Observation of the Noncovalent Quaternary Associations of Proteins by Electrospray Ionization Mass Spectrometry

Karen J. Light-Wahl, Brenda L. Schwartz, and Richard D. Smith*

Contribution from the Chemical Methods and Separations Group, Chemical Sciences Department, Pacific Northwest Laboratory, Richland, Washington 99352

Received November 17, 1993. Revised Manuscript Received April 7, 1994®

Abstract: The noncovalent tetrameric active forms of avidin, concanavalin A (Con A), and adult human hemoglobin (Hb A_o) can be observed intact in the gas phase by electrospray ionization mass spectrometry (ESI-MS). The atmosphere–vacuum ESI interface conditions strongly influence the retention of these weak noncovalent solution associations into the gas phase, as well as the average extent of charging for the subunits upon dissociation. The known solution pH dependence of the dimer–tetramer equilibrium of Con A was observed by ESI-MS, and the intact heterodimeric and -tetrameric active forms of adult human Hb A_o, $(\alpha\beta)$ and $(\alpha\beta)_2$, with the prosthetic heme groups could also be characterized by ESI-MS. Under harsher interface conditions a species corresponding to a trimer was observed for each of the proteins, a species not known to be formed under physiological conditions. Differences in the relative stabilities of these tetrameric proteins, formed from the known solution structures, are also qualitatively consistent with the gas-phase stability observed with ESI-MS by adjusting the atmosphere–vacuum interface conditions. The hemoglobin tetramer was found to be less stable in the gas phase than either the Con A or avidin tetramer, consistent with solution dissociation constants.

Introduction

Noncovalent associations which are highly structurally specific in nature are of fundamental biological interest. The physiologically active forms of many proteins are multimeric and the active sites are often at the interfaces between subunits. The strengths of such noncovalent associations, that generally arise from a multitude of relatively weak bonds, can vary widely and are reflected by the dissociation constants (K_D) typically determined for a specific set of solution conditions. The detection of specific noncovalent associations from solution in the gas phase has recently been shown to be possible by ESI-MS.[1] ESI-MS is a rapid and highly sensitive method for such investigations and can provide information on structurally specific and biologically important interactions involving multimeric proteins, as well as receptor–ligand, antibody–antigen, enzyme–substrate, and other structurally specific liquid-phase associations.[1] Initial studies have also used ESI-MS to investigate the thermodynamics for dissociation of such complexes by manipulation of solution conditions (e.g., the thermodynamics of the ribonuclease S, S-protein–S-peptide association) which were compared to known values obtained by more traditional solution techniques.[2] These results suggest that ESI-MS has potential for the study of such relatively weak but highly specific noncovalent associations and thus may provide a major tool for biochemical research by enabling much faster screening and/or identification of such interactions and their relative stabilities.

Most ESI-MS studies with proteins have been carried out in acidic solutions (or alkaline solutions for negative ion formation) which generally have the effect of increasing the extent of multiple charging. Such conditions often lead to a loss of elements of higher order structure, a process that is correlated with increased charging by ESI.[3] Acidic solutions, in addition to the use of organic solvents or heating,[4] can be effective in denaturing biopolymers and result in a sufficient net charge so as to be detected within the m/z range of most commercial quadrupole mass spectrometers (maximum m/z < 2000–3000). However, the recent application of ESI-MS to the study of biologically important noncovalent associations[1] and higher order protein structure[5] has indicated the need to use solution conditions closer to those of physiological interest,[6] and more suitable for preserving weak structural associations (which often correspond to near neutral pH solutions). Conformational constraints implicit in the retention of higher order structure under these solution conditions can reduce the number of available charge sites for the resulting gas-phase molecular ion. In addition, the more compact structures generally existing under these conditions might also be expected to exhibit increased Coulombic contributions and tend to decrease the maximum extent of charging in the gas phase. The maximum charge based upon the Rayleigh stability criterion for a compact spherical structure increases as (mass)$^{2/3}$ and has been estimated previously to be ≤20 for a globular protein of ~6 nm diameter.[3a] Thus, ESI-MS with neutral pH solutions of proteins generally

* Abstract published in *Advance ACS Abstracts*, May 15, 1994.

(1) (a) Ganem, B.; Li, Y.; Henion, J. D. *J. Am. Chem. Soc.* **1991**, *113*, 6294–6296. (b) Ganem, B.; Li. Y.-T.; Henion, J. *J. Am. Chem. Soc.* **1991**, *113*, 7818. (c) Katta, V.; Chait, B. T. *J. Am. Chem. Soc.* **1991**, *113*, 8534. (d) Baca, M.; Kent, S. B. H. *J. Am. Chem. Soc.* **1992**, *114*, 3992–3993. (e) Ganguly, A. K.; Pramanik, B. N.; Tsarbopoulos, A.; Covey, T. R.; Huang, E.; Fuhrman, S. A. *J. Am. Chem. Soc.* **1992**, *114*, 6559–6560. (f) Light-Wahl, K. J.; Springer, D. L.; Winger, B. E.; Edmonds, C. G.; Camp II, D. G.; Thrall, B. D.; Smith, R. D. *J. Am. Chem. Soc.* **1993**, *115*, 803–804. (g) Ganem, B.; Li, Y.-T.; Henion, J. D.; *Tetrahedron Lett.* **1993**, *34*, 1445–1448. (h) Ogorzalek Loo, R. R.; Goodlett, D. R.; Smith, R. D.; Loo, J. A.; *J. Am. Chem. Soc.* **1993**, *115*, 4391–4392. (i) Jacquinod, M.; Leize, E.; Potier, N.; Albrecht, A.-M.; Shanzer, A.; Van Dorsselaer, A. *Tetrahedron Lett.* **1993**, *34*, 2771–2774. (j) Huang, E. C.; Pramanik, B. N.; Tsarbopoulos, A.; Relchert, P.; Ganguly, A. K.; Trotta, P. P.; Nagabhushan, T. L.; Covey, T. R. *J. Am. Soc. Mass Spectrom.* **1993**, *4*, 624–630. (k) Li, Y.-T.; Hsieh, Y.-L.; Henion, J. D.; Ganem, B.; Senko, M. W.; McLafferty, F. W.; *J. Am. Chem. Soc.* **1993**, *115*, 8409. (l) Loo, J. A.; Ogorzalek Loo, R. R.; Andrews, P. C. *Org. Mass Spectrom.* **1993**, *28*, 1640–1649. (m) Schwartz, B. S.; Light-Wahl, K. J.; Smith, R. D. *J. Am. Soc. Mass Spectrom.* **1994**, *5*, 201–204.

(2) Goodlett, D. R.; Wahl, J. H.; Udseth, H. R.; Ogorzalek Loo, R. R.; Loo, J. A.; Smith; *Proceedings of The 40th ASMS Conference on Mass Spectrometry and Allied Topics*; Washington, D.C., May 31–June 5, 1992, pp 422–423. (b) Goodlett, D. R.; Ogorzalek Loo, R. R.; Loo, J. A.; Wahl, J. H.; Udseth, H. R.; Smith, R. D. *J. Am. Soc. Mass Spectrom.*, in press.

(3) (a) Loo, J. A.; Udseth, H. R.; Smith, R. D. *Anal. Biochem.* **1989**, *179*, 404–412. (b) Chowdhury, S. K.; Katta, V.; Chait, B. T. *J. Am. Chem. Soc.* **1990**, *112*, 9012.

(4) Volkin, D. B.; Klibanov, A. M. *Protein Function*: A Practical Approach; Creighton, T. E., Ed.; IRL Press: Oxford, 1990; p 4.

(5) Suckau, D; Shi. Y.; Beu, S. C.; Senko, M. W.; Quinn, J. P.; Wampler, F. M., III; McLafferty, F. W. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 790–793.

(6) Bohinski, R. C. *Modern Concepts in Biochemistry, 4th ed.*, Allyn and Bacon, Inc.: Boston, 1983; pp 36–50.

0002-7863/94/1516-5271$04.50/0

demands a more extended *m/z* range mass spectrometer than typically required for ESI-MS using conventional solvent conditions.

A primary consideration in the study of noncovalent associations is determination of their specificity, i.e., establishing that the species being detected in the gas phase are both structurally specific and derived from those in solution. There are several ways to provide convincing evidence that the associations observed in the gas phase do correspond to the specific associations present in solution prior to electrospray and are not formed during the electrospray process.[7] For example, the solution conditions (e.g., pH) can be adjusted to affect the associations present in solution, which should produce a corresponding change in the mass spectra. In addition, adjustment of the interface conditions should allow for the observation of ions indicative of **specific** complexes of defined stoichiometry (i.e., A·A, B·B) to dominate over ions due to dissociated subunits or random aggregation (i.e., A, B, $A{\cdot}B_2$, $A_2{\cdot}B$, $A{\cdot}B_3$, A_3, etc.). Noncovalent associations should be readily dissociated under more severe interface conditions, providing further support for the weak nature of the putative associations. Further convincing evidence for the observation of a **specific** association by ESI-MS can be derived from the ability to chemically modify one component of the complex which affects the complex formation in solution and results in a corresponding change to the mass spectra. Finally, MS/MS experiments are very useful for determining whether observed complexes are noncovalent based on their ease of dissociation, and can provide additional information of their composition based on the subunit dissociation at higher energy collisions. While it may be impossible to prove beyond a doubt that a specific association present in solution is the same as that observed with ESI-MS, meeting combinations of these criteria provides convincing evidence for the existence of specific noncovalent associations in the gas phase.

In a recent communication, the preservation of the tetrameric form of concanavalin A (Con A) into the gas phase and its detection by ESI-MS was reported,[8] utilizing the same extended *m/z* range instrument as for these studies. The ions indicative of the tetrameric species were observed in the *m/z* range of 4500–5500 at charge states of 22+ to 20+. This result established that ESI can be used to maintain tetrameric noncovalent associations into the gas phase and emphasizes the need for a higher *m/z* range mass spectrometer to observe some of these biological complexes. Similar results have been obtained with a sector mass spectrometer.[11]

Presented here are ESI-MS results showing that specific solution associations can be maintained in the gas phase for a range of multimeric proteins including avidin, Con A and adult human hemoglobin (Hb A_o). Avidin and Con A are both composed of four identical polypeptide chains, while the more complex Hb A_o,[9] important for O_2 transport, is composed of two hetero dimers in addition to hemoporphyrins in the active site of each of the four polypeptide chains. We show that under mild interface conditions, only the biologically relevant complexes present in solution are observed in the gas phase, namely the dimers and tetramers, to the exclusion of trimers, pentamers, and other higher order aggregates. We also report several surprising observations regarding the dissociation of the tetrameric proteins under harsher interface conditions and discuss such observations in the context of the ESI process. In addition, we show that ESI-MS can be used to monitor the solution pH dependence of the noncovalent dimeric and tetrameric forms of such proteins. Finally, correlation of the differences in solution behavior for a chemically modified form of Con A (succinyl Con A) to gas-phase behavior is demonstrated as well as the ability of ESI-MS to determine relative stabilities of the tetrameric species of these proteins.

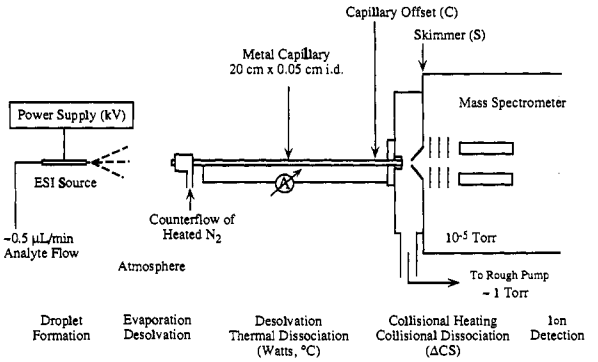

Figure 1. Schematic diagram of the atmosphere–vacuum interface showing the regions of droplet formation to ion formation and the variables important for observation of noncovalent associations.

Experimental Section

All mass spectra were obtained with a previously described[10] low-frequency single quadrupole mass spectrometer with an *m/z* range of approximately 45000. Limitations of this instrument include low resolution (~100) and discrimination against the low *m/z* range (*m/z* < 500). The lens elements were tuned for optimization of the higher *m/z* ions, resulting in some additional discrimination at low *m/z* (i.e., for higher charge state monomer ions). The atmosphere–vacuum interface consisted of a resistively heated stainless steel capillary (1.59 mm o.d., 0.5 mm i.d., 20–cm long) similar to the design of Chait and co-workers.[11] A schematic of the atmosphere–vacuum interface region is shown in Figure 1. Many variables in this region can affect the ability to observe noncovalently associated species. However, the most important variables seem to be the heating of the inlet capillary and the capillary–skimmer offset voltage. In the results presented here, capillary heating is reported as watts applied across the capillary and the approximate temperature as measured on the external surface of the capillary (near the center). In addition, the voltage offset between the capillary and the skimmer lens is given (as ΔCS). A heated coaxial flow of N_2 was utilized to assist in desolvation. The electrospray source (i.e., emitter) implemented in these studies was a metal capillary tip with only coaxial $SF_{6(g)}$ or $N_{2(g)}$ flow. The sample flow rate was 0.5 mL min^{-1}. All samples used in this study were obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Sample concentrations were 1 mg mL^{-1} in pH-adjusted (with either acetic acid or ammonium hydroxide) aqueous 10 mM ammonium acetate (NH_4OAc) solutions unless otherwise noted. A Teknivent Vector One data system was used to acquire the mass spectra. Typical scan ranges were from *m/z* 500 to 10500 at 5 *m/z* step sizes and approximately 2 min per scan. Most of the spectra shown are an average of five scans. Mass axis calibration was performed with bovine cytochrome *c* in 10 mM NH_4OAc using the 12+ to 1+ charge state ions. There is some uncertainty in the accuracy of the high *m/z* region due to the difficulty in forming high *m/z* ions of known molecular weight without residual solvation or adduction. However, for the data presented here, the calibration appears to be adequate for the low-resolution capabilities of this instrument.

Studies examining the relative stability of the avidin, Con A, and Hb A_o tetramer species were all performed on the same day and under the same interface conditions to avoid day-to-day variations in instrument performance. In addition, the ESI source position was kept constant relative to the vacuum interface inlet during these experiments. All the proteins for these experiments were dissolved in aqueous 10 mM NH_4OAc, pH adjusted to 8.4 with NH_4OH.

(7) Smith, R. D.; Light-Wahl, K. J. *Biol. Mass Spectrom.* **1993**, *22*, 493–501.

(8) Light-Wahl, K. J.; Winger, B. E.; Smith, R. D. *J. Am. Chem. Soc.* **1993**, *115*, 5869–5870.

(9) Human hemoglobin is primarily a tetramer with the subunits $\alpha_2\beta_2$ (Hb A_o) with a small component (~2%) of and Hb A_2 which contains the subunits $\alpha_2\delta_2$. (Stryer, L. *Biochemistry*; W. H. Freeman & Co., San Francisco, CA, 1981; pp 57–58.) The sample used here is the isolated Hb A_o form as obtained from Sigma Chemical Co.

(10) (Winger, B. E.; Light-Wahl, K. J.; Ogorzalek Loo, R. R.; Udseth, H. R.; Smith, R. D. *J. Am. Soc. Mass Spectrom.* **1993**, *4*, 536–545.

(11) Chowdhury, S. K.; Katta, V.; Chait, B. T. *Rapid Commun. Mass Spectrom.* **1990**, *4*, 81.

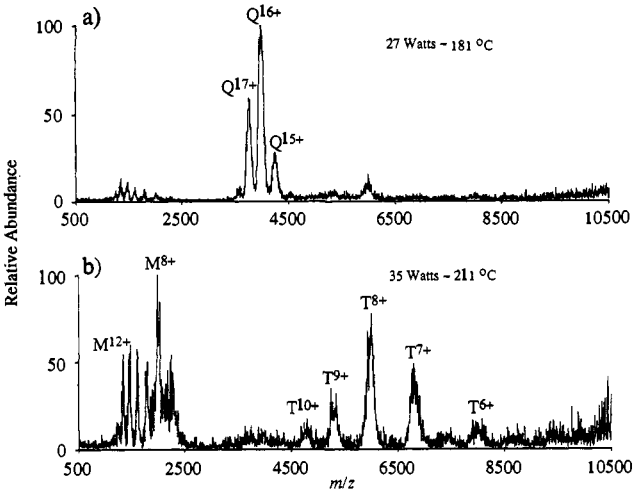

Figure 2. Positive ion ESI-MS of egg white avidin in 10 mM NH_4OAc (pH 6.7). The capillary–skimmer offset voltage (ΔCS) was 84 V. The resistive heating applied to the inlet capillary varied for the two spectra: (a) 27 W applied (external surface temperature ~ 181 °C) and (b) 35 W applied (external surface temperature ~ 211 °C). The labeling scheme for all spectra is as follows: M = monomer, D = dimer, T = trimer, and Q = tetramer with the superscript denoting the charge state of each ion.

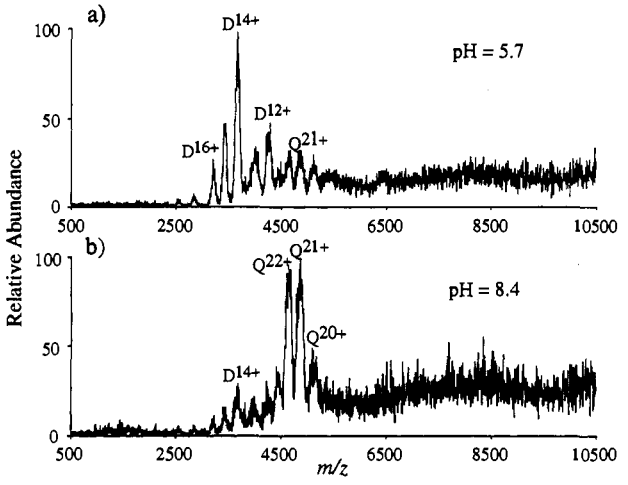

Figure 3. Positive ion ESI-MS of concanavalin A in 10 mM NH_4OAc at (a) pH 5.7 and (b) pH 8.4. For both spectra ΔCS = 120 V and 17 W was applied to the inlet capillary (external surface temperature ~ 135 °C).

Results

Avidin—Interface Effects on Noncovalent Associations. Avidin (M_r 64 kDa) is a glycoprotein found in egg white whose active form is a tetramer composed of identical subunits. Each subunit contains a polypeptide chain of 128 residues with a carbohydrate moiety linked to asparagine 17.[12] The tetramer species in solution is relatively stable throughout a wide pH range,[13] and observation of this protein by ESI-MS might therefore be less sensitive to solution conditions compared to the tetrameric forms of either Con A or Hb A_o.

Figure 2a illustrates intense ions (Q^{17+} to Q^{15+}) corresponding to the intact tetramer for a pH 6.7 solution of 10 mM NH_4OAc at a capillary heating of 27 W (~181 °C) and a ΔCS = 84 V. The M_r of 63 950 ± 30 Da determined experimentally from data for the three charge states correlates well with the expected M_r value of 63 870 Da. The somewhat high experimental M_r observed could be due in part to the difficulties in calibration of this high m/z region but is most likely due to solvation and adduction effects present at these relatively mild capillary heating conditions, i.e., the preservation of a range of specific and nonspecific interactions with small species from solution. The center of the tetramer ion distribution at ca. m/z 4000 demonstrates the advantages of an extended m/z range in detecting the intact quaternary structure. Previous experiments conducted with a more limited range quadrupole instrument (m/z < 1400) only allowed detection of the monomeric species but did reveal the heterogeneity of the intact subunit from the presence of different glycoforms (this inhomogeneity would not be resolvable at the low resolution characteristic of the low frequency quadrupole instrument).[14] Also evident in Figure 2a is a small monomer ion contribution at low m/z. The monomer ion contribution becomes more prominent with increased capillary heating, as the tetramer dissociates into individual subunits. The ESI mass spectrum for somewhat harsher interface conditions (35 W, ~211 °C) is shown in Figure 2b. Ions corresponding to the monomer (M^{12+} to M^{7+}) are observed over an m/z range of 1300–2300, in addition to a series of intense peaks (labeled T^{10+} to T^{6+}) at significantly higher m/z (4800–8000) that, surprisingly, can only be attributed to a trimer species. Trimers are not known to have physiological significance or be formed under the solution conditions used in this study. The fact that their appearance is correlated with the disappearance of the tetramer species under increased interface heating indicates they most likely arise from gas-phase dissociation. The formation of these low charge state, high m/z trimer ions also occurs in the ESI mass spectra of Con A and Hb A_o at harsher interface conditions and will be discussed later.

The observation of the intact tetramer of avidin in the gas phase is important for the utility of mass spectrometry as an analytical tool for biochemical problems since this represents the active form of avidin in solution. The good correlation of the known solution behavior of these species to the corresponding ions observed in the gas phase is evident. In addition to the avidin tetramer, the noncovalent association of biotin to avidin has also been observed with similar charge state distributions in the high m/z range, which explains why this relatively stable noncovalent complex has remained elusive to mass spectrometric analysis with limited m/z range quadrupole mass spectrometers. These avidin/biotin studies are described in more detail elsewhere.[1m] Much more subtle examples of solution condition effects, such as pH, on the formation of multimeric forms of proteins are illustrated by results for Con A and Hb A_o.

Concanavalin A—Solution pH Dependence of Associations Observed. Concanavalin A (M_r 102 kDa) is the jack bean lectin composed of four identical 237 amino acid polypeptides[15] that exists primarily as the tetramer in solution at pH > 7, and primarily as the dimer in solution at pH ~5.[16] No trimer species are known to be present in solution at either pH. A comparison of pH dependence on the ESI mass spectra of Con A in pH adjusted 10 mM NH_4OAc solutions are shown in Figure 3a for pH 5.7 and Figure 3b for pH 8.4 solutions. Ions indicative of the tetramer (Q^{22+} to Q^{20+}, ~ m/z 5000) dominate the mass spectrum obtained at pH 8.4 (Figure 3b). As expected based on solution behavior, the mass spectrum obtained at pH 5.7 (Figure 3a) contains peaks primarily due to the dimer species labeled D^{16+} to D^{12+} in the m/z range of 3500, although a smaller contribution from the tetramer is still evident. These spectra were obtained with the same relatively mild interface conditions (17 W, ~135 °C, ΔCS = 120 V). From this data, the experimentally determined molecular weights are 51.2 ± 0.2 kDa and 102.4 ± 0.5 kDa for the dimer and tetramer species, respectively. When a 5% acetic acid solution

(12) DeLange, R. J.; Huang, T.-S. *J. Biol. Chem.* **1971**, *246*, 698–709
(13) Green, N. M. *Adv. Protein Chem.* 1975, 29, 85–133.
(14) Smith, P. B.; Snyder, A. P.; Simmons, T. A.; Vestling, M. M. *Proceedings of The 41st ASMA Conference on Mass Spectrometry and Allied Topics*; San Francisco, CA, May 31–June 4, 1993; pp 76a–b.
(15) (a) Kalb, A. J.; Lustig, A. *Biochem. Biophys. Acta*, **1968**, *168*, 366–367. (b) Wang, J. L.; Cunningham, B. A.; Edelman, G. M. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 1130–1134.
(16) (a) McKenzie, G. H.; Sawyer, W. H.; Nichol, L. W. *Biochem. Biophys. Acta* **1972**, *263*, 283–293. (b) Huet, M. *Eur. J. Biochem.* **1975**, *59*, 627–632.

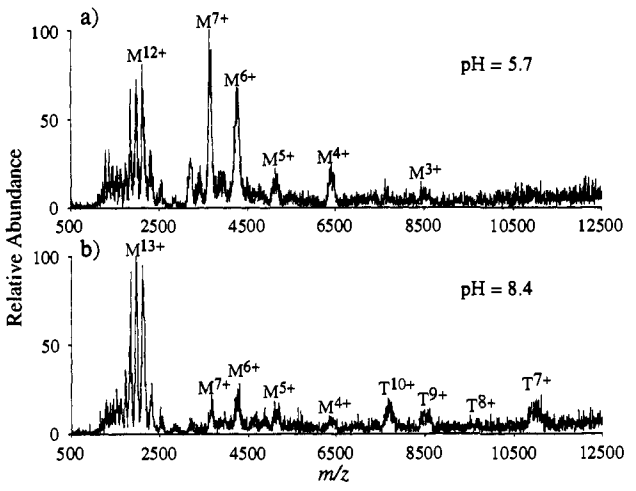

Figure 4. Positive ion ESI-MS of concanavalin A in 10 mM NH_4OAc at (a) pH 5.7 and (b) pH 8.4. The capillary heating was increased to 36 W or ~225 °C with the same ΔCS = 120 V.

(pH 2.5) of Con A is electrosprayed, only a broad distribution of peaks indicative of the monomer species at relatively high charge states (low m/z) is observed regardless of the interface conditions (data not shown). Only the monomer species is expected to be present in solution at this pH. The presence of the specific dimer and tetramer species under the appropriate solution conditions, and particularly in the absence of nonspecific trimer, pentamer, etc., suggests that these dimeric and tetrameric species observed by ESI-MS arise due to specific associations in solution as opposed to random aggregation during the electrospray process.

As the interface conditions are made increasingly harsh (i.e., capillary heating and/or capillary offset voltage are increased) these weak complexes dissociate to form primarily the monomeric subunits labeled M^{20+} to M^{4+}. This can be observed in Figure 4 for both pH solutions for 36 W capillary heating, ~225 °C, and ΔCS = 120 V. Figure 4a suggests contributions due to two different charge state distributions for the monomer ions, perhaps arising from different mechanisms of formation. The dimer ions were observed with an average charge state of 14+, and yet some of the monomer ions are observed with a charge state greater than 14+. This suggests that in some cases dissociation occurs prior to the charge state being "locked in" during the evaporation/ion formation process. The lower charge state monomer ions, on the other hand, most likely arise from dissociation of the dimer at a later stage of the desolvation process after the charge state of the ions has been determined. Indeed the lower average monomer charge state is almost half that for the dimer species under the more gentle interface conditions (Figure 4a). In the pH 8.4 solution mass spectrum shown in Figure 4b, a similar bimodal distribution of monomer species is observed. In addition, there are a few peaks that appear with increased energy added in the interface region at higher m/z (7000–11000) labeled T^{10+} to T^{7+}. These ions are attributed to the trimer of the polypeptide subunit of Con A, which is not known to be present in solution. These peaks are observed to have relatively low charge states suggesting they are formed after the initial ion formation process (i.e., in the gas phase). In addition they are only observed to a significant extent when a sufficient amount of tetramer is initially present in solution (as evident using gentler interface conditions) and with increased heating and/or activating collisions in the interface region. These observations will be discussed in more detail later.

Succinylconcanavalin A—Chemically Modified Subunit Effect on Quaternary Associations. Further support for the Con A observations were obtained with succinyl-Con A which is a

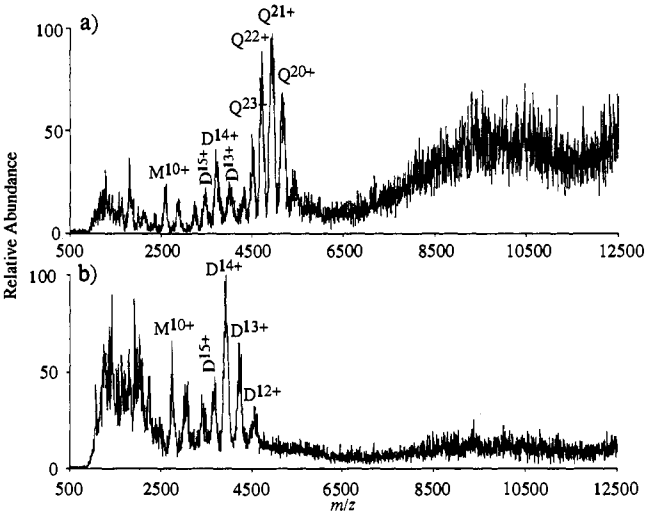

Figure 5. Comparison of the positive ion ESI-MS of (a) concanavalin A and (b) succinylconcanavalin A in 10 mM NH_4OAc at pH 8.4. For both spectra ΔCS = 71 V and 20 W was applied to the inlet capillary.

derivative of Con A that only forms a dimer in solution.[17] Therefore, if the tetrameric ions observed for Con A are related to specific solution associations, the tetrameric form should not be observed for succinyl-Con A by ESI-MS. Figure 5 shows a comparison of the ESI-MS of Con A and succinyl-Con A in 10 mM NH_4OAc solutions at pH 8.4 with identical atmosphere–vacuum interface conditions. In Figure 5a the dominant ions observed correspond to the tetramer of Con A in the m/z range of 4400–5400 for the 23+ to 19+ tetramer ions. However, for the succinyl-Con A solution, no ions corresponding to a tetramer are observed upon ESI-MS analysis (Figure 5b). Only ions corresponding to the dimer and monomer species are observed as labeled by D^{n+} and M^{n+}, respectively. This further supports our contention for Con A that the associations observed under mild interface conditions are specific, noncovalent, and derived from the solution associations.

Human Hemoglobin A_o—More Complex Tetrameric Associations. In contrast to the identical subunits composing avidin and Con A, adult human hemoglobin (Hb A_o, M_r 64.5 kDa) is a more complex noncovalent tetrameric protein composed of two different polypeptide chains, two identical α subunits (141 amino acids, 15.1 kDa), and two identical β subunits (146 amino acids, 15.9 kDa), in addition to the heme moiety (616 Da) noncovalently bound to each chain. Similar to Con A, the tetramer of Hb A_o is composed of two dimers, which for hemoglobin are hetero $(\alpha\beta)_2$. Previous studies have shown that it is possible to maintain the heme–globin complex during the electrospray process for myoglobin[1c] and the individual hemoglobin subunits[18] under appropriate solution and interface conditions. However, no observations of the physiologically significant dimeric or tetrameric forms of hemoglobin with heme have been reported by MS. Figure 6a shows the noncovalently heme bound αβ heterodimer of Hb A_o obtained by ESI-MS of a 10 mM NH_4OAc (pH 5.7) solution. The interface conditions used represented a compromise between the gentle conditions required to preserve the noncovalent associations while still providing sufficient desolvation to detect the ions. Three main charge states for the heme bound Hb A_o heterodimer are observed between m/z 2500 and 3500 for the 12+ to 10+ multiply protonated ions. These three charge states give an experimentally determined M_r of 32 600 ± 60 Da which is somewhat higher than the calculated M_r of 32 225 Da. It is very likely that residual solvation/adduction is

(17) Gunther, G. R.; Wang, J. L.; Yahara, I.; Cunningham, B. A.; Edelman, G. M. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 1012–1016.
(18) (a) Li, Y.-T.; Hsieh, Y.-L.; Henion, J. D.; Ganem, B. *J. Am. Soc. Mass Spectrom.* **1993**, *4*, 631–637. (b) Ganem, B.; Henion, J. D. *Chemtracts-Org. Chem.* **1993**, *6*, 1–22.

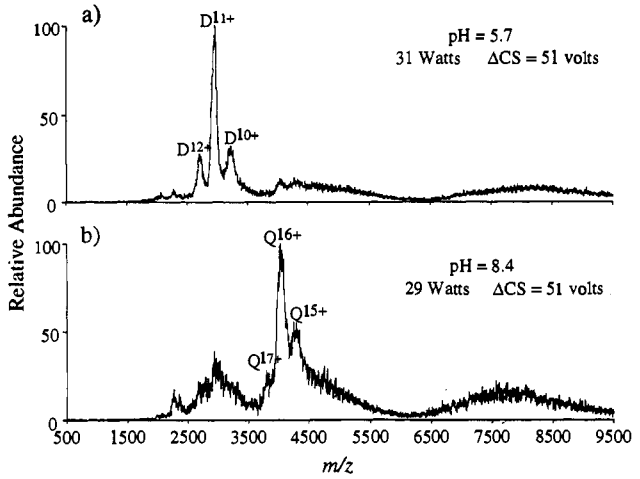

Figure 6. Positive ion ESI-MS of adult human hemoglobin A_o in 10 mM NH_4OAc at (a) pH 5.7 and (b) pH 8.4. For both spectra ΔCS = 51 V with only slightly different capillary heating to optimize for the observation of the noncovalent associations (a) 31 W and (b) 29 W.

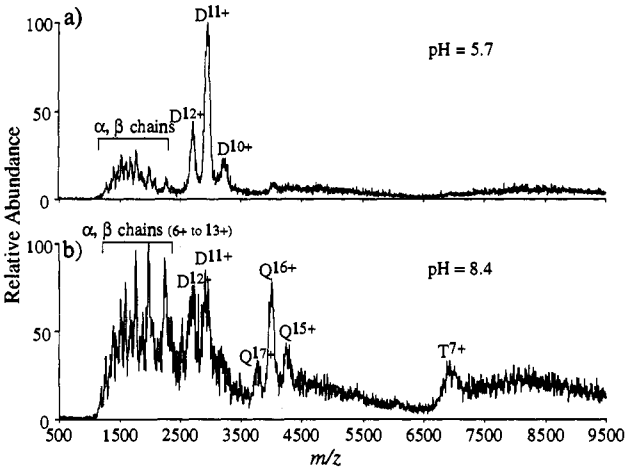

Figure 7. Positive ion ESI-MS of adult human hemoglobin A_o in 10 mM NH_4OAc at (a) pH 5.7 and (b) pH 8.4. For both spectra ΔCS = 61 V with 31 W applied to the heated inlet capillary.

present due to the mild interface conditions utilized in order to preserve the weak dimer associations. When the solution is adjusted to pH 8.4 (10 mM NH_4OAc) under very similar interface conditions, the dominant species corresponds to the intact heme bound tetramer of Hb A_o between *m/z* 3700 and 4300 at the 17+ to 15+ charge states (Figure 6b). The experimentally obtained M_r from these three ions is 64 500 ± 100 Da, which is close to the calculated value for the tetramer (64 450 Da). In addition there appears to be a small dimer contribution around *m/z* 3000.

Increasing the heating and/or collisional excitation in the interface region is expected to dissociate the dimeric and tetrameric species if they are due to the specific noncovalent associations present in solution. This is shown in Figure 7a,b for the pH 5.7 and 8.4 solutions of Hb A_o, respectively, where the increased capillary–skimmer voltage offset (ΔCS = 61V) induces partial dissociation of the noncovalent dimers and tetramers. A comparison of parts a and b of Figure 7 shows the tetramer species (Q) to be less stable than the dimer (D) species, as evidenced by the relative abundance of these respective ions compared to the monomer subunit ions under the same interface conditions, consistent with solution behavior. The ions indicative of the α and β subunit chains are now observed with and without heme attached at the lower *m/z* range of 1000–2500. The low resolution of this instrument limits the exact identification of the single chain ions due to the overlapping of the peaks for the two chains with and without heme. Results obtained for these solutions

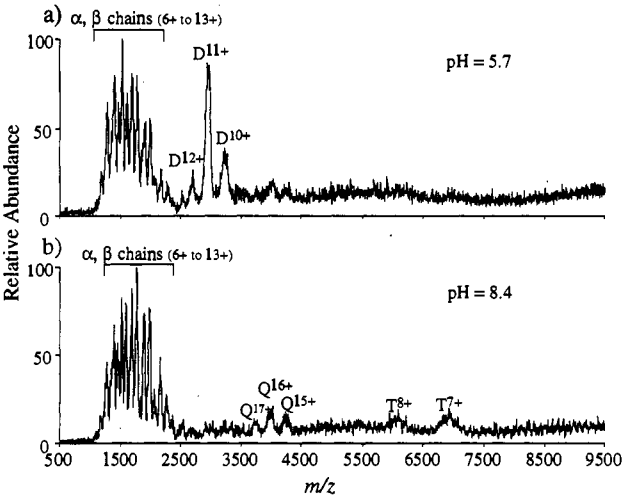

Figure 8. Positive ion ESI-MS of adult human hemoglobin A_o in 10 mM NH_4OAc at (a) pH 5.7 and (b) pH 8.4. For both spectra ΔCS = 106 V with 31 W applied to the inlet capillary.

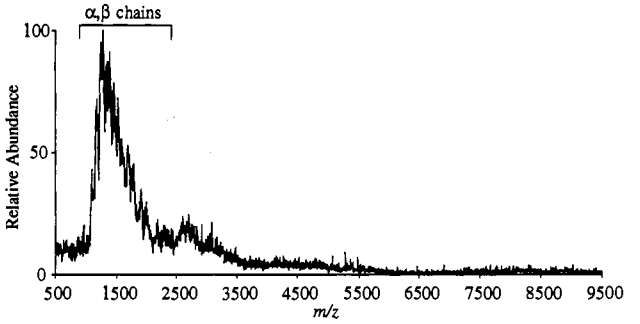

Figure 9. Positive ion ESI-MS of adult human hemoglobin A_o in 5% HOAc with ΔCS = 71 V with 31 W applied to the inlet capillary. The interface conditions are very similar to those utilized to obtain the spectra shown in Figure 6 for the 10 mM NH_4OAc solutions.

with a Sciex TAGA 6000E triple quadrupole mass spectrometer with a *m/z* range of 10–1400 showed resolvable peaks representative of both the α chain and the β chain species with and without heme (data not shown), similar to those reported by other workers.[18]

Increasing activation in the interface further (ΔCS = 106 V) results in more dissociation of the noncovalent complexes, as shown in Figure 8a for the dimeric species (pH 5.7) and Figure 8b for the tetrameric species (pH 8.7). Under these interface conditions the tetramer is almost completely dissociated and a new series of peaks emerges at higher *m/z* . These ions correspond to the trimer ($\alpha\beta\beta$ and/or $\alpha\alpha\beta$ with three heme moieties) even though there is no evidence for the existence of these species in solution. This observation is very similar to that for avidin and Con A where these nonphysiological "trimer" species are detected at higher *m/z* than the corresponding tetramer ions (therefore, at lower charge states) and are only observed from solutions containing the tetramer species and when extra energy (either thermal or collisional) is added in the interface region. In addition, as more energy is input in the interface region, these Hb trimer ions shift to lower *m/z*, by an amount corresponding to the apparent loss of the heme groups. Further dissociation of the trimers has not been observed by increasing the interface conditions (capillary heating and/or collisional heating (ΔCS)). Similar gas-phase stability is observed for the trimer ions of avidin and Con A, and the evidence suggests these trimers arise from a gas-phase dissociation process of the tetrameric species.

Neither the tetramer ions nor the dimer ions of Hb A_o are observed in the mass spectra under "conventional" electrospray conditions (i.e., a 1–5% acetic acid solution), regardless of the interface conditions. Figure 9 shows the ESI mass spectrum

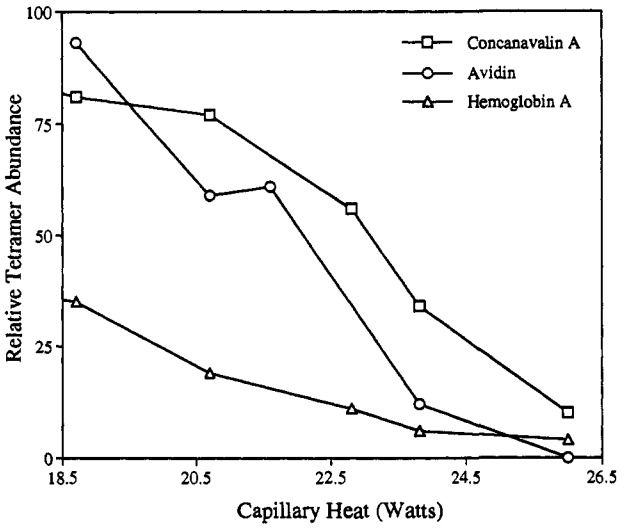

Figure 10. Qualitative plot of the relative abundance of the tetramer species of avidin, concanavalin A and hemoglobin A_o as a function of inlet capillary heating obtained on the same day with identical operating conditions and the same ESI source position.

obtained for Hb A_o in 5% HOAc (pH 2.5) with interface conditions similar to that utilized to obtain the spectra of the pH 8.4 and 5.7 solutions shown in Figure 7, where the noncovalent associations are observed. The only significant ion current is at the low *m/z* region from the unresolvable distribution of charge states for the α and β subunits of Hb A_o. This is expected based on solution behavior as the tetramer and dimer species are unstable in solution at acidic pH.[19]

Relative Stability of the Tetrameric Forms of Avidin, Con A, and Hb A_o. In addition to observing the tetrameric proteins by ESI-MS, it is also of interest to determine the relative stabilities of these tetramers utilizing mass spectrometry. As shown here, the interface conditions of the mass spectrometer can greatly influence the mass spectra obtained for a given solution. However, under mild interface conditions it is possible to maintain noncovalent associations; therefore, it may be possible to use mass spectrometry to determine the relative stability of these specific complexes. In these studies, the metal inlet capillary heating and the capillary offset voltage were the variables used to affect the associations. Five different capillary heating conditions and four different capillary offset voltages were used to study the effects of these two variables on the observation of the tetrameric forms of avidin, Con A, and Hb A_o in 10 mM NH_4OAc at pH 8.4. These experiments indicated that the hemoglobin tetramer is much more labile with increasingly harsh interface conditions (i.e., increasing ΔCS or the inlet capillary temperature), whereas the avidin and concanavalin A tetramers are relatively stable and behave very similarly (Figure 10). This behavior qualitatively mirrors that in solution where the dissociation constant of the tetrameric Hb A_o under similar solution conditions is much higher than that for either avidin or Con A.[20] Another difference evident in Figures 11 and 12 involves the relative abundance of the tetramer and monomer forms of Con A and Hb A_o, as a function of the capillary offset voltage. For high *m/z*, low charge state ions, ΔCS should be less effective than capillary heating for inducing dissociation. Increasing ΔCS at a constant capillary heating induces dissociation of the tetramer of Hb A_o into the monomeric α and β chains (Figure 12). In contrast, ΔCS had little effect on the tetrameric forms of Con A (Figure 11) or avidin (data not shown) under relatively mild capillary heating conditions. Once again, the Hb A_o tetrameric form dissociates more readily than Con A or avidin based on this mass spectrometric study. These results suggest that it may be possible to obtain qualitative information on relative stabilities with ESI-MS using carefully controlled experiments.

(19) Chiancone, E. Gilbert, G. A. *J. Biol. Chem.* **1965**, *240*, 3866–3867.

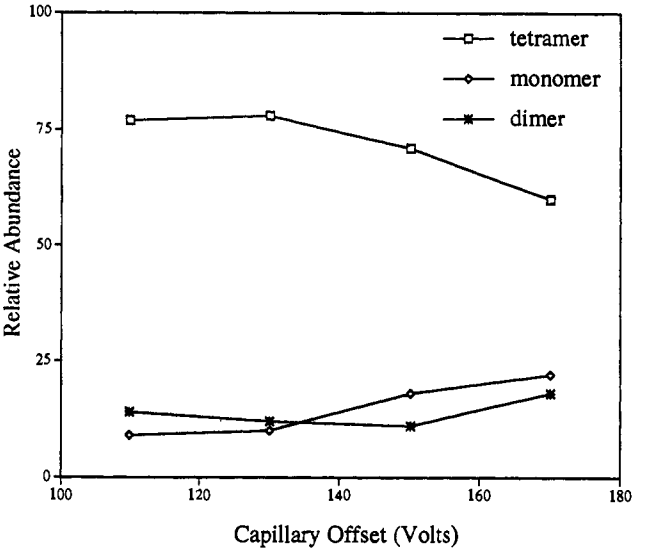

Figure 11. Plot showing the small change in the relative abundance of the tetrameric, dimeric, and monomeric species of concanavalin A when increasing the capillary offset voltage at a constant mild capillary heating (18.7 W).

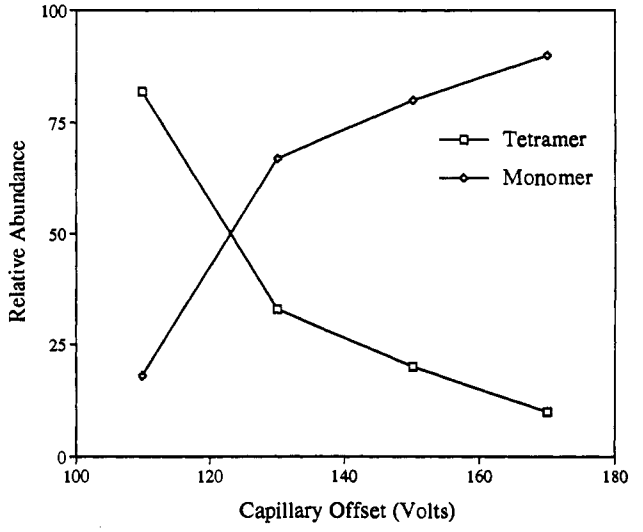

Figure 12. Plot showing the dramatic change in the relative abundance of the tetrameric and monomeric species of hemoglobin A_o when increasing the capillary offset voltage at a constant mild capillary heating (18.7 W).

Discussion

The determination of protein quaternary structure as well as its subunits has been demonstrated to be feasible by ESI-MS. The results presented here show that the specific noncovalent associations of avidin, Con A, and Hb A_o in solution can be probed by ESI-MS under carefully controlled interface conditions using an extended *m/z* range ($m/z > 3000$) quadrupole instrument. Adjusting the atmosphere–vacuum interface conditions allows the observation of the intact noncovalent complexes or the dissociation of these weak complexes into their individual subunits. The pH dependence of the ESI mass spectra obtained for Con A agrees well with its known solution behavior; that is the tetramer is more prevalent at pH > 7 and the dimer is the primary species at pH $\sim$ 5. The pH dependence of the Hb A_o ESI spectra can be related to the known solution behavior of Hb A_o as well. In the oxidized form, the Hb A_o tetramer is more stable at higher pH, and the dimer is more stable at lower pH.[20c] The commercially

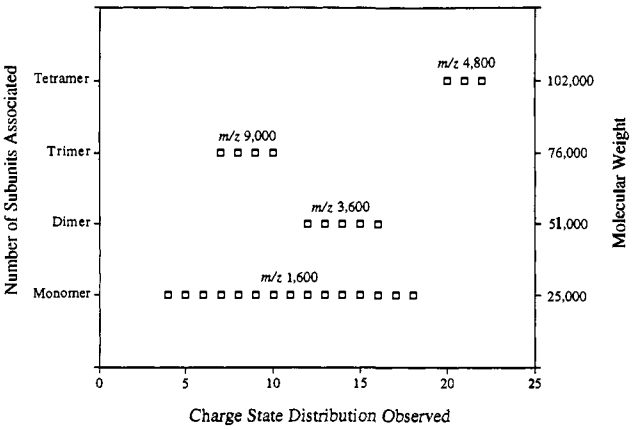

Figure 13. Plot of the charge states observed vs the number of subunits associated for the four species observed for Con A. In addition, the average *m/z* value where each species is observed is provided above each charge distribution.

obtained samples of Hb A_0 are most likely to be primarily in the oxidized form without careful sample handling.[18a] Therefore, as expected based on solution behavior,[20c] the tetramer is the primary species observed from the ESI-MS analysis of the pH 8.4 solution and the heterodimer of Hb A_0 is the main species observed upon ESI-MS analysis of the pH 5.7 solution. In addition, the succinylated form of Con A, which is known to exist as a dimer in solution even at the higher pH conditions where Con A is primarily tetrameric, is observed only as a dimer in the gas phase with gentle interface conditions. Qualitatively, the relative stabilities of the tetrameric forms of avidin, Con A, and Hb A_0 determined by ESI-MS closely resemble the known behavior in solution based on their dissociation constants.[20] Therefore, at least at a qualitative level, the solution behavior can be probed by ESI-MS under appropriate interface conditions. This correlation of noncovalent solution behavior with ESI mass spectra may lead to the development of ESI-MS as a rapid screening technique for determining relative strengths of noncovalent associations in solution.

Careful inspection of the spectra presented here provides further insight into the electrospray ion formation process. The first general observation is that for all three tetrameric proteins studied here, the multimeric forms (i.e., dimer, trimer, and tetramer) are observed to have very narrow charge state distributions of approximately three to four charge states, whereas the monomer species are observed over a broader distribution of more than eight different charge states (like typical ESI spectra obtained under acidic conditions). This is shown in Figure 13 for the plot of the charge states observed for the different species of Con A (compiled from the data shown in Figures 3 and 4). Also, there is a general trend of increasing *m/z* with an increase in the number of subunits associated, with the exception of the trimer species. Therefore, the average charge per subunit observed has the order of monomer > dimer > tetramer. For example, the monomer species is observed with an average charge state of 11+, whereas the tetramer is observed at approximately 20+ which is an average of five charges per subunit. The narrow charge state distribution and lower average charge per subunit for the multimeric species is ascribed to the remaining higher order structure (at least at the time of charge state determination) and therefore to restrictions in the number of available protonation sites as well as indirect Coulombic restraints on the proximity of charge sites in a more compact structure. This is consistent with observations of lower charge states for a "native" monomeric protein that has retained some higher order (secondary) structure compared to the unfolded "denatured" form.[21] These results suggest that the narrow charge state distribution evident for the intact quaternary associations can be at least partially attributed to preservation of a well defined structure during ion formation. The corollary of this suggestion is that the typically broad charge state distribution observed for denatured proteins may arise, in part, due to structural heterogeneity of the protein during ion formation.

The observation of the trimer species for all three proteins studied is intriguing. There is no evidence to suggest the presence of these trimer species under the solution conditions utilized. The trimer species, formed only with increased activation in the interface, is observed at higher *m/z* and lower average charge per subunit than the tetramer species. In Figures 3b and 4b, the tetramer ions of Con A are observed at approximately the 21+ charge state, and, upon dissociation, the monomer ions are produced with an average charge state of 13+ and trimer ions of approximately 8+. The tetramer is composed of four equivalent polypeptide chains, and it seems unlikely that it would dissociate with such an asymmetric distribution of charge and mass, even though the observations suggest this. If the corresponding trimer and monomer species are in fact complementary ions from the dissociation of tetramers, it is surprising that one subunit could strip away approximately 60% of the charges, leaving a trimer with the remaining 40% of initial charges. Without the advantage of MS/MS experiments on the dissociation of the tetramer and trimer ions it is difficult to determine the pathway of forming these ions and their dissociation characteristics. There is additional unresolved ion current at much higher *m/z* that may also contribute to the interface dissociation spectra and the formation of these unexpected trimer ions. However, since the trimer ions are only observed when analyzing solutions that exhibit strong contributions of the tetramer species and under interface conditions where the tetramer dissociates, it follows that the tetramers are the precursors to the trimer. Also the trimer ions are observed at higher *m/z* than the tetramer ions and thus could have been formed by dissociation of the tetramer ions at the later stages of ion formation/desolvation and after charge state determination for the complex. These observations suggest that trimer ions are formed during a gas-phase dissociation process.

The other interesting feature of the trimer species common to the proteins investigated is their stability. Increasing the capillary–skimmer voltage offset or the inlet capillary heating does not lead to the dissociation of these trimer species. However, it must be noted that there is less effective collisional activation due to ΔCS as *m/z* increases, and, therefore, there may be insufficient collisional activation to dissociate even weak noncovalent associations at such high *m/z* values where these trimer ions are observed. In addition, it is possible that these ions are formed late in the ion transport process and therefore may have little opportunity for additional heating. It is unclear why this stable trimer species would be formed upon gas-phase dissociation of the noncovalent tetramer species. We speculate that dissociation of the tetramer may occur by a Coulombically driven process in which a monomer species becomes "unraveled" and ejected from the aggregate (i.e., the remaining is a compact trimer) with a disproportionately large share of the charge. Such a process would be analogous to asymmetric droplet breakup at larger size scales, and the low charge trimer produced by such a process would be more stable to subsequent activation in the interface due to its relatively high *m/z* .

It appears that more than one ion formation mechanism may contribute to production of the ions observed in the dissociation spectra shown in Figures 2b, 4b, and 8b for avidin, Con A, and

(20) (a) The K_D of avidin complexed to biotin in a 1:1 noncovalent complex of each avidin subunit within the avidin tetramer is 10^{-15} M. (b) K_A of Con A in a phosphate buffer with pH > 7.5 is 10^{12}. Therefore, the K_D is 10^{-12} M. Senear, D. F.; Teller, D. C. *Biochemistry* **1981**, *20*, 3076–3083. (c) K_D of oxyhemoglobin tetramer at pH 8.5 = 3×10^{-8} M. Atha, D. H.; Riggs, A. *J. Biol. Chem.* **1976**, *251*, 5537–5543.

(21) (a) Loo, J. A.; Ogorzalek Loo, R. R.; Udseth, H. R.; Edmonds, C. G.; Smith, R. D. *Rapid Commun. Mass Spectrom.* **1991**, *5*, 101. (b) Le Blanc, J. C. Y.; Beuchemin, D.; Siu, K. W. M.; Guevremont, R.; Berman, S. S. *Org. Mass Spectrom.* **1991**, *26*, 831–839.

Hb A_o, respectively. If the trimer ions and monomer ions were complementary, then the number of different charge states of each species would be expected to be the same. In all three cases, there is a wider charge state distribution for the monomer ions observed than for the trimer ions observed (as shown in Figure 13). Therefore, other mechanisms of monomer ion formation must be proposed to help account for the different charge state distributions of the monomer species. One possible explanation for forming relatively high charge state monomer ions is that some fraction of the tetramer species dissociates in the electrospray process ***prior*** to complete desolvation and before the charge state is determined. Once in the monomer state in solution, more charging per subunit could presumably occur, perhaps approaching the high charge state situation observed for the denatured monomer under acidic conditions. These high charge state monomer ions are observed for avidin, Con A, and Hb A_o. In addition to this high charge state monomer ion distribution, the Con A dissociation spectrum also contains a lower charge state distribution of monomer ions that may originate from the dissociation of the tetramer *after* charge state determination. This additional monomer ion distribution is observed for dissociation of both the dimer and the tetramer species.

Unfortunately, these tetrameric and trimeric species have only been observed, to date, on instrumentation that is incapable of performing MS/MS experiments, necessary to trace the dissociation mechanisms. Until these tandem mass spectrometry experiments are possible, only speculations on the ion formation processes can be presented. A simplified overview of some of the possible pathways of forming the ions observed here for avidin, Con A, and Hb A_o is outlined in Figure 14. In bulk solution, the monomer, dimer, and tetramer forms of the multimers are known to exist depending on the solution conditions. The first step, as shown in Figure 14, is the transfer of the solution-phase species to the vapor phase via the electrospray process. Based on the pH studies presented, the species present in solution can be transferred to the gas phase by electrospray ionization. Careful selection of the atmosphere–vacuum interface conditions is clearly crucial in preserving the weak noncovalent associations, as demonstrated here. Under some conditions these noncovalent complexes may dissociate prior to complete desolvation (Figure 14, step 2). Dissociation of these multimers can occur at any stage of the ion formation/ion desolvation process; for instance within the droplet before the charge state has been determined, which we believe may lead to higher charge state monomer ions, or after the desolvation process and charge state determination (Figure 14, step 3), which may result in relatively lower charge state species. Further studies, including the use of tandem mass spectrometry will be necessary to gain a better understanding of the ion formation pathways relevant to the dissociation processes of multimeric proteins.

Conclusions

The successes obtained here for preserving the tetrameric proteins into the gas phase by ESI-MS stress the exciting potential of ESI-MS for probing biochemically complex systems with mass spectrometry and the need for instrumentation with an extended *m/z* range. They also highlight the broad potential for ESI-MS studies of noncovalent associations and provide further suggestions that a surprising degree of higher order structure may be preserved upon transfer to the gas phase. The present studies have also established a definite correlation between the associations present in solution and those observed upon carefully controlled ESI-MS experiments. Changes in solution associations by pH adjustment or chemical modifications to the subunit (succinyl-Con A) have been shown to result in corresponding changes in the ESI mass spectra. Therefore, ESI-MS can be used to probe relatively weak noncovalent solution associations under appropriate interface conditions. In addition, our results indicate that relative stabilities of noncovalent complexes in solution may be obtainable (at least for widely varying stabilities) by comparing their relative stabilities in the gas phase via ESI-MS.

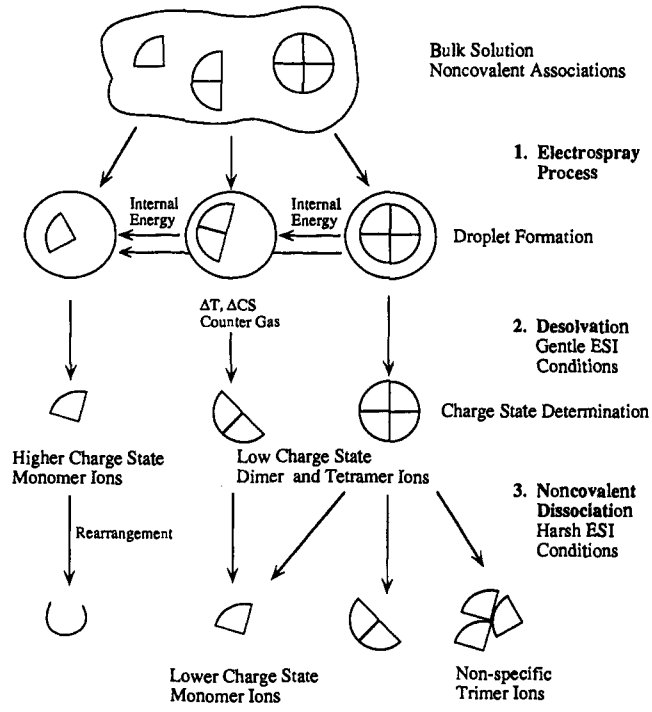

Figure 14. Possible dissociation pathways of the different noncovalent associations present in solution to produce the ions observed by ESI-MS.

Acknowledgment. We are grateful for the helpful discussions with Drs. D. R. Goodlett and H. R. Udseth and the encouragement and initiation of these studies by Dr. B. E. Winger. We thank the U.S. Department of Energy through Laboratory Directed Research and Development of Pacific Northwest Laboratory for support of this research. Pacific Northwest Laboratory is operated by Battelle Memorial Institute for the U.S. Department of Energy, through contract DE-AC06-76RLO 1830.