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Studies of the *Ras*-GDP and *Ras*-GTP Noncovalent Complexes by Electrospray Mass Spectrometry†

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Abstract: A novel MS based methodology utilizing electrospray ionization is described for the detection of the noncovalent interaction between a host protein (*ras*) and its guest ligands (GDP and GTP). The *ras* proteins are regulatory guanine nucleotide binding proteins which serve as signal transducers controlling cell proliferation or differentiation. They can exist in two interconvertible states of GDP-bound (inactive form) and GTP-bound (active form). The presence of the noncovalent complexes of *ras*-GDP and *ras*-GTP, as well as the unbound apo-*ras* protein, in various sample solutions containing biological buffers were confirmed by the observed average molecular weights of 19295, 19374, and 18852 Da, respectively. The stability of the observed GDP-bound and GTP-bound complexes is a combined function of solution pH and organic modifier content.

INTRODUCTION

Three *ras* genes found in the human genome named Harvey, Kirsten and Neuroblastoma-*ras*, code for 21 kDa guanine nucleotide binding proteins which are believed to play a central role in signaling events of cell growth and differentiation.[1,2] *Ras* proteins can exist in two interconvertible states: guanosine diphosphate (GDP)-bound and guanosine triphosphate (GTP)-bound representing the inactive and active forms, respectively. The *ras* family of oncogenes are of particular interest since they have been found in a wide range of human tumors.[3] In oncogenic *ras*, the rate of hydrolysis of the GTP ligand to GDP by the GTPase activating protein is dramatically reduced, which "locks" the *ras* in the active state resulting in unregulated cell growth. Therefore, the detection and differentiation of the *ras*-GDP and *ras*-GTP noncovalent complexes under physiological conditions is important to the study of abnormal cell growth, as well as to the discovery of organic molecules as anticancer drugs which will displace the nucleotide (GDP or GTP) in the *ras* protein.

Conventional techniques used for direct detection of noncovalent complexes (e.g; gel permeation chromatography, centrifugation, and gel electrophoresis) provide limited or no information about the molecular weight of the complex and its binding stoichiometry. Advanced spectroscopic techniques such as X-ray and NMR have been used to solve 3-D structures of various proteins. In the case of the *ras*-GDP, the 3-D structure shows that the protein consists of 6 β-sheets, 4 α-helices, and 9 connecting loops[4,5] with the GDP molecule bound in a pocket formed by four of the loops. Recently, the structure of the *ras*-GTP and *ras*-Mn^{II}GDP complexes have also been studied by X-ray[6] and electron paramagnetic resonance.[7] In spite of these studies, direct proof of the noncovalent complex based on molecular weight information had yet to be demonstrated.

†Dedicated to Professor Sir Derek Barton on the occasion of his 75th Birthday.

In recent years, mass spectrometry (MS) has become an indispensable tool for biomedical research involving protein and peptide structural analysis mainly due to the development of various soft ionization methods.[8-12] Nevertheless, the detection of noncovalent complexes by MS methods has been a challenging task. To successfully perform direct MS analysis of the pre-formed noncovalent complexes, the MS ionization technique utilized must satisfy the following criteria: (1) the ionization method must be capable of generating intact gas phase protein ions from a solution containing biological buffer; (2) the internal energy transfer to the macromolecule during the ionization process must be minimal to prevent complex dissociation; (3) the MS instrumentation must have sufficient mass range to observe the ionized complex.

The newly developed technique of electrospray MS allows the formation of gas phase macromolecular ions directly from solution at atmospheric pressure via ion evaporation.[13,14] The process takes place during the electrostatic and pneumatic nebulization of a solution of charged analyte ions forming liquid droplets at atmospheric pressure. Convective heat exchange and interaction with air causes rapid size reduction of the droplets to a point where the repulsive coulombic forces approach the level of droplet cohesive forces. When the Rayleigh limit is reached, the large droplets appear to "explode" to produce a cascade of fission products comprising smaller and smaller droplets. The repulsive forces within these tiny droplets appear to cause a field-induced "ion evaporation" of the dissolved analyte ion which effectively transfer the ion from the condensed phase to the gas phase.[14] These gas phase ions are subsequently focused and transmitted into the quadrupole mass filter for mass analysis. The immediate advantage of this ionization mode is that a protein sample can be introduced at its physiological solution in which the biological activity is maintained.

The first applications of using electrospray MS for the detection of noncovalent interactions were reported by Ganem et al.[15,16] in which the enzymatic reaction of hen egg-white lysozyme with various substrates, and the binding between FKBP (an immunosuppressive binding protein) and immunosuppressive agents (FK506 and rapamycin) were studied. The ternary complex between the dimeric enzyme HIV-1 protease and a substrate-based inhibitor, as well as a small oligonucleotide duplex, has also been detected by electrospray MS.[17,18] We have been studying the noncovalent interaction of *ras* protein with its ligands GDP and GTP using electrospray MS, and a preliminary report of our findings to the successful detection of *ras*-GDP has been recently published as a communication.[19] In this paper we provide the details of our investigation in using electrospray MS to probe the existence of noncovalent complex of C-terminally truncated human *ras* (1-166) with GDP, which was further extended to the study of the *ras*-GTP complex. The effect of the solution pH and organic modifier content to the stability of these noncovalent complexes is also discussed.

EXPERIMENTAL SECTION

Preparation of ras-GDP Complex

C-terminally truncated normal human *ras* (1-166; the protein sequence is shown in **Figure 1**) was expressed in *E. coli* from a synthetic *ras* gene under the control of both the *lpp* and tac promoters.[20] The noncovalently bound *ras*-GDP complex was subsequently purified from fermentor-grown, IPTG-induced cells by a reported procedure.[21] The activity of the *ras* protein in GDP binding assays and GTPase assays was comparable to published data.[21-23] The final apo-*ras* protein was stored in 750 mM ammonium sulfate, 50 mM Hepes buffer pH 6, 10 mM 2-mercaptoethanol at a concentration of 6.15 mg/mL. The FPLC isolated *ras*-GDP

sample was kept in the solution containing 64 mM Tris HCl pH 7.6, 10 mM $MgCl_2$, 1 mM NaN_3, 0.5 mM DTE, and 250 mM NaCl. All sample solutions were stored at 4°C prior to MS analysis.

	10	20	30	40
1	MTEYKLVVVG	AGGVGKSALT	IQLIQNHFVD	EYDPTIEDSY
41	RKQVVIDGET	CLLDILDTAG	QEEYSAMRDQ	YMRTGEGFLC
81	VFAINNTKSF	EDIHQYREQI	KRVKDSDDVP	MVLVGNKCDL
121	AARTVESRQA	QDLARSYGIP	YIETSAKTRQ	GVEDAFYTLV
161	REIRQH			

Figure 1. Sequence of C-terminally truncated (1-166) human *ras* protein.

Preparation of ras-GTP Complex

An aliquot (100 µL) of apo-*ras* stock solution was diluted in Hepes buffer (50 mM, pH 7.5, 1 mM DTT) to two mL. To this, 10.2 µL of GTP were added (i.e., 2 - 3 molar excess) from solution of GTP in Hepes buffer. The concentration of the GTP solution was 3.34 mg/mL. This solution was refrigerated overnight and then concentrated to roughly 200 - 250 µL using a centrifuge concentrator. It was found that a small amount of the protein was lost during the concentration step. In order to minimize this loss, a modified procedure was adopted where the dilution of stock solution was eliminated. To an aliquot (100 µL) of apo-*ras* stock solution, 10.2 µL of GTP solution was added directly. The mixture was then taken up to 200 - 250 µL volume by adding Hepes buffer. All samples were further treated with 1 mM EDTA and 1 mM $MgCl_2$ prior to MS analysis.

Mass Spectrometry

A SCIEX API III triple quadrupole mass spectrometer equipped with a standard atmospheric pressure ionization source (Sciex, Inc., Thornhill, Ontario, Canada) was used. Details of the interface has been described previously.[24] The mass analysis was carried out by scanning either the first quadrupole (Q1) or the third quadrupole (Q3) from 300 to 2400 Da at a typical scan rate of 2 sec/scan. Sample solution was introduced either through direct infusion of the protein solution or via Rheodyne external loop injector with a preselected solvent system. In the latter case, an Applied Biosystems Inc. (Foster City, CA) Model 140A dual-syringe micro LC pump was used to deliver a constant liquid flow of 5-100 µL/min.

RESULTS AND DISCUSSION

In general, protein samples upon electrospray MS analysis generate a "bell-shaped" ion distribution of multiply-charged molecular ions arising from multiple protonation or other cation attachment (in the positive ion mode). **Figure 2** shows the electrospray mass spectrum of the noncovalent complex of *ras*-GDP obtained under the typical electrospray MS conditions (i.e., 1/1 0.1% aqueous TFA/acetonitrile, v/v, pH 2.4). Under this condition, a distribution of multiply-charged ions at m/z 2357, 2095, 1886, 1714, 1571, 1450, 1347, 1257, 1179, 1110, 1048, 993, 943, 899, and 858 was observed. The corresponding charge states and the average molecular weight thus obtained can be determined by solving two simultaneous equations derived from series of

adjacent ion pairs, as described previously.[25] Therefore, the ions observed in **Figure 2** were assigned as a series of integral charged states from +8 to +22, respectively, with the apex of the ion distribution centered around the +18 charge state. This provided an average molecular weight of 18,852±0.8 Da which correlates well with the theoretical value of 18,853.3 Da for apo-*ras* protein. Hence, the protein was unfolded in the solution and the GDP had been released from its bound state.

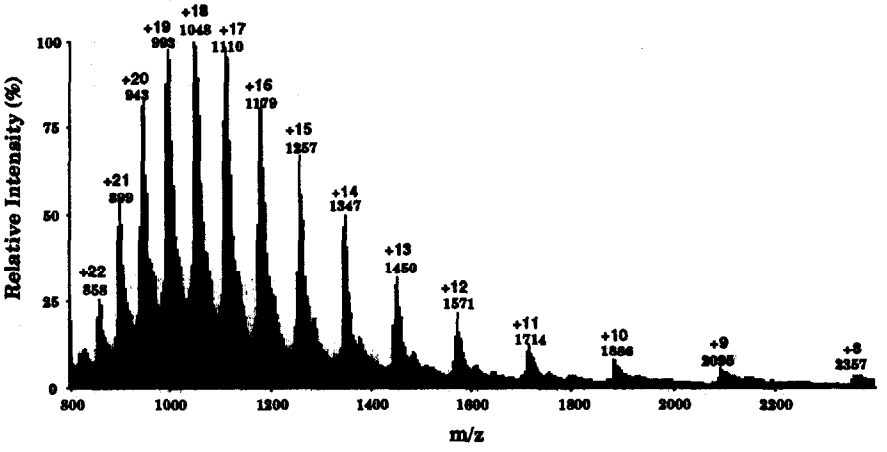

Figure 2: Electrospray mass spectrum of *ras*-GDP complex obtained from infusion (5 μL/min flow rate) of protein solution. Mass spectrometer was scanned from 800 to 2400 Da in 4 seconds with a "peak-hopping" of 1Da. The experimentally determined molecular weight of this free *ras* protein is 18,852 Da.

It is well known that the conformation of a protein in solution is highly dependent on the solution matrix. Denaturation of a protein from its native conformation can be achieved by the addition of acid, base, organic solvent, salt, as well as by the application of heat. The challenge for a successful MS analysis of noncovalent complexes is how to preserve the active protein conformation. During the course of this study, we have found that the control of solution pH, organic solvent and stabilizing buffer additives is crucial to the detection of noncovalent complexes.

The effect of solvent pH on the electrospray MS detection of the *ras*-GDP noncovalent complex was examined by adjusting the pH of the running solvent with acetic acid. The results are summarized in **Table 1**. Instead of using the typical electrospray solvent system as shown in **Figure 2**, an aqueous solvent system (i.e., 2 mM NH_4OAc, pH 5.8) was employed to prevent the organic solvent-induced protein denaturation. In the resultant mass spectrum (**Figure 3**) the characteristic "bell-shaped" distribution of ion signals was shifted upward to a higher m/z region since fewer basic sites were protonated in the fully folded protein. The signals at m/z 2396, 2145, and 1931 corresponded to an average molecular weight of 19,295 Da. These ions were assigned as the integral charge states of +8, +9, and +10, respectively, of the *ras*-GDP noncovalent complex with 1:1 stoichiometry. (Note: The average molecular weight of protein sample shown in the deconvoluted spectrum is derived from all ions detected. Even though the multiple charges resulting from solving two simultaneous equations are non-integral values in most cases, the charge state assigned to a particular m/z signal is an integral number.) Closer examination of **Figure 3** with the aid of a computer deconvolution procedure

revealed the presence of a second species with an average molecular weight of 19,344 Da, a component that could have resulted from the addition of two Mg^{+2} ions to the *ras*-GDP complex. Nevertheless, this assignment has not yet been confirmed experimentally. We would like to point out that no signals corresponding to the free *ras* protein were observed from this freshly prepared *ras*-GDP solution, in contrast to our earlier observation[19] from an aged (1.5 month) sample, where signals corresponding to the free *ras* protein were detected possibly due to the decomposition of *ras*-GDP complex over time.

Table1. The pH Effect on MS Observation of *ras*-GDP Noncovalent Complexation under Aqueous Condition.

Solvent	pH	Observation on *ras*-GDP Complex System
2 mM NH_4OAc	5.8	- Observe *ras*-GDP complex - Charge distribution of complex is centered on ±9 charge - No unbound *ras* protein is observed
2 mM NH_4OAc	4.0	- Observe *ras*-GDP complex - Charge distribution of complex is centered on ±11 charge - No unbound *ras* protein is observed
2 mM NH_4OAc	3.3	- Observe *ras*-GDP complex - Charge distribution of complex is centered on ±11 charge - Protein denaturation occurred
2 mM NH_4OAc	2.8	- Near complete denaturation of *ras* protein - Charge distribution of free *ras* is centered on ±17 charge - Almost no *ras*-GDP complex is left in the solution

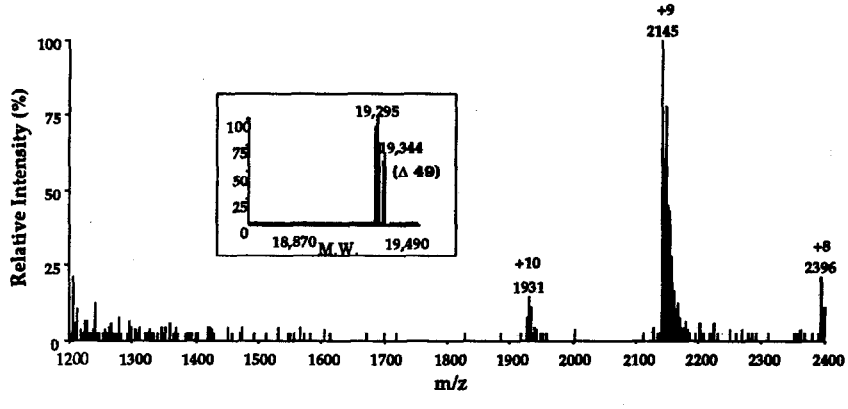

Figure 3: Electrospray mass spectrum of ras-GDP complex obtained from a 2 mM NH_4OAc solution, pH 5.8. The inset shows the deconvoluted spectrum. The 19,295 Da component corresponds to the 1:1 association ratio between ras protein and GDP ligand, and the component with MW of 19,344 is presumably the adduct of ras-GDP complex with 2 Mg atoms.

When the pH of the electrospray running solvent was lowered from 5.8 to 4.0, the electrospray spectrum shown in **Figure 4** was obtained in which two distinct changes were noted. First, the apex of the "bell-shaped" ion distribution was shifted from +9 charges to +11 charges, and second, a weak signal (18,852 Da) representing the apo-*ras* was observed in the deconvoluted spectrum. However, this signal was so weak that it was not readily identifiable from the raw mass spectrum.

The observation of the higher charge states is attributed to the *accessibility* of more protonation sites at lower pH. It has been suggested[26] that the multiply charged positive ions observed in the electrospray mass spectra of proteins are produced by proton attachment to basic amino acids (e.g., lysine, arginine, and histidine) and, therefore, reflect the degree of protonation in solution.[27,28] The *accessibility* of the potential protonation sites is determined by the protein conformation in the solution which, in turn, is affected by the solution pH. Lower pH induces protein unfolding, thus resulting in more protonation of the potential sites and change in the multiply charged ion distribution in the observed electrospray mass spectrum.[28,29] For example, electrospray mass spectra of hen egg white lysozyme (a ~14 kDa protein containing four disulfide bonds) over the pH range

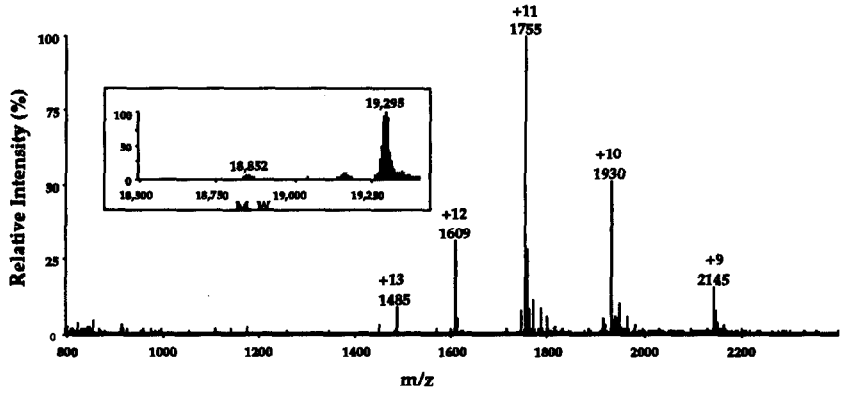

Figure 4: Electrospray mass spectrum of *ras*-GDP complex obtained from aqueous solution with pH adjusted to 4.0.

3-10 have been reported;[30] the data indicates that decreasing solution pH has little effect on the observed charge distribution, while the spectrum of reduced lysozyme taken under the same solution conditions showed a dramatic shift in the charge distribution toward lower m/z (i.e., higher charge state). This implies that unfolding the protein exposes more basic sites for protonation; thus, the apex of the "bell-shaped" ion distribution is expected to shift towards a lower m/z ratio (i.e., a higher charge state) when the solution pH is decreased.

It should be noted that in the *ras* protein bound to GDP, the signal corresponding to 19,344 Da (**Figure 3**) disappeared when the solution pH was decreased from 5.8 to 4.0. Further decrease of the pH to 3.3 resulted in partial release of GDP, leading to the observation of both the *ras*-GDP complex and the apo-*ras* signals (**Figure 5**). The relatively higher degree of GDP released from the *ras*-GDP complex at pH 3.3 (**Figure 5**) could be due to the weakening of protein-ligand binding affinity.

When the pH was adjusted to 2.8, the observed mass spectrum (**Figure 6**) showed signals corresponding to apo-*ras* as the major component, with weak signals representing a small amount of the *ras*-GDP complex remaining in solution. The resistance to complete the protein unfolding at pH 2.8 could be overcome by adding a trace amount of organic modifier to this solution. The resultant spectrum showed no signals corresponding to the *ras*-GDP complex (*vide infra*).

A common tertiary structure of protein in aqueous solution consists of hydrophilic surface area with apolar amino acids oriented away from water molecules forming a hydrophobic interior pocket. Addition of

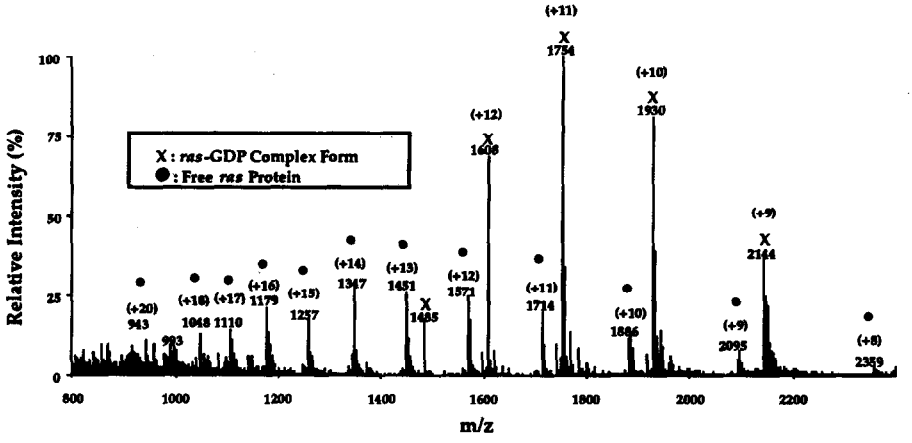

Figure 5: Conditions are the same as described in Figure 4, except that the pH was adjusted to 3.3.

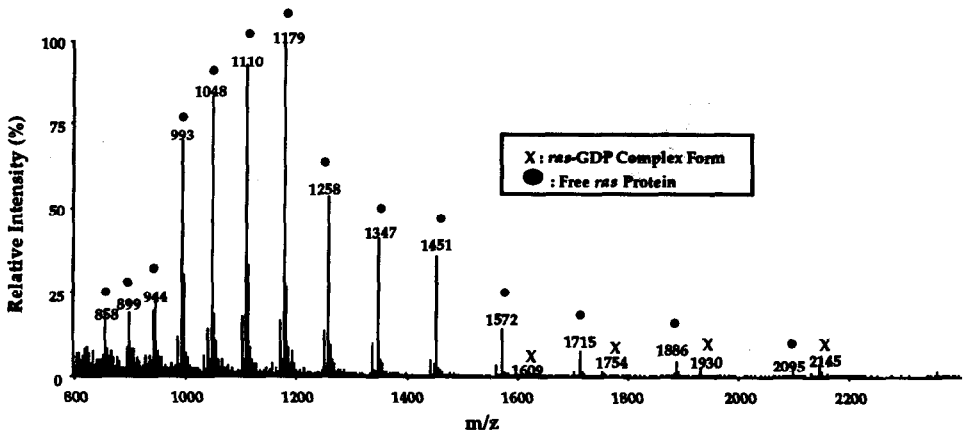

Figure 6: Conditions are the same as described in Figure 3, with the pH lowered to 2.8.

organic solvent disrupts the well-ordered water molecules allowing organic molecules to penetrate into the hydrophobic interior and subsequently interact with these buried hydrophobic residues. The disruption of internal hydrophobic interactions could lead to a more extended protein conformation. **Table 2** summarizes the effect of the organic solvent content on the mass spectral detection of the *ras*-GDP complex. The effect of adding 10% MeOH to an aqueous solution of pH 5.8 is shown in **Figure 7**. Comparison of the mass spectra shown in **Figure 3** and **7** revealed that a substantial degree of protein denaturation had occurred. The multiply-charged ion distribution of the apo-*ras* was found centered around the +9 charge state, and similarly the noncovalent *ras*-GDP association was also centered at the +9 charge state. This observation suggests that the apo-*ras* protein generated under this condition probably assumes a similar conformation as that of the original *ras*-GDP complex in which the GDP is "stripped" away from its binding pocket.

Table 2. Organic Solvent Effect on MS Observation of *ras*-GDP Noncovalent Complexation at pH 5.8.

Aqueous	MeOH	Observation on *ras*-GDP Complex System
100%	0%	- Observe *ras*-GDP complex - Charge distribution of complex is centered on ±9 charge - No unbound *ras* protein is observed
90%	10%	- Observe *ras*-GDP complex - Charge distribution of complex is centered on ±9 charge - Some denatured free *ras* protein is observed
50%	50%	- Observe small amount of *ras*-GDP complex - Near complete denaturation of *ras*-GDP complex
10%	90%	- Neither apo-*ras* protein nor *ras*-GDP related signals are observed under this condition

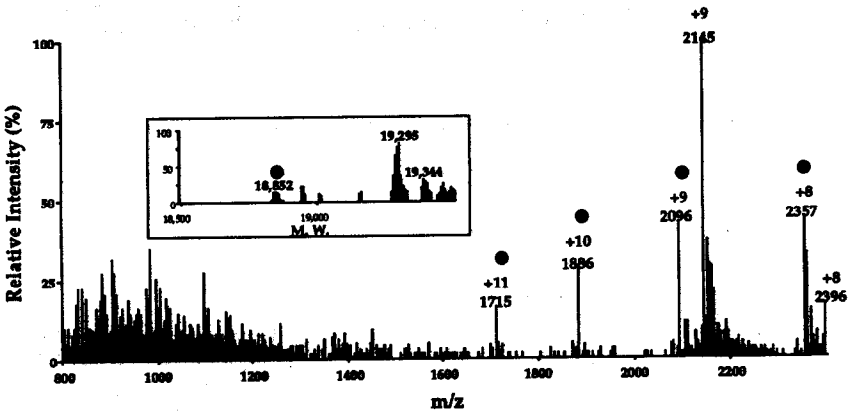

Figure 7: Electrospray mass spectrum of *ras*-GDP complex obtained from protein solution containing 10% MeOH, pH 5.8.

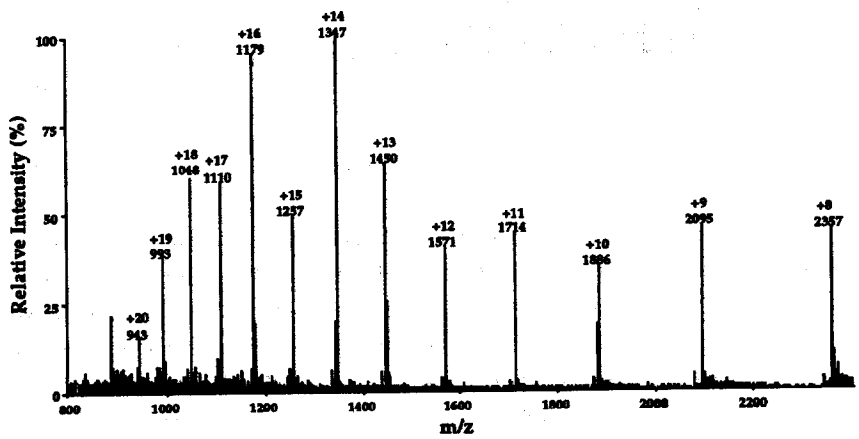

Figure 8: Electrospray mass spectrum of *ras*-GDP complex obtained from protein solution containing 50% MeOH, pH 5.8.

At a 50% MeOH content, a state of complete release of GDP was reached. The mass spectrum corresponding to this state is illustrated in **Figure 8**, where no signals corresponding to the *ras*-GDP complex were observed. Under this condition, ion signals with a charge distribution spread from +8 to +20 charges were generated. Comparison of **Figure 2** and **Figure 8** indicates a distinct difference in the observed protein charge distribution. In the first, the multiply-charged ion profile is centered around the +16 charge, thus indicating a more extended structure with more basic sites exposed for protonation, and in the second the multiply-charged ions detected at higher m/z (lower charge states) indicate a tighter protein structure (fewer protonation sites available). This experiment was repeated with the apo-*ras* protein under the same denaturing conditions and the multiply-charged ion distribution observed (**Figure 9**) was similar to that shown in **Figure 8.** Further increase of the organic content from 50% MeOH to 90% MeOH in the solution, resulted in the precipitation of the protein, and neither free *ras* protein nor *ras*-GDP complex related signals were observed in the spectrum.

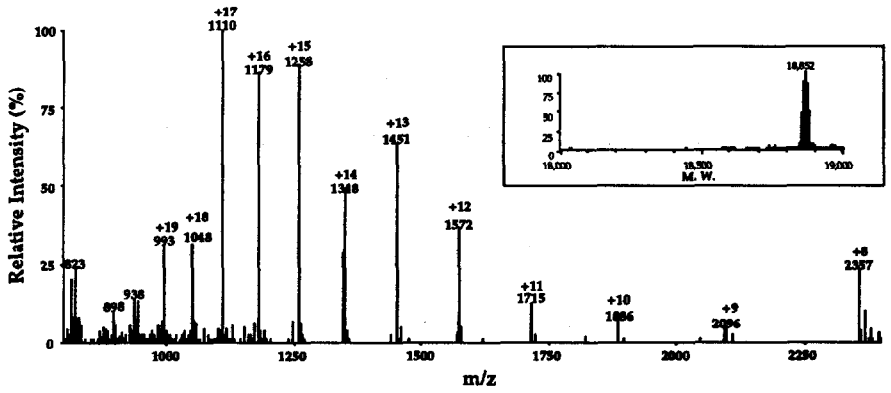

Figure 9: Electrospray mass spectrum of apo-*ras* protein obtained from solution containing 50% MeOH, pH 5.8. The inset shows the deconvoluted spectrum.

The methodology developed for the detection of the *ras*-GDP complex was also applied to monitor the formation of the GTP-bound *ras* protein complex. The electrospray mass spectrum taken from infusion of the aqueous solution of *ras*-GTP complex at pH 4.0 is given in **Figure 10.** A series of ions at m/z 2154, 1938, 1762, 1616, and 1491 were detected, yielding an average molecular weight of 19,374 Da indicating the formation of an 1:1 *ras*-GTP noncovalent complex. These multiply-charged ions corresponded to the +9, +10, +11, +12, and +13 charge states of the *ras*-GTP complex, respectively. It was found that treatment of the protein sample with concentrated EDTA (10 mM) and $MgCl_2$ (100 mM) solution prior to the MS analysis was important to the formation, as well as the MS observation, of the *ras*-GTP complex. This observation is consistent with a previous report[31] that nucleotides bind to *ras* in the absence of Mg; this interaction, however, is much tighter in its presence. It should be noted that the multiply charged ion distribution profiles shown in **Figures 10** and **4** are identical. In both cases, the +11 charge state was at the apex of its "bell-shaped" multiply-charged ion distribution profile. In solution, the conformational difference between the "*on*" and "*off*" states of the *ras* protein was significant enough that the epitope motifs for receptor recognition was altered leading to drastic difference in its biological activity. However, this difference does not necessarily affect the net charge of protein

in solution which was transmitted into the gas phase via the ion evaporation mechanism and subsequently detected by MS. Everything being the same for MS analysis of *ras*-GDP and *ras*-GTP complexes, the data suggest that even though there is a significant difference in biological activity, the change in conformation during the transition between the "*on*" and the "*off*" state is not significant enough to allow the differentiation to be made by the MS methodology currently employed.

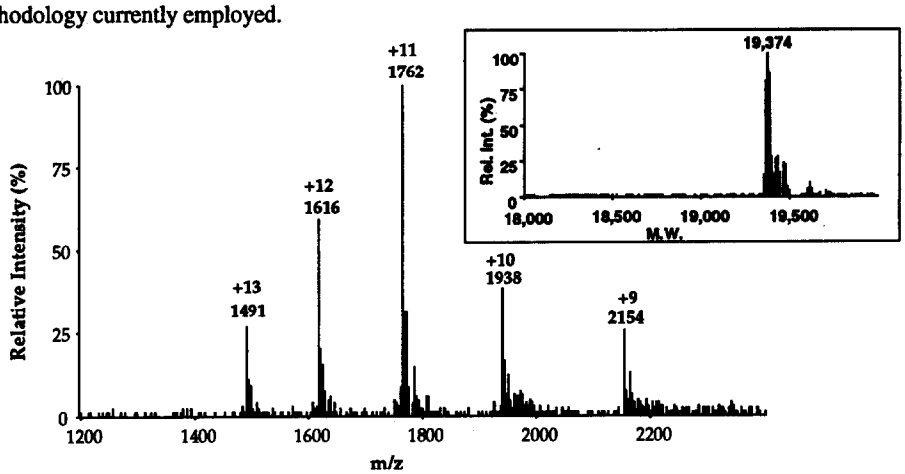

Figure 10: Electrospray mass spectrum of ras-GTP obtained from protein solution (contains no organic modifier), pH 4.

The pH effect in the noncovalent interaction between protein and ligand observed previously for *ras*-GDP was also examined for *ras*-GTP. Results are shown as deconvoluted spectra in **Figure 11A-C**. As indicated

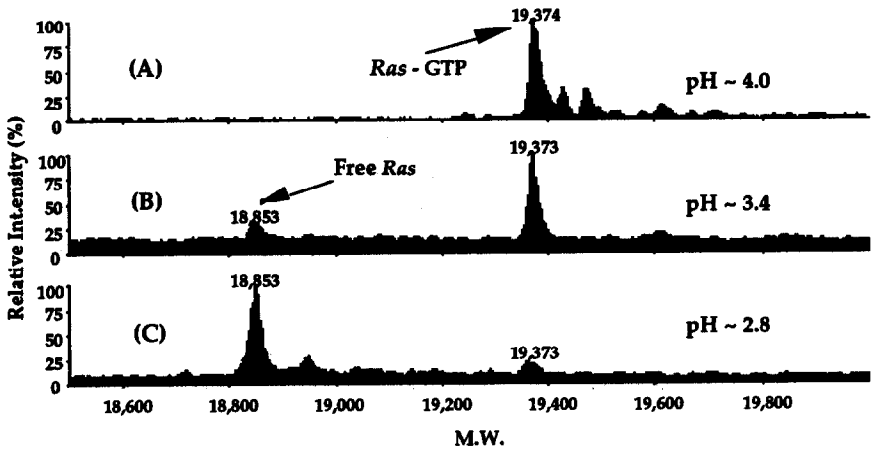

Figure 11: Deconvoluted electrospray mass spectra of ras-GTP complex obtained from protein solution (no organic modifier) with (A) pH 4.0, (B) pH 3.4, (C) pH 2.8.

in **Figure 11A** no free *ras* protein was present at pH 4.0. Decreasing the pH from 4.0 to 3.4, resulted in the

partial dissociation of the *ras*-GTP complex which was evident by the detection of signals corresponding to the free *ras* protein (**Figure 11B**). The majority of the GTP-bound *ras* complex was still preserved. Further decrease of the pH to 2.8 gave rise to an almost complete release of its guest ligand GTP as shown in **Figure 11C**. These data suggested that the *ras*-GDP and the *ras*-GTP noncovalent complexes possessed the same behavior upon electrospray MS analysis, with the solution pH having the same effect on *ras* protein denaturation regardless of the conformational difference originating from the different binding ligands.

CONCLUSION

The GDP-bound and GTP-bound noncovalent complexes of the C-terminally truncated human *ras* protein were successfully detected by electrospray MS. The method can be used with aqueous solution containing biological buffer additives. This important result suggests that noncovalently-bound species with suitably high association constants could be detected directly in a complex mixture. This observation also indicates that the protein-ligand complex can be desorbed, at its native or very close to its native solution conformation, as multiply-charged ions and maintained in the gas phase. From the molecular weight information obtained by this soft ionization method, one can readily provide information regarding the stoichiometry of protein-ligand interactions. The capability of the methodology employed here can be further extended beyond molecular weight determination. In fact, protein unfolding processes associated with the acid-induced, as well as organic solvent-induced, denaturation can be studied. For the system studied here, changes in the physical properties of the solution environment and the consequent effects on protein conformation were directly observed as changes in the multiply-charged ion distribution of the protein sample. Correlation of these changes with specific events in the unfolding is not possible. However, it is reasonable to say that as the protein solution environment is gradually altered toward conditions favoring protein unfolding, the folded conformation initially changes very little, if at all. There undoubtedly are some changes in structural or conformational flexibility, but the overall structure remains unchanged. The protein then becomes fully unfolded within a rather limited range of conditions. The ability to observe such noncovalent interactions in solution highlights the potential of using electrospray MS for protein-protein interaction studies, kinetic studies of protein-ligand interactions by measuring dissociation constants, and for screening enzymes and receptors for inhibitors and substrates of pharmaceutical interest.

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