secondly no transthyretin dimer or trimer is observed in the absence of RBP. Furthermore, the fact that no species containing two RBP molecules with transthyretin monomers could be detected is in accord with the X-ray structure which shows that RBP interacts with different transthyretin monomers. Moreover, each RBP makes contact with up to three transthyretin molecules, consistent with the X-ray structure of the chimeric complex[14] where one transthyretin dimer and one of the monomers from the opposite dimer interacts with one RBP molecule. A comparison of the measured masses with those predicted for loss of the transthyretin monomers from the complexes shows that water molecules are lost from the assembly during the high-energy collisions which disrupt the complex. This is in agreement with the proposal that the transthyretin channel is partially occupied with solvent, even in the gas phase, hence disruption of the transthyretin tetramer leads to loss of the mass associated with water.

Comparison of the dimensions of the symmetrical transthyretin tetramer (55 Å × 70 Å)[11] with those of the asymmetric 3ttr:RBP:retinol complex (90 Å × 70 Å)[31] suggests that the more compact transthyretin tetramer would be more likely to survive high-energy collisions. These results suggest, therefore,

that the interactions in the complex are the dominant factor in determining the survival of the protein subunit complexes rather than the cross section of the protein complex. This demonstration, that gas-phase dissociation reveals the interactions in the multi-protein complex, is compelling and suggests a general method for the analysis of such complexes by varying the energy of gas-phase collisions and monitoring the subunit composition of the ions formed.

## CONCLUSIONS

Many features of protein interactions are exemplified by this mass spectral analysis of the physiologically important complexes of transthyretin and RBP. The fact that these species survive in the gas phase of the mass spectrometer and can be assigned by simulation of the overlapping charge states enables an evaluation of solution phenomena, such as an estimate of Kd measurements and non-cooperativity of binding. Comparison of the charge states of the monomeric proteins with those of the complexed proteins suggests the number of ionic interactions made by the protein subunits. The opportunity to probe subunit contacts by increasing the energy of collisions to induce dissociation of protein complexes allows the overall architecture of the assembly to be examined. This detailed information from an electrospray time-of-flight mass spectrum, coupled with the minimal sample requirements (1–2 µL of 5 µM solution) and tolerance of mixtures, ensures that mass spectrometry will play a significant role in the structure elucidation of protein assemblies, especially those which currently lie beyond the reach of the structural biologist's armory.

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