Observation of a Noncovalent Ribonuclease S-Protein/S-Peptide Complex by Electrospray Ionization Mass Spectrometry

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Electrospray ionization mass spectrometry (ESI-MS) investigations of noncovalently-bound complexes are of great interest because of their relevance to solution biochemistry.[1-7] ESI-MS is particularly well suited to studies of weak interactions because it is very gentle and it allows studies to be performed under physiological or near-physiological solution conditions. However, it is not yet fully understood which weakly-bound complexes known to exist in solution will be observable by ESI-MS, or what minimum binding strength may be required for ESI-MS observation. Also unclear is whether complexes observed by ESI-MS reflect *only* species present in the bulk solution, or whether complexes also reflect aggregation in electrospray-generated microdroplets.[5,6,8] We address some of these issues by employing ribonuclease S (RNase-S) as a test of the applicability of ESI-MS for analysis of noncovalent complexes.

Bovine pancreatic RNase-S (M_r 13 700) is a modified form of RNase-A (M_r 13 682) in which the peptide bond between residues 20 and 21 is enzymatically cleaved with subtilisin to yield two polypeptides: S-peptide (residues 1–20, M_r 2166) and S-protein (residues 21–124, M_r 11 534).[9] S-peptide and S-protein associate under selected solution conditions to form RNase-S, with a crystal structure and enzymatic activity very similar to those of RNase-A.[9-11]

Figure 1 illustrates ESI mass spectra obtained with a nozzle–skimmer interface from solutions of RNase-S in deionized water (pH 5.3) and in 5% acetic acid/deionized water (pH 2.4), respectively.[12,17] Multiply charged ions for the RNase-S (hetero dimer) complex are the dominant species from a water solution (Figure 1a) at a low nozzle–skimmer bias (ΔNS) of 0 V. No

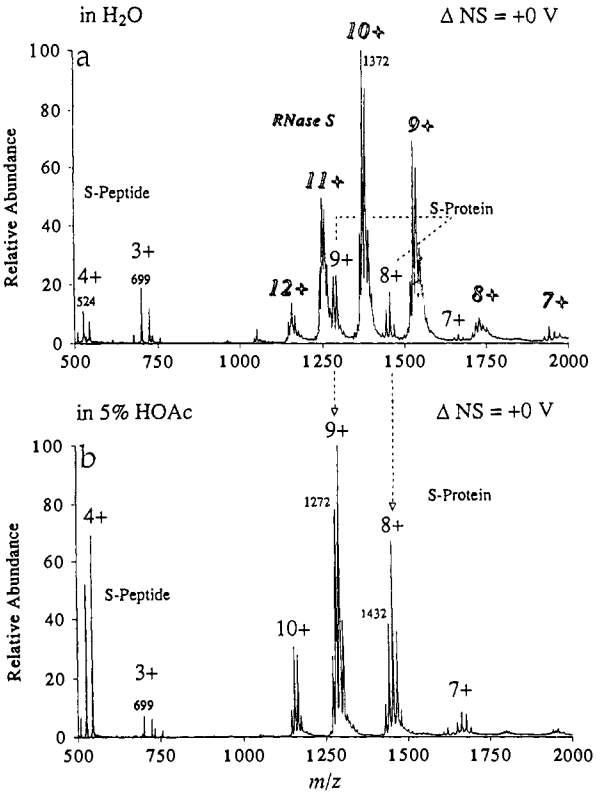

Figure 1. ESI-MS of RNase-S in deionized water without (a) and with (b) 5% acetic acid.

peaks due to S-peptide (homo) dimers were observed, and only low-intensity S-protein dimers were detected, demonstrating that the observed complex reflects specific binding rather than random aggregation in solution or from the ESI process. Extensive 98-Da adducts,[18b,c,19] observed under the gentle interface conditions, likely arise from phosphate or sulfate anions reflecting buffers employed in the purification of RNase-S.[9] Mitchinson and Baldwin[11c] have shown that phosphate stabilizes the RNase-S complex in solution. No complex was observed from ESI-MS of the acidic solution (Figure 1b), consistent with earlier measurements,[20,21] establishing the strong pH dependence of K_D, the

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(12) The electrospray ionization source and the quadrupole mass spectrometer[13] employed in these studies have been described previously. Except where indicated, all data were obtained with a nozzle–skimmer inlet and unheated countercurrent nitrogen.[13-15] Bovine pancreatic RNase-S and S-peptide (product numbers R6000 and R6125, respectively) were obtained from Sigma Chemical Company (St. Louis, MO) and were used without further purification. Commercial S-protein (Sigma R6250, Lot No. 125C 8045) was found to contain mainly reduced S-protein; binding studies employed material obtained from dithiothreitol oxidation of the commercial product.[16] All studies employed deionized water as the ESI source sheath liquid.
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RNase-S dissociation constant[22] (at 0 °C, pH 2.7, $K_D = 10^{-6}$ M, and at pH 7.0, $K_D = 2 \times 10^{-10}$ M[17]). At an elevated ΔNS (*e.g.*, +200 V), only multiply protonated S-peptide and S-protein ions were observed from ESI-MS of the deionized water solution, due to collisional dissociation of the complex. Except for detachment of 98-Da adducts, the spectrum from the acidic solution was unchanged at +200 V. No dissociation of RNase A is observed under these interface conditions since dissociation of covalent bonds would be required.

ESI-MS has been applied to studies of thermal denaturation in proteins.[23] K_D for the RNase-S complex is strongly temperature dependent;[24] optimum stability for the truncated S-peptide (1–15) RNase-S complex is attained at 0 °C.[25] The thermal stability of the RNase-S complex and its effect on the ESI mass spectrum were investigated by heating the continuous infusion capillary. These temperature studies yielded a phenomenological enthalpy of dissociation of $\Delta H_{diss} = +48$ kcal/mol. The difference with solution enthalpy (29 kcal/mol)[26] is most probably due to our use of an unbuffered, low ionic strength RNase-S solution.

To evaluate the selectivity of complex formation, we examined by ESI-MS equimolar solutions containing the natural S-protein (Figure 2a) and truncated S-peptide analogues. Residues Met-13 and Phe-8 are thought to be particularly important for RNase-S binding.[24] Because residues 15–20 of the S-peptide are not clearly defined in the crystal structure of RNase-S,[10,27] are not important for binding the S-peptide to the S-protein,[10,25,27,28] and do not affect the activity of the RNase-S complex, we selected S-peptide 1–15 (S15) and Gly-13 S-peptide (M13G) for the comparison.[25,29] Solution measurements at 25 °C and pH 6.0 yield $K_D \sim 1.1 \times 10^{-7}$ and 5×10^{-4} M for S15/RNase-S and M13G/RNase-S, respectively.[28] Consistent with these measurements, we observed significant complex formation of S-protein with S15 (Figure 2b). The data for complexation of S-protein with M13G (Figure 2c) was complicated by spectral overlap of the S-protein charge states, but less binding was observed.[30]

Clearly, the detection of weakly bound, thermally sensitive complexes requires gentle ESI interface conditions. Application of heat and/or collisions to assist in desolvation may significantly perturb the ratio of complexed to free molecules. To better understand these issues, we compared data obtained with the nozzle–skimmer interface and unheated countercurrent nitrogen flow[13,15] to data obtained with a heated stainless steel capillary interface at capillary temperatures from 50 to 220 °C and capillary–skimmer biases (ΔCS) from 0 to 400 V.[31–33] Both interfaces require a compromise between "gentleness" and sensitivity. Observing the complex was difficult with the heated capillary inlet when countercurrent nitrogen was not employed due to the higher temperature and/or ΔCS required to desolvate the ions sufficiently for detection. The highest complex:S-protein ratio (1.5:1) achieved with the heated capillary interface was acquired at 110 °C with a ΔCS of 0 V. Employing countercurrent N_2 for additional desolvation enabled use of lower capillary temperatures and yielded a complex:S-protein ratio similar to

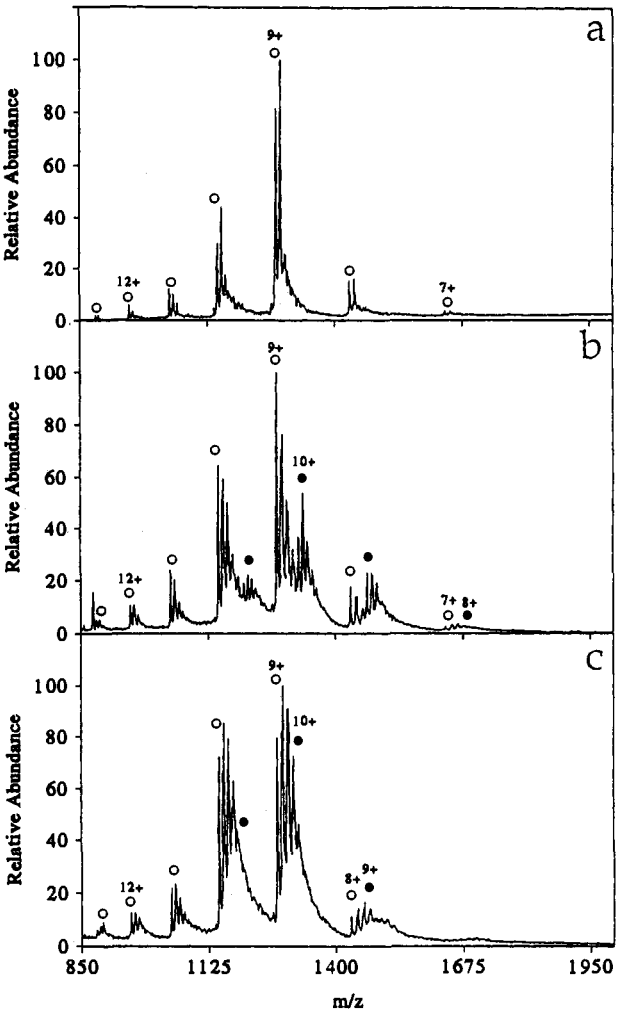

Figure 2. ESI mass spectra of aqueous solutions of (a) reoxidized S-protein,[11,16] (b) S-protein and S-peptide 1–15 (KETAAAKFERQH-MDS-NH_2), and (c) S-protein and Gly-13 S-peptide (KETAAAKFER-QHGDS-NH_2). The open circles (○) identify S-protein ions, and the closed circles (●) identify ions for the S-protein–peptide complex.

that achieved with the nozzle–skimmer inlet (Figure 1a). This result was obtained at a capillary inlet temperature of 60 °C and a ΔCS of +250 V. Thus, the observation of noncovalent complexes is not limited to nozzle–skimmer inlets; it may also be made in heated capillaries provided that a low enough capillary temperature is maintained to avoid thermal dissociation of the complex while still obtaining adequate desolvation.

These results show that the ESI mass spectra of RNase-S can reflect the specificity of binding in solution. More detailed studies comparing K_Ds, measured by ESI-MS as a function of pH, temperature, and buffer composition, to values obtained by other solution techniques should allow quantitative evaluation of how closely such measurements reflect solution chemistry.

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