Several factors, attributable to the ESIMS mechanism, can affect the assumptions of the titration method are examined: (1) The assumption that the concentrations in solution of the protein P, the ligand L, and the complex PL are proportional to the respective ion intensities observed with ESIMS, is examined with experiments in which ion intensities of two non-interacting proteins are compared with the respective concentrations. The intensities are found to be approximately proportional to the concentrations. The proportionality factors are found to increase as the mass of the protein is decreased. Very small proteins have much higher intensities. The results suggest that it is preferable to use only the intensity ratio of PL and P, whose masses are very close to each other when L is small, to determine the association constant \( K_A \) in solution. (2) From the charge residue model (CRM) one expects that the solution will experience a very large increase of concentration due to evaporation of the precursor droplets, before the proteins P and PL are produced in the gas phase. This can shift the equilibrium in the droplets: P + L = PL, towards PL. Analysis of the droplet evaporation history shows that such a shift is not likely, because the time of droplet evaporation is very short, only several \( \mu s \), and the equilibrium relaxation time is much longer. (3) The droplet history shows that unreacted P and L can be often present together in the same droplet. On complete evaporation of such droplets L will land on P leading to PL and this effect will lead to values of \( K_A \) that are too high. However, it is argued that mostly accidental, weakly bonded, complexes will form and these will dissociate in the clean up stages (heated transfer capillary and CAD region). Thus only very small errors are expected due to this cause. (4) Some PL complexes may have bonding that is too weak in the gas phase even though they have \( K_A \) values in solution that predict high solution PL yields. In this case the PL complexes may decompose in the clean up stages and not be observed with sufficient intensity in the mass spectrum. This will lead to \( K_A \) values that are too low. The effect is expected for complexes that involve significant hydrophobic interaction that leads to high stability of the complex in solution but low stability in the gas phase. The titration method is not suited for such systems. (J Am Soc Mass Spectrom 2004, 15, 1424–1434) © 2004 American Society for Mass Spectrometry
The Electrospray Mechanism for Proteins

Recent research and reviews [7–10] dealing with the ESI mechanism have presented very good evidence that the small ions (such as the inorganic ions Na\(^+\), NH\(_4\)^+, in the positive ion mode) are produced by the ion evaporation model (IEM) while macroions like the non-denatured proteins are produced by the charge residue model (CRM).

The small droplets produced at the tip of the electrospray capillary are positively charged (ESI in the positive ion mode). The charge Ze, where Z is the number of elementary charges +e, is due to an excess of positive ions in the droplets. The ion excess is created by an electrolytic process that takes place at the positive electrode in the sprayed solution [11, 12]. For nano ESI, that would be the inert Pt wire in the solution at the spray tip or the metal coating of the spray tip when coated tips are used. An inert electrode leads to the formation of H\(_3\)O\(^+\) ions [11, 12] when water is the major solvent. The production of H\(_3\)O\(^+\) can lead to significant decrease of the pH of the sprayed solution as demonstrated recently for nanospray with Pt electrodes by Van Berkel and coworkers [12].

The excess ions are distributed near the surface of the droplets minimizing thus the coulombic repulsions between them. The concentration of buffer salts such as ammonium acetate, NH\(_4\)Ac, generally used in millimolar concentrations, is much higher than the H\(_3\)O\(^+\) concentration produced by the electrolysis. Therefore, the charges on the surface will be predominantly attributable to NH\(_4^+\) ions because of exchange of cations in solution and the surface. Rapid droplet evaporation at constant charge leads to droplet instability and fission when the droplet radius R comes close to a critical value given by the Rayleigh stability limit

Rayleigh Stability limit: \(Z_{RS} = (\gamma e_0 R^3/8\pi)\) 0.5 where \(Z_{RS}\) is the number of elementary charges at the surface of the droplets, \(R\) is the droplet radius, \(\gamma = \) surface tension, \(e_0 = \) the electrical permeability of vacuum, and \(e = \) the elementary charge. The fission, which is generally asymmetric, leads to release of several small (offspring) droplets that carry off some 2% of the (parent) droplet volume and some 15–20% of the parent charge [13]. The measurements by Gomez and Tang [13] were made with solvents that evaporate slowly so that the droplets are more easily observed.

More relevant are the recent determinations by Beauchamp and coworkers [14, 15] with the rapidly evaporating solvents, water, methanol, and acetonitrile [14], used in ESI. The droplet fission does not occur exactly at the Rayleigh stability limit for all solvents. Thus, water droplet fission at some 90% of the limit and immediately after the fission the parent droplets are at about 70% of the limit while methanol and acetonitrile fission is closer to the stability limit [14].

Since solvents in which water is the major component are most often used to produce non-denatured proteins, we restrict our discussion to nanospray and water as solvent. Evaporation of the parent droplet after the first fission leads to subsequent fissions producing successive litters of first generation offspring droplets. With nanospray the initial parent droplet size is already very small so that the first generation offspring have radii that are in the 30–10 nm range. (For source of numerical data see Results and Discussion). Small ions are expected to be produced in the gas phase by the ion evaporation mechanism (IEM) when the droplets reach a radius in the neighborhood of 10–5 nm. The excess ions at the droplet surface begin to continually “evaporate” from the droplet as the droplet radius decreases by solvent evaporation [7]. The IEM process is supported also by recent results of computer modeling of clusters of water molecules charged by excess ions [10]. These results indicate processes that resemble quite closely IEM, however additional interesting insights are provided also.

The macro ions, such as the folded proteins, are produced from very small droplets by the charge residue model (CRM). The radius of the droplets decreases because of solvent evaporation until it reaches the size of the protein. The charge due to small ions at the surface of the droplet “lands” on the protein and leads to the charging of the protein. The major evidence for the validity of CRM was provided by de la Mora [8] who showed that the great majority of folded proteins observed under conventional ESI conditions had charge states where the charge was close to the Rayleigh limit evaluated with eq 1 when a radius R equal to the radius of the protein was used. This approximate equality is expected because the charge of the droplets stays at all times close to the Rayleigh charge. For example, the observations of Beauchamp and coworkers [14] quoted above, show that charged water droplets stay mostly within 90–70% of the Rayleigh Charge.

Work from this laboratory [16–18] extended the CRM for proteins by examining how the charge of the small ions at the surface of the droplet is transferred to the protein. When ammonium acetate is used, the charges landing on the protein will be predominantly NH\(_4^+\) ions. These lead to charging by adding themselves to residues at the surface of the protein. In the clean up stage, the adducts dissociate by release of NH\(_3\) [18].

The Titration Method

An aqueous solution of the protein P and the ligand L at given initial concentrations, which has reached equilibrium, is sprayed. Nano spray is used most often and in most applications the ligand is a protein inhibitor. The ion intensities \(I_P, I_L\), and \(I_{PL}\) are determined. The titration method is based on the following assumptions. The association constant between the protein P and the ligand L in solution is