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Mass Spectrometric Analyses of the Activation Products of the Third Component of Complement

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I. Introduction

The complement system constitutes an immune defense mechanism that originated early in evolutionary history. The system is comprised of a collection of effector and control proteins that interact in a highly intricate and specific manner that leads to both proteolytic fragmentation and association of proteins that provide known effector functions of humoral immunity and inflammation in addition to other not yet defined biological functions. Of the 25 or so proteins that comprise the complement system , one protein in particular plays a central role, the third component identified known as C3, a 187 kD glycoprotein (for reviews, see Refs. 1 and 2). This protein is synthesized as a single chain and is posttranslationally cleaved into two chains, alpha (α) and beta (β), that are disulfide bonded through a single linkage between Cys^{816} of the α and Cys^{559} of the β. In connection with the immunobiological role of C3, the protein is remarkable in that the 187 kD form is relatively inactive and acquires a diverse multifuntionality expressed through specific ligand binding upon further specific fragmentation by proteolysis. C3 also contains a unique internal thioester bond that becomes involved with covalent "fixations" to other molecules often resulting in "surface bound" C3 - derived species.

Specific proteolytic events and subsequent conformational change in the resulting C3-derived fragments appear to be the mechanism for the tight regulatory control that is necessary for this system to function properly. Biophysical methods employing spectral and scattering techniques using various chemical probes have revealed in low-resolution various fragment specific conformational states in the fluid phase (1). Immunological probing with monoclonal antibodies has been employed to probe conformational states in both surface bound fragment and fluid phase situations and has identified areas in higher resolution if the epitope for the monoclonal antibody has been defined (1). Although much information is known at the protein level about C3 and its fragments (1), a fine detail map of the structure at the protein level using protein chemistry techniques is not yet available because of its large molecular size. The deduced amino acid sequence of human C3 has been obtained (3) and has only been partially verified with protein chemistry methods. We have begun to employ mass spectrometry to explore the structures comprising the functional

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life-cycle of C3 at the protein level. The goal is to fully characterize the C3 molecule and its fragments at the protein level cataloging any post-translational covalent modifications using mass spectrometric methods. Here we reveal the results of our initial attempts to characterize C3 and its activation fragments. The initial endeavor also indicates that the mass spectrometric approach is also yielding information about the profound conformational changes that exist in this family of protein derivatives.

II. Experimental

A. Preparation of C3 and fragments

C3 was isolated from EDTA-plasma using precipitation and chromatographic methods as previously described (4). The final step of purification employs ion exchange chromatography on Mono-Q HR10. C3 fragments were generated "experimentally" (in vitro) using protease treatment: a mild trypsin treatment for the generation of C3b form C3 and an elastase treatment of purified C3 to generate C3c . C3dg was derived as described previously (5) from chromatographic isolation employing ion exchange and gel filtration from whole plasma that had been incubated for 6 days at 37°C in taking steps to eliminate plasmin activity. The final purification step employed reverse phase HPLC using a PE-ABI Model 130 microbore system and a Brownlee Aquapore BU-300 C4 (2 X 220 mm) column. Acetonitrile gradients in approximately 0.1% TFA were used for elution of components. Typically fragments are initially evaluated by SDS-PAGE and immunoblotting and bioassayed by methods described previously (6).

B. Disulfide Reductions

Reductions were performed with tributylphosphine (TBP) (7). In connection with MALDI-MS analysis proteins in the 10 to 50 μM range were diluted 1/10 in 0.05% ammonium bicarbonate. TBP dissolved in MeOH was added to either a final 200 μM or 2 mM concentration. Molar ratios of TBP to disulfide ranged from 3 to 300. When the molar ratio was increased several-fold using 2 mM TBP concentrations, no further changes in reduction patterns were observed. Reductions were allowed to proceed for at least 30 min at room temperature unless specified otherwise. When full and complete reduction was desired for isolation of the separate chains heat (100 °C for 3 min.) was applied.

C. Mass Spectrometry

Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS)

(8) was performed on a Fisons Instruments (Beverly, MA) VG TofSpec time-of-flight mass spectrometer (0.6 m flight tube) outfitted with a N_2 (337 nm) laser. Mass calibrations were established using BSA. Data were analyzed using Fisons Instruments OPUS software. Two matrices were used. For C3 and the C3b fragments 4 to 20 μM solutions of polypeptide in PBS were diluted in 1/5 in sinapic acid so that each target site contained sample in the 1 to 5 pmole range. For reduction studies and for higher sensitivity when needed 2-(4-hydroxyphenylazo)benzoic acid (HABA) (9) was the matrix of choice. Acceleration voltage was set to 29000 volts and the multichannel plate detector was set to 1900 volts.

Electrospray ionization mass spectrometry (ESI-MS) (10) was performed on a Fisons Instruments (Beverly, MA) VG Quattro triple quadrupole mass spectrometer outfitted with the manufacturer's electrospray source. Samples were diluted into 50% acetonitrile 1% formate in water and injected as a 10 μL bolus into a stream of matrix consisting of 50% acetonitrile, 1 or 5 mM in triethylamine (TEA) to permit a family of lower charge state distributions that is essential for analysis of large molecular weight proteins. Matrix flow was established at 12 μL/min using a microliter syringe pump. In most cases 10 scans were acquired and averaged. The instrument was calibrated using equine myoglobin and data was processed using Fisons Instruments MassLynx software.

III. Results

A. Mass Spectrometric Analysis of C3 and the activation fragments

The C3 molecule and various preparations of activation fragments were analyzed by MALDI-MS to survey whether the observed masses would approximate theoretical masses. Typical spectra obtained for the larger fragments C3 and C3b are shown in Fig. 1. The upper panel of Fig.1 is the mass spectral result showing the ion signals for +1, +2 and +3 charge states of C3. These charge states suggest an average mass of 186400 for C3 which is 0.5% lower than the theoretical mass of 187443 based on amino acid sequence deduced from cDNA sequence and average carbohydrate composition (11). The carbohydrate micro-heterogeneity has the potential to be an issue in the mass analysis of these proteins even though the percent carbohydrate is low 1.7 %. The observed mass is also within the experimental error arising from external calibration using BSA. Given this moderate level of uncertainty, we conclude cautiously that this preparation of C3 approximates the theoretical construct. It is of interest to note that an ion signal having a mass assignment of 8980 was observed in the low mass end of the mass spectrum as shown in the upper panel of Fig. 1. This ion signal could represent a minor amount of C3a that has been

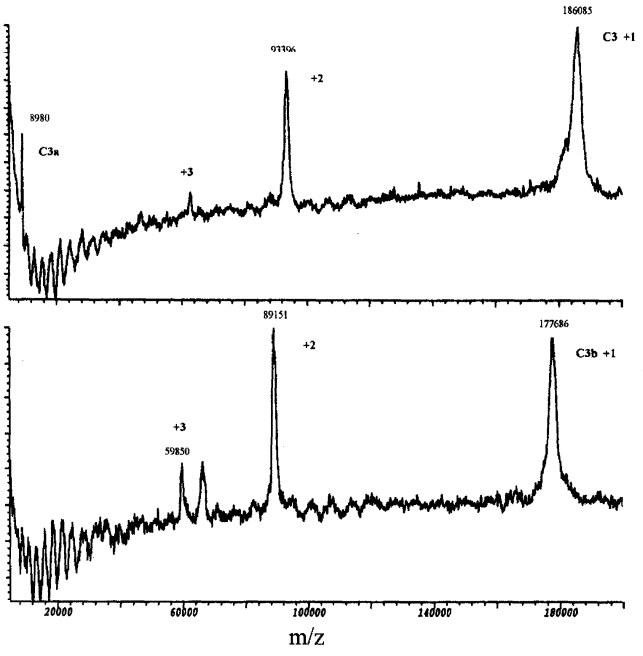

Figure 1. MALDI-MS analysis of C3 (upper panel) and C3b (lower panel)

generated by proteolysis of this intact C3 preparation. It should be noted that minor amounts of smaller molecular weight peptides may present as exaggerated signals in the MALDI-MS analyses of proteins and that the signal heights of the peptide signals do not quantitatively reflect the actual content. The lower panel of Fig. 1 shows the mass spectrum of C3b, the C3 activation fragment that arises from the proteolytic removal of the 9 kD C3a peptide from the N-terminal region of the alpha subunit of C3. The average mass for C3b deduced from the three charge states shown in the lower Panel of Fig. 1 is 178500 a value that agrees very well with that for the theoretical construct (178510.5). Again these results superficially suggest that the theoretical model of C3 developed over the years may be close to reality.

B. Disulfide reduction studies

To check the theoretical construct more stringently we chose to mass analyze the individual chains of C3 (2 interchain disulfide bonded polypeptides) and the subsequent activation products: C3c (3 interchain disulfide bonded polypeptides) and the single chain C3dg product. The rationale was that mass analysis of this collection of fragments would permit a more fine-detailed evaluation of most of the molecule. We chose tributylphosphine (TBP) because

it is a potent reducing agent working at nearly equimolar concentrations of disulfide. Micro-molar concentrations of TBP reagent were thought to minimally interfere with the MALDI-MS ionization process. It was feared that the mmolar concentrations of other reducing reagents such as DTT and β–mercaptoethanol would possibly interfere with MALDI-MS analysis and require a greater dilution of sample and subsequent loss of sensitivity. Before proceeding to C3 and C3 fragment analysis, the TBP reagent was used on purified rabbit IgG and bovine insulin and was found to be completely effective in reducing the disulfides that held the polypeptide chains and permitted MALDI-MS visualization of the respective chains (data not shown). The MALDI-MS analysis of the TBP reduction of C3 is shown in Fig. 2. The upper panel of Fig. 2 is the nonreduced control and the lower panel is the result for reduced material. TBP treatment resulted in highly modified spectrum that yielded ion signals that are consistent with the visualization of the two disulfide α and β bonded chains of C3. The predominant ion signals observed in the reduced material (Fig. 2, lower panel) were for the multi-charge state species for the β chain. An average mass of 72544 for the β chain was derived from the multicharge state family shown in the lower panel of Fig. 2. This value is within 0.1% of the theoretically derived mass (72630.8). The other major but relatively weaker ion signals in the spectrum are derived from the α chain, the +1 of which was mass assigned at 113959 (theoretical = 114813) and some

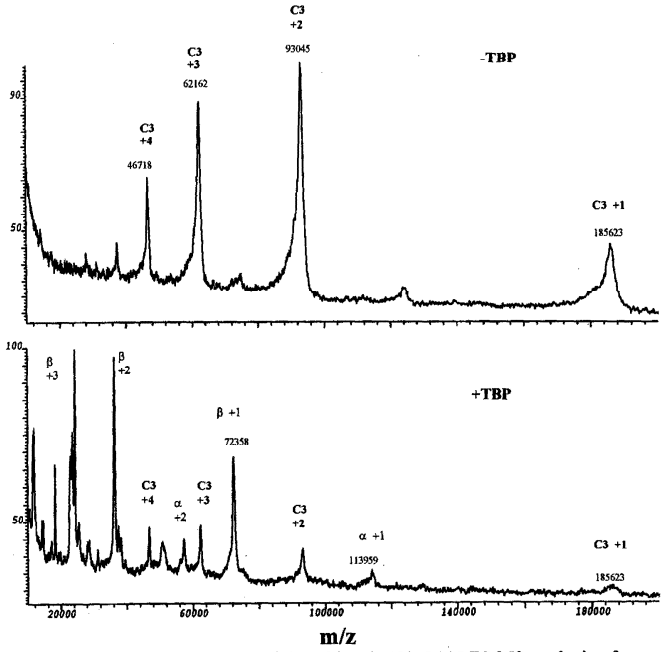

Figure 2. Reduction of C3 with Tributylphosphine (TBP). MALDI-MS analysis of nonreduced control (upper panel) and MALDI-MS analysis of TBP-reduced C3 (Lower panel).

unreduced C3 mass assigned at 186233. The poor peak shapes for both of these ions led to appropriate but less accurate mass assignments for these fragments.

To achieve an additional refinement in the mass analysis, an elastase-generated C3c preparation that had been characterized by N-terminal sequencing and subjected to an identical TBP treatment was examined with the expectation that the three chains would be observed. C3c is essentially a C3b molecule with the middle segment of the alpha chain (C3dg) removed. The three chains are held together by two disulfides. An N-terminal region of the alpha chain designated α_1 having a theoretical mass of 24533 is bonded to the C-terminal region of the alpha chain designated α_2 having a theoretical mass of 36305. The beta chain having a theoretical mass of 72630 is bonded to the α_1 chain through the disulfide formed between αCys^{816} and βCys^{559} major ion. The MALDI-MS results for the reduction of C3c are shown in Fig. 3. The non reduced control is presented in the upper panel of Fig. 3 and the spectrum for the TBP-treated material is presented in the lower panel. An unexpected and paradoxical result was achieved. No beta chain signal was obtained and signals having masses at 96 kD and 36 kD were observed indicating that the reduction was partial in this case. This result was the same for C3c treated with TBP at a low concentration of TBP (data not shown) as used in the C3 experiment (Fig. 2) and for a reduction experiment where nearly a hundred fold of the amount of TBP was employed as shown in the lower panel of Fig. 3. Only two ion signal

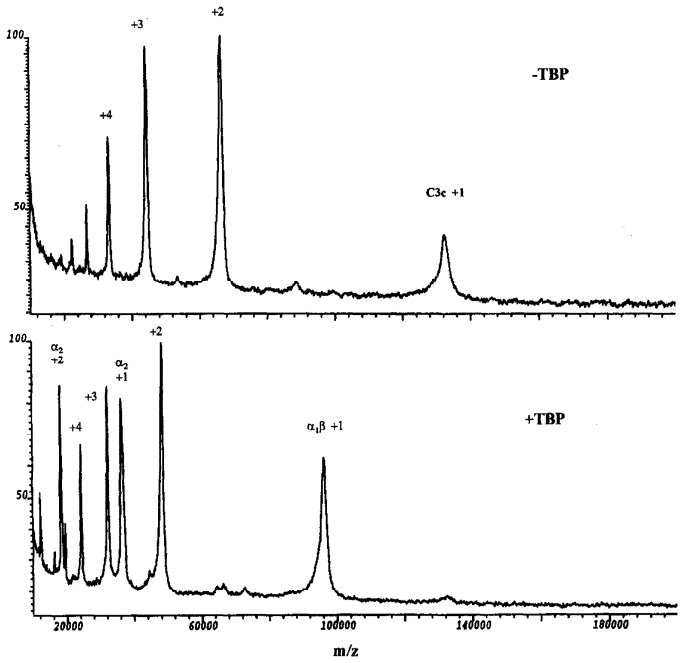

Figure 3. Reduction of C3c with Tributylphosphine (TBP). MALDI-MS analysis of nonreduced control (upper panel) and MALDI-MS analysis of TBP-reduced C3c (lower panel).

multicharge state families are evident; one for the 96188 representing the disulfide bonded $\alpha_1\beta$ species and the other 36430 species representing the α_2 species. The conclusion of these results are schematically portrayed in Fig. 4. There is a differential reactivity of the disulfide formed between αCys^{816} and βCys^{559} depending whether it exists in the C3 molecule or the C3c molecule. The suggestion is that there are extreme differences in conformation that exist in C3 and C3c that affect reagent access to this region of the molecule surrounding the αCys^{816}- βCys^{550} disulfide. We propose that coupling reduction with a mass spectrometric evaluation may be an additional approach to probing conformational states in this family of molecules as well as validating theoretically proposed structure at the protein level.

In order to test the resistance of this disulfide resistance further and to rule out a MALDI-MS related artifact, we analyzed C3c by ESI-MS. Although C3c is at the upper mass limit of this mass spectrometric method, the resulting reduced fragments would fall within. The ESI-MS analysis of the C3c reduction is shown in Fig. 5. Similar conclusions can be drawn from the ESI-MS data shown in Fig. 5. The ESI-MS pattern for untreated C3 as shown in the upper panel of Fig. 5 yielded a low quality multicharge state ion pattern that appears to arise from an ion having a mass of 138123, a value around that for C3c but much higher than expected and observed in MALDI-MS analysis (Fig. 3, upper panel). We believe this inaccuracy is related to the molecular weight of the protein being at or over the upper limit for this ionization technique. Treatment of this C3c preparation with TBP at room temperature resulted in a distinct change in the electrospray ion pattern as shown in the middle panel of Fig. 5. This family of charge states transformed or deconvoluted to a mass of 96606 a

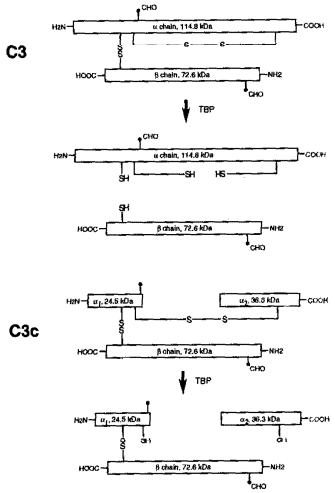

Figure 4. Schematic presentation of the differential reduction of αCys^{816}-βCys^{559} in C3 and C3c by TBP.

value consistent with that for the $\alpha_1\beta$ species observed in MALDI-MS (Fig. 2, lower panel). It is of interest to note that the 96.6 kD ion pattern is the dominant signal in the spectrum and that the 36kD signal MALDI-MS detectable (Fig. 2, lower panel) was not observed in the ESI-MS analysis (Fig. 5, middle panel) . If the TBP reduction is carried out in the presence of heat (100°C for 3 min) and the protein is electrosprayed (Fig. 5, bottom panel) another distinct multicharge state ion pattern is observed that yields a mass of 72697 upon transformation or deconvolution of the multicharge state data. Again the observed mass is the dominant species and is in very good agreement with the theoretical mass of the β chain (72630). This result indicates that with the addition of heat and subsequent thermal denaturation of the protein exposes the αCys^{816}- βCys^{559} disulfide to reagent and complete reduction is achieved as has been indicated by SDS-PAGE analysis after reduction with β–mercaptoethanol in the presence of SDS and heat (5). It is of interest to note that the MALDI-MS analysis gave a more complete picture of the reduction results than the electrospray analysis. However the electrospray result, although incomplete, fortuitously yielded spectra for pertinent fragments that permitted interpretation of the reduction of C3c at the two different temperatures. These results also represent a case where complex mixtures of proteins are more readily assessed by MALDI-MS than ESI-MS.

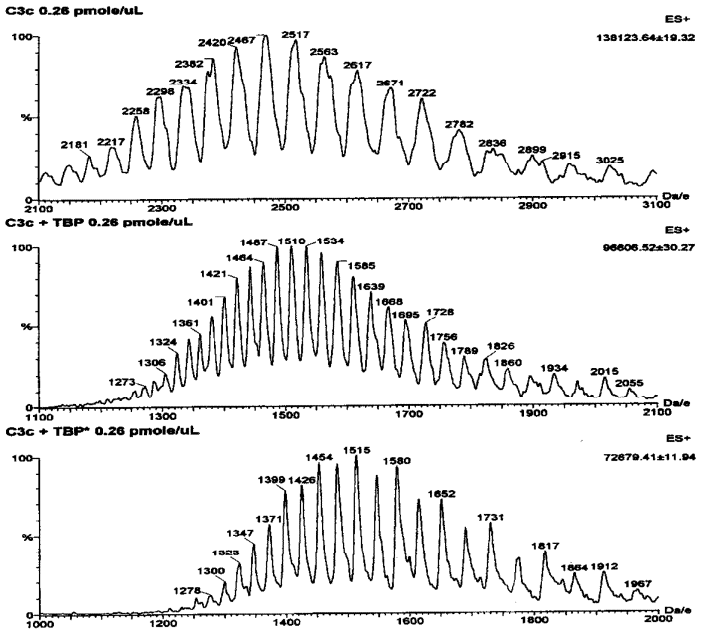

Figure 5. ESI-MS analysis of the TBP reduction of the C3c molecule. Nonreduced C3c control (upper panel); Room temperature TBP treatment of C3c (middle panel) and TBP treatment at 100°C/3 min (lower panel).

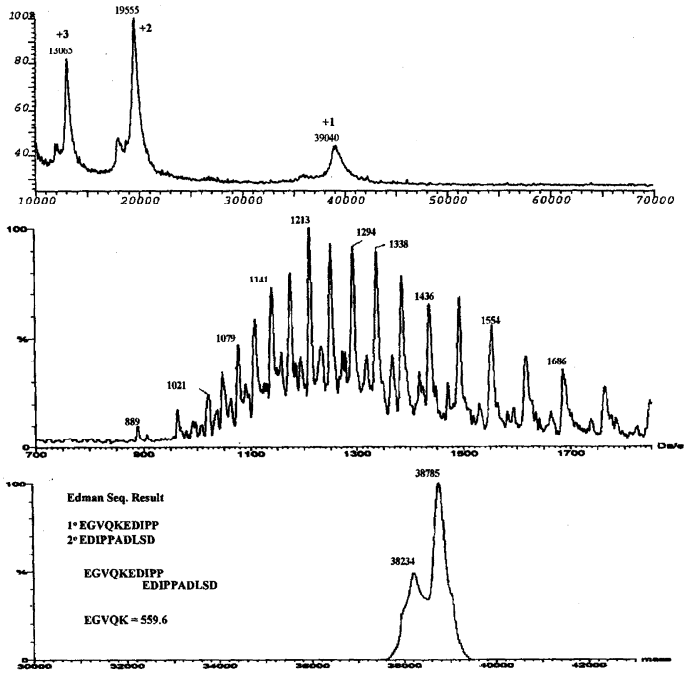

Figure 6. Mass spectrometric analysis of C3dg. MALDI-MS analysis of C3dg (upper panel); ESI-MS raw data for C3dg (middle panel) and deconvoluted or transformed raw data spectrum for C3dg (lower panel).

C. C3dg characterization

Our ultimate goal is to mass spectrometrically fine-map each fragment of C3 to survey and clarify the status of post translational modifications if any and to verify the theoretically proposed primary and secondary structure of the complete C3 molecule. In addition the physiological proteolytic processing sites need clarification as well as those created in the laboratory by enzyme treatment. Clarification of these are essential for structure-function relationship studies employing fragments. Along these lines we have characterized a "physiological" C3dg fragment that has been generated in plasma and isolated from plasma treated as described previously (5). The final product was obtained by fractionation over reverse phase column as described in the Experimental section. MALDI-MS and ESI-MS analyses of the RP-HPLC derived product are shown in Fig. 6. The upper panel of Fig. 6 indicates the MALDI-MS analysis of the C3dg preparation. The observed major ion signal mass assigns at 39000 in close agreement with the theoretical proposed mass of 38906. In the MALDI-MS analysis another minor signal was evident at 36000 (possibly C3d) that has not yet been identified or characterized. When this preparation was analyzed by ESI-MS as shown in the middle panel of Fig. 6, two sets of

multicharge state ion signals were observed. The major ion multicharge state pattern deconvoluted or transformed to a mass of 38785 and the secondary multicharge state pattern deconvoluted to a mass of 38235 as shown in the bottom panel of Fig. 6. The mass difference between these two ion signals is 547 u. The basis to this mass difference became apparent when the C3dg preparation was subjected to Edman sequencing. A primary and secondary sequence was found as shown in the bottom panel of Fig. 6 indicating that the minor species was truncated from the N-terminus by the first 5 residues accounting for the 547 mass difference in the two major ion signals observed in the ESI-MS spectrum. It is of interest to note that MALDI-MS did not reveal the presence of the species identified by Edman sequencing and ESI-MS.

IV. Conclusions

This preliminary study demonstrates that biological mass spectrometry offers complement immunologists and biochemists an additional protein chemsitry level perspective on the C3 problem and in general to large glycoproteins. MALDI-MS and ESI-MS analyses in a complementary sense along with other protein chemistry techniques provide a comprehensive and definitive evaluation of C3 molecules and the derived fragments. A fine-detail analysis of a C3 molecule or ligand at the protein chemistry level should now be addressable in a reasonable time frame. Employing reducing agents such as TBP to generate individual polypetide chains for mass spectrometric characterization led to an interesting observation concerning one particular disulfide in the C3 molecule formed between αCys^{816} and βCys^{559}. The use of this reagent in conjunction with mass spectrometric techniques allows a probing of the conformational structure around this particular region in the molecule. The present data suggest that profound differences must exist around this disulfide in the C3 and C3c fragments.

Acknowledgments

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