Analysis of the Stoichiometry of the T4 Gene 45 Protein by Ion Spray Mass Spectrometry

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Abstract: The synthesis of bacteriophage T4 DNA is catalyzed by a complex of five proteins that includes the T4 DNA polymerase protein, one single-stranded DNA binding protein, and three T4-coded polymerase accessory proteins. The accessory proteins form two subassemblies, one consisting of a tightly-bound complex of gene 44 and 62 proteins and the other an oligomer of the gene 45 protein (product of gene 45, gp45). On the basis of equilibrium sedimentation, velocity sedimentation, and chemical cross-linking studies, aqueous gp45 was thought to exist as a noncovalent trimer at pH 7. However, independent studies employing a combination of gel filtration and sucrose gradient sedimentation suggested that gp45 exists as a dimer in solution. We report an investigation into the stoichiometry of gp45 association in solution using a combination of ion spray mass spectrometry (MS) and microbore size exclusion chromatography. In 10 mM NH_4OAc (pH 2.5), both homodimer and trimer were observed by ion spray MS. Further experiments on two representative gp45 dimer ions using tandem mass spectrometry confirmed their structures as noncovalent gas-phase association complexes. Judging from size exclusion chromatography, a gp45 homotrimer was the predominant species in 10 mM sodium phosphate at pH 6.8, although a corroborating mass spectrum could not be obtained at that pH.

Introduction

DNA synthesis on a primed DNA substrate by bacteriophage T4 can be reconstituted *in vitro* by an assembly of a core replication complex consisting of the T4 DNA polymerase (product of gene 43, gp43), a single-stranded DNA binding protein (gp32), and three accessory proteins (products of genes 44, 62, and 45). Termed the T4 DNA polymerase holoenzyme, this multiprotein assembly replicates DNA with greater efficiency, speed, and fidelity than the polymerase enzyme alone.[1] Because the accessory proteins confer unique structural and functional characteristics to the DNA polymerase, it is of interest to determine how they are incorporated into the overall complex. Several laboratories have established that the holoenzyme can be reconstituted from its individual parts with full activity, suggesting that closer scrutiny of the supporting proteins and their functional stoichiometry might provide insight into the overall replication process.

The accessory proteins in the holoenzyme form two subassemblies, one consisting of a tightly-bound complex of gene 44 and 62 proteins and the other an oligomer of the gene 45 protein (product of gene 45, gp45). Amino acid sequences of each accessory protein are known from DNA sequences, and average molecular weights (MWs) have been calculated. Jarvis *et al.*[2] have studied the stoichiometry of the gp45 complex using equilibrium sedimentation, velocity sedimentation, and chemical cross-linking studies. On the basis of these measurements, the apparent MW of gp45 was determined to be ca. 77 000 at pH 7. From the calculated MW of 24 710 Da for monomeric gp45,[3] it was concluded that aqueous gp45 existed as a noncovalent trimer at pH 7. However, independent studies by Morris *et al.*[4] and Rush *et al.*[5] employing a combination of gel filtration and sucrose gradient sedimentation concluded that gp45 existed as a dimer in solution. It should be noted that the sedimentation coefficient determined by Rush *et al.* was lower than that determined by Jarvis *et al.* and that buffers and temperatures differed significantly in the two investigations. Identifying the stoichiometry of gp45 association is important, however, since several new lines of evidence suggest a direct interaction between gp45 and the polymerase (gp43) on primer-template DNA.[6] The crystal structure of the β subunit in the *Escherichia coli* system (analogous to the T4 gp45) indicates that this protein is a dimer that encircles the DNA.[7] Because of the MW difference between the β subunit and gp45, gp45 would have to exist as a trimer to adopt the same topology.

Here we report a novel use of ion spray mass spectrometry (MS),[8] an analytical technique capable of detecting and identifying macromolecular noncovalent complexes,[9-11] to investigate the stoichiometry of gp45 association in solution. Our findings confirm previous reports that gp45 is largely dimeric at pH 2.5. In the absence of added salts or buffer, an apparent homodimer (previously observed by gel filtration[5] and now reconfirmed by microbore size exclusion chromatography) was characterized by

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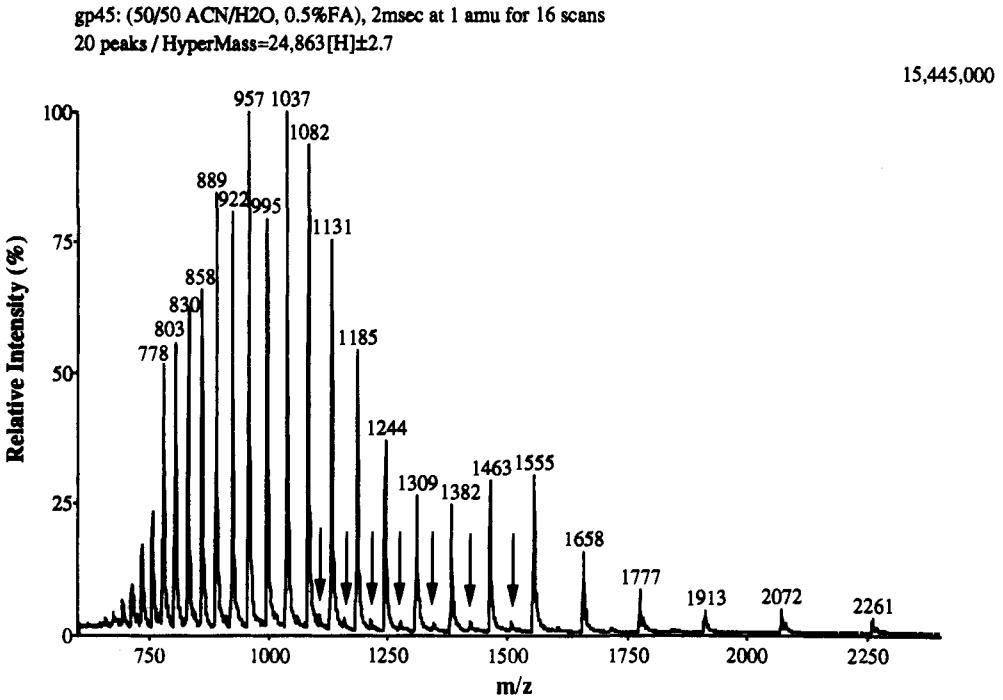

Figure 1. Ion spray mass spectrum of gp45 recorded on a Sciex TAGA 6000E atmospheric pressure ionization (API) triple quadrupole mass spectrometer. The spectrum was recorded by infusion of gp45 (1 μg/μL in 1:1 CH_3CN–H_2O containing 0.5% formic acid, pH 2.5) at 2 μL/min through the ion spray interface. Gas-phase ions were formed at atmospheric pressure by the electrospray process and sampled into the high vacuum region of the mass spectrometer. The spectrum is an averaged sum of 16 scans at a scan rate of 2 ms/scan.

ion spray MS. In 10 mM NH_4OAc (pH 2.5), both homodimer and trimer were observed by MS. Judging from size exclusion chromatography, a gp45 homotrimer was the predominant species in 10 mM sodium phosphate at pH 6.8, although a corroborating mass spectrum could not be obtained at that pH.

Experimental Procedures

Materials. T4 gene gp45 was overexpressed using the plasmid pTL45W, a generous gift of Dr. William Konigsberg, and purified according to the method described by Nossal.[12] Prior to all chromatographic and mass spectrometric measurements, gp45 was desalted three times at 5000 × *g* (2 °C) in a Sorvall Model RC2-B centrifuge (Norwalk, CT) using a Centricon-10 centrifuge microconcentrator having a cutoff MW of 10 000 Da (Amicon Division, W. R. Grace & Co., Beverly, MA).

Methods. Microbore size exclusion liquid chromatography was performed using BioSep columns purchased from Phenomenex (Torrence, CA). Studies at pH 2.5 were conducted using a 2.1 × 300 mm BIOSEP-SEC-S2000 column, while studies at pH 6.8 used a 4.6 × 300 mm BIOSEP-SEC-S3000 column. Separation of an authentic mixture of MW standards consisting of rabbit muscle phosphorylase B (97 400 Da), bovine serum albumin (66 200 Da), egg white ovalbumin (45 000 Da), bovine carbonic anhydrase (31 000 Da), soybean trypsin inhibitor (21 500 Da), and lysozyme (14 300 Da) was accomplished by isocratic size-exclusion HPLC using either 0.1% TFA (pH 2.5) or 50 mM sodium phosphate buffer (pH 6.8) as the mobile phase. With the relatively slow flow rates used (150 μL/min at pH 2.5; 200 μL/min at pH 6.8), a typical analysis time was 60 min. A 2.5-μL sample was injected directly into a Rheodyne Model 9125 injector (Cotati, CA) equipped with a 20-μL sample loop. A dual-syringe Brownlee Labs micro LC pump (Santa Clara, CA) was used as the mobile phase delivery system for these experiments. An Applied Biosystems Inc. (ABI, Foster City, CA) Model 783 Spectroflow absorbance detector equipped with a 2.4-μL flow cell was used to detect the peptides at a UV wavelength of 280 nm.

Ion spray mass spectrometry (MS) was performed using a Sciex (Thornhill, Ontario) TAGA 6000E atmospheric pressure ionization (API) triple quadrupole mass spectrometer (Thornhill, Ontario) upgraded to an API-III with an upper mass limit of 2400 Da. Samples of gp45 were dissolved in 1:1 acetonitrile–H_2O containing 0.5% by weight HCO_2H or in 1:1 acetonitrile–aqueous 5 mM NH_4OAc containing 0.5% by weight HCO_2H, or in 0.5% HCO_2H alone. Sample solutions were continuously introduced (flow rate = 2 μL/min) at ambient temperature via a Harvard Model 22 microsyringe infusion pump (South Natick, MA) for MS and tandem mass spectrometry (MS/MS) experiments. The ion spray interface was positioned off-axis about 1 cm away from the orifice and nitrogen was used as the nebulizing gas at 55–60 psi. Spraying was achieved at room temperature via charging the spray needle at 3.6 kV. Polypropylene glycol (PPG) in 80/20 CH_3CN–H_2O (3 mM NH_4OAc) was used to calibrate each mass-resolving quadrupole (Q_1 and Q_3). All experiments were performed at a declustering potential of 30 V. The collision gas (argon) was introduced into the collision cell (Q_2) for the MS/MS experiments and the listed collision energies (laboratory frame) were used for all collision-induced dissociation (CID) experiments. Mass spectra were obtained at a dwell time of 2 s per scan with 10 scans summed.

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Results

Before attempting to determine the state of aggregation of gp45, it was of interest to confirm the purity of the protein and establish its monomer MW by MS. The full-scan ion spray mass spectrum of gp45 at pH 2.5 (Figure 1) displayed a nested series of multiply-protonated parent ions ranging in charge state from 11^+ to 36^+. Two overlapping Gaussian distributions of charge states were evident, suggesting the presence of two discrete conformations or folded states of the protein. Analysis of the spectral data confirmed the presence of a single polypeptide, having an average MW of 24 863 ± 2.7 Da, as computed from 20 independent MW calculations using individual gas-phase multiply-protonated, multiply-charged parent ions. The high internal consistency of this data set was typical of MW determinations by ion spray MS. However, the value obtained by MS differed significantly both from the published value of 24 710 Da[3] (deduced from the corresponding DNA sequence of gene 45) and from the average MW of 24 745 Da (calculated from the published amino acid sequence). The discrepancies (153 ± 3 or 118 ± 3 Da, respectively) between observed and calculated MWs may be due to a single amino acid deletion or, in the case of the larger discrepancy, to two post-translational phosphorylations.

Detection of gp45 Dimers and Trimers by Microbore Size Exclusion Chromatography. Purified gp45 was first analyzed by microbore size exclusion chromatography at pH 2.5 in the absence of salt or buffer (Figure 2A). Three peaks were evident, with

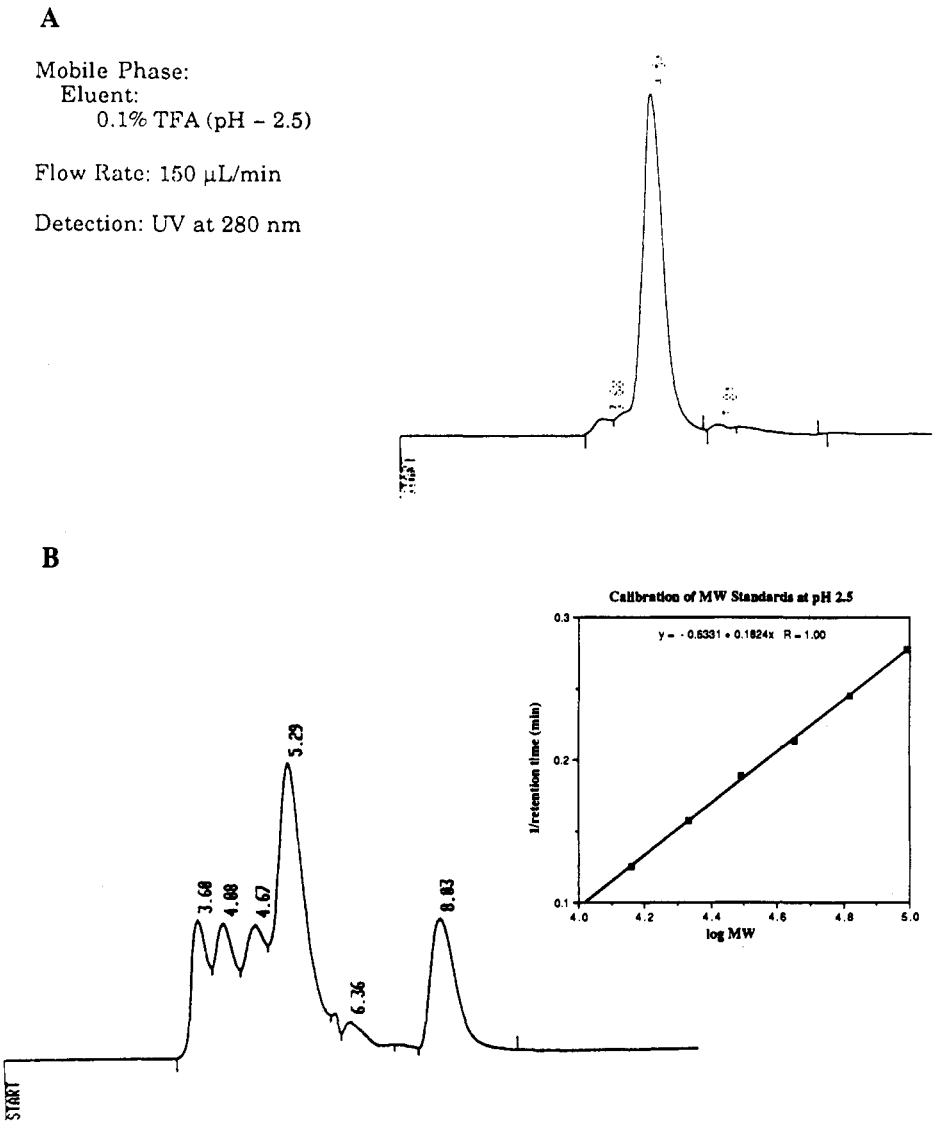

Figure 2. Microbore size exclusion liquid chromatography at pH 2.5 using a 2.1 × 300 mm BIOSEP-SEC-S2000 column. The mobile phase consisted of 0.1% trifluoroacetic acid, at a flow rate of 150 μL/min: (A) gp45 and (B) MW standards—rabbit muscle phosphorylase B (97 400 Da, 3.60 min), bovine serum albumin (66 200 Da, 4.08 min), egg white ovalbumin (45 000 Da, 4.67 min), bovine carbonic anhydrase (31 000 Da, 5.29), soybean trypsin inhibitor (21 500 Da, 6.36 min), and lysozyme (14 300 Da, 8.03 min). Inset: MW calibration plot of (retention time)$^{-1}$ versus log MW for the standards in part B.

two minor components flanking a dominant fraction eluting at 4.54 min. The same column and solvent conditions were used to elute six reference proteins of known MW, first individually and then as a mixed sample (Figure 2B). A calibration plot of log MW versus (retention time)$^{-1}$ revealed good linear behavior over the MW range studied (Figure 2B, inset) and was used to estimate the MW of each component in the gp45 chromatogram. The predominant peak at 4.54 min corresponded to a protein of MW 47 000 Da and was consistent with the dimeric form of gp45 at pH 2.5

Smaller, weakly-resolved components eluting at 3.68 and 5.81 min in Figure 2A were consistent with trimer and monomer, respectively, at pH 2.5. The apparent MW of gp45 monomer (26 000 Da) was in good agreement with the value determined by MS. The apparent gp45 trimer (91 000 Da, based on the calibration in Figure 2B) was significantly larger than the calculated MW, although macromolecular weights cannot be unambiguously determined without shape information. The observed value was in fair agreement with earlier gel filtration results (MW 82 000 Da)[5] and was consistent with cross-linking studies on the trimer which revealed an apparent molecular mass of 89 000 Da as determined on a denaturing gel.[2]

Since equilibrium ultracentrifugation studies by Jarvis *et al.*[2] suggested that gp45 self-associated predominantly to a trimer under near-neutral conditions (25 mM Tris–HCl, pH 7.4), it was of interest to study the effect of pH on purified gp45 by microbore size exclusion chromatography. When analyzed on a BIOSEP-SEC-S3000 column at pH 6.8 buffer (10 mM Na_2HPO_4, Figure 3A), purified gp45 displayed one major peak having a retention time of 14.61 min, along with several minor components which flanked the dominant component (Figure 3A). The same column and solvent conditions were used to elute five reference proteins of known MW, first individually and then as a mixed sample (Figure 3B; for practical purposes phosphorylase B was eluted separately at a retention time of 14.51 min). The calibration plot in this instance was nonlinear, with steep curvature in the high MW domain (Figure 3B, inset). Nevertheless, interpolation could be used to establish an approximate MW of 84 000 Da for the major component in the gp45 chromatogram.

The apparent MW of gp45 trimer, when measured by size exclusion chromatography, was larger than expected. However, it should again be noted that without corroborating sedimentation data, MW information based only on size exclusion gels can be ambiguous. The minor peak having a retention time of 17.70 min in Figure 3A probably corresponds to monomeric gp45, and there is no evidence of any dimeric gp45, which should be bracketed between ovalbumin (15.62 min) and BSA (14.90 min). Alternatively, one cannot rule out at least the possibility that the major

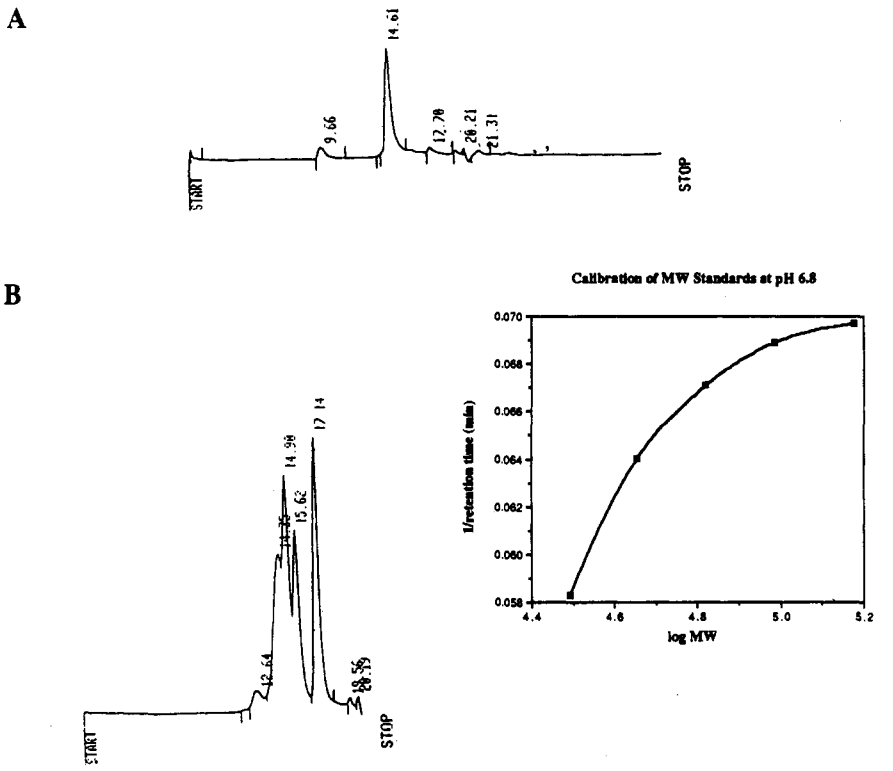

Figure 3. Size exclusion liquid chromatography at pH 6.8 using a 4.6 × 300 mm BIOSEP-SEC-S3000 column. The mobile phase consisted of 50 mM sodium phosphate buffer, at a flow rate of 200 μL/min: (A) gp45 and (B) MW standards—IgG (150 000 Da, 14.35 min), rabbit muscle phosphorylase B (97 400 Da, 14.51 min), bovine serum albumin (66 200 Da, 14.90 min), egg white ovalbumin (45 000 Da, 15.62 min), and bovine carbonic anhydrase (31 000 Da, 17.14 min). Inset: MW calibration plot of (retention time)$^{-1}$ versus log MW for the standards in part B.

peak at 14.61 min may represent gp45 dimer. However, this hypothesis would require a large change in shape between the dimeric species noted at pH 2.5 and a dimeric species at pH 6.8. We conclude from size exclusion chromatographic analysis that at lower pH, the dimeric form of this accessory protein predominates, while a homotrimer of gp45 likely becomes the major species near neutral pH.

Detection of gp45 Dimers and Trimers by MS. Our main interest was to ascertain whether the effect of pH on the stoichiometry of gp45 self-association might also be detected by ion spray MS. When ion spray and electrospray MS were first developed to analyze proteins, low pH was employed to ensure that the macromolecules were sufficiently protonated for MS. However, ion spray mass spectra of the FKBP-FK506 drug-receptor complex[9] and of noncovalent leucine zipper peptide dimers[11] were recently obtained under neutral conditions. The ability to detect a dimeric HIV protease-inhibitor ternary complex[13] further indicated that the mild ion evaporation conditions of ion spray MS were compatible with multisubunit protein analysis.

The differentiation of monomers from symmetrical dimers and trimers by MS must be approached with care. It should be noted that the observed mass-to-charge ratios in Figure 1 correspond not only to distinct, multiply-protonated, multiply-charged parent ions M^{n+} but also to symmetrical dimer $(2M)^{2n+}$ or trimer $(3M)^{3n+}$ ions composed of subunits in the same charge state ($n+$). In fact, the data presented in Figure 1 alone do not differentiate monomeric gp45 from *any* of its higher oligomers.

By contrast, an even number of charges on one gp45 peptide chain combined with an odd number of charges on the other would result in an odd-charged, gas-phase gp45 dimer ion $(2M)^{2n+1}$ whose mass-to-charge ratio would be readily distinguishable from that of a monomer. In other words, gas-phase ions of gp45 dimer possess additional, odd-charge states that are not degenerate with monomeric gp45 charge states. Likewise, gas-phase ions of gp45

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Table 1. Calculated Mass-to-Charge Ratios for Selected Monomers, Dimers, and Trimers of gp45[a]

m/z	MONOMER charge	DIMER charge	TRIMER charge
2073.5	12+	24+	36+
2017.5	--	--	37+
1990.6	--	25+	--
1964.4	--	--	38+
1914.1	13+	26+	39+
1866.2	--	--	40+
1843.0	--	27+	--
1820.8	--	--	41+
1777.4	14+	28+	42+
1736.1	--	--	43+
1716.0	--	29+	--
1696.7	--	--	44+
1659.0	15+	30+	45+
1622.9	--	--	46+
1605.5	--	31+	--
1588.5	--	--	47+
1555.4	16+	32+	48+
1523.7	--	--	49+
1508.0	--	33+	--
1493.2	--	--	50+
1463.9	17+	34+	51+
1435.8	--	--	52+
1422.0	--	35+	--
1408.7	--	--	53+
1382.7	18+	36+	54+

[a] Highlighted charge states (see boxes) correspond uniquely to either dimer or trimer as indicated in each column and would be observed at the m/z ratios calculated on the left.

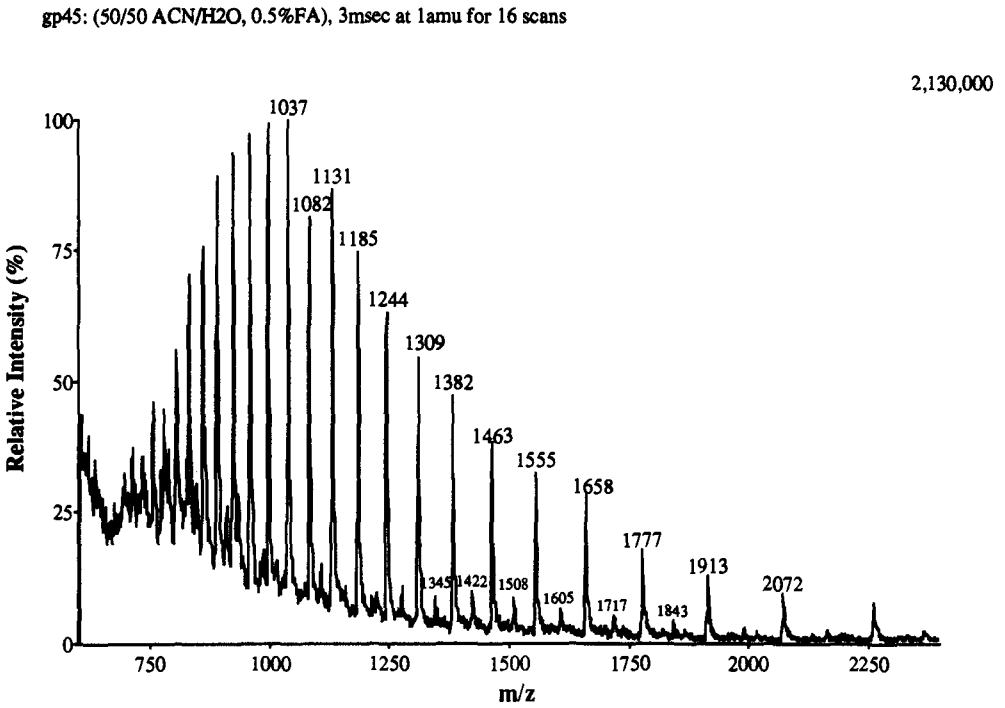

Figure 4. Ion spray mass spectrum of gp45, recorded by infusion of gp45 (1 μg/μL in 10 mM NH_4OAc, pH 2.5) at 2 μL/min through the ion spray interface. The spectrum is an averaged sum of 16 scans at a scan rate of 3 ms/scan.

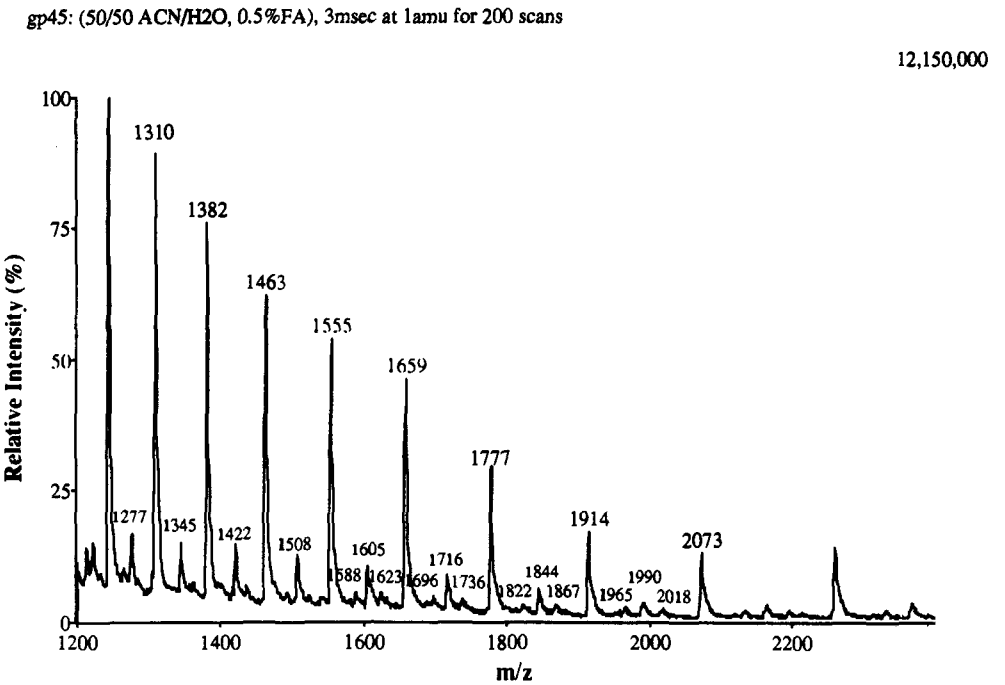

Figure 5. Expanded view of the ion spray mass spectrum of gp45 at pH 2.5, recorded as in Figure 4.

trimer possess unique charge states (i.e. nonintegrally divisible by 3) which, if detectable, would differentiate the trimer from dimer or monomer. Table 1 presents a partial sequence of calculated mass-to-charge ratios for monomer, dimer, and trimer ions based on the experimentally determined MW of 24 863 Da for monomeric gp45. Note that the unique, diagnostic charge states for gp45 dimer and trimer (indicated in boxes in Table 1) are predicted to appear at m/z values which are bracketed by the strong ion current signals observed in Figure 1.

Closer inspection of the full-scan mass spectrum of gp45 obtained at pH 2.5 indeed revealed smaller peaks in the regions corresponding to noncovalent gp45 dimers (cf. arrows, Figure 1). Those minor peaks, which were most evident in the mass range from m/z 1107 to 1508, were shown to occur at m/z values precisely midway between each principal charge state, just as would be expected for odd-charge-state dimer ions.

Higher ionic strength would be expected to enhance hydrophobic interactions between subunits of the accessory protein; therefore, solutions of gp45 in 10 mM NH_4OAc (1:1 CH_3CN–H_2O, pH 2.5) were analyzed by ion spray MS. As expected, the minor dimer ion signals in FIgure 1 increased in intensity (Figure 4). Under these conditions, a partial scan from m/z 1200–2250 (Figure 5) revealed even more detail. A recurring, three-signal motif is evident which is repeated between the 12+ and 18+ monomer charge states at m/z 2073 and 1382, respectively. The most abundant peak in each motif, which was observed midway between each principal charge state, was characteristic of odd-charge-state gp45 dimer ions. The remaining two peaks of each

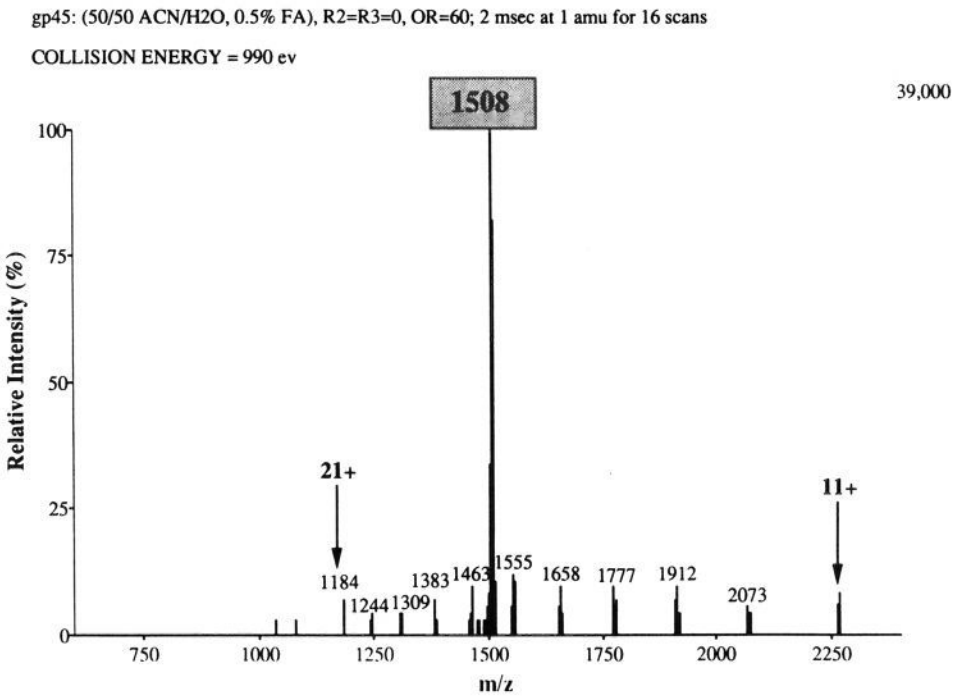

Figure 6. Tandem mass spectrometric (MS/MS) product ion mass spectrum of the precursor ion at m/z 1508 (Figure 5). The collision energy was 990 eV.

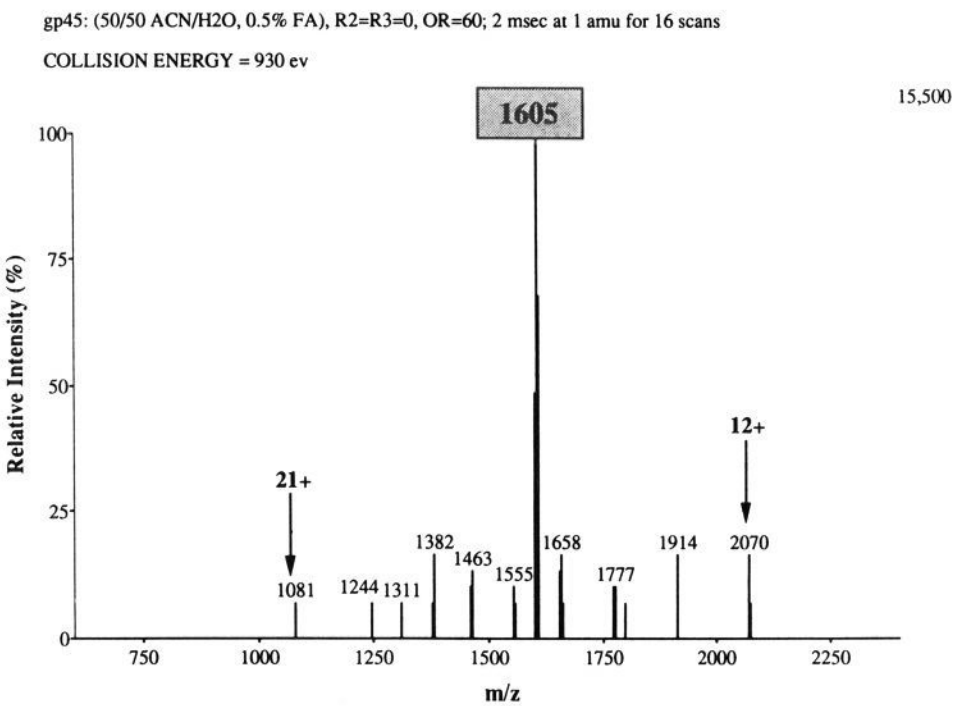

Figure 7. Tandem mass spectrometric (MS/MS) product ion mass spectrum of the precursor ion at m/z 1605 (Figure 5). The collision energy was 930 eV.

triad corresponded to unique gp45 trimer ions. Experimentally observed m/z ratios in each case agreed well with calculated values in Table 1.

Control experiments with several other proteins of comparable size established that the gas-phase gp45 dimer and trimer ions reflected true solution behavior and were not artifacts of clustering in the mass spectrometer. Lysozyme, calmodulin, soybean trypsin inhibitor, trypsin, α-casein, and δ-chymotrypsin (MWs ca. 14 500–25 500 Da) all are known to be monomeric in solution. Ion spray mass spectra of these proteins (not shown) displayed no dimer or trimer ions whatsoever when measured under the same experimental conditions as gp45.

Further experiments on two representative gp45 dimer ions depicted in Figure 5 using tandem mass spectrometry (MS/MS) confirmed their structures as noncovalent gas-phase association complexes. In traditional applications of MS/MS, rapid vibrational energy redistribution normally limits the method to molecules of ca. 3000 Da; however, MS/MS was here used only to ascertain whether the energy required to dissociate the gp45 dimer was comparable to or significantly less than the energy required to break covalent bonds in the protein.

In one MS/MS experiment (Figure 6), the signal at m/z 1508 in Figure 5, corresponding to the family of gp45 noncovalent dimers in the 33+ charge state, was mass selected and then

accelerated at 990 eV into the collision cell of the mass spectrometer, where it was collided with argon gas. Subsequent collision-induced decomposition produced a range of product ions corresponding to individual gp45 monomers in the 11+ to 21+ charge states. These ions would be expected from the dissociation of a series of noncovalent dimer ions in the 33+ charge state, ranging from $(gp45)^{17+}(gp45)^{16+}$ to $(gp45)^{22+}(gp45)^{11+}$. Similar results were obtained (Figure 7) when the same experiment was performed on the m/z 1605 dimer ion in Figure 5. Not surprisingly, no significant new ion current signals were observed when the MS/MS experiment was performed on the principal charge state ions, since most of the ion current in the major, degenerate peaks arises from monomer.

Although the peak tentatively identified by size exclusion chromatography as a gp45 trimer was more abundant at neutral pH, its mass spectrum could not be obtained by ion spray MS. Moreover, we were unable to detect any parent ions for gp45 at pH 5–7, in either the presence or the absence of NH_4OAc. In fact, acidic conditions are usually required to obtain acceptable mass spectra of proteins, otherwise insufficient protonation results in mass-to-charge ratios for $(M)^{n+}$ that fall outside the observable range of typical spectrometers (up to 2400 Da).[14,15]

The present study demonstrates that noncovalent association of gp45 into dimeric and trimeric species may be detected by ion spray MS. Weak noncovalent binding is largely due to hydrophobic interactions, which are greatly diminished in the gas phase,[16] thus accounting for the low abundance of unique noncovalent gp45 dimer and trimer ions in the mass spectrum of this protein.

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