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Formation of the serine octamer: Ion evaporation or charge residue?

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Abstract

The mechanism of formation for clusters of serine generated by electrospray ionization is hypothesized to play a critical role in determining their ultimate properties. Under carefully manipulated electrospray source conditions, two distinct and well-separated distributions of clusters can be observed. The characteristics of the two cluster populations are consistent with different formation mechanisms, namely ion evaporation and charge residue. Upon further inspection, it is proposed that the magic number intensity, homochiral selectivity, and unique formation of the serine octamer are best explained within the context of the ion evaporation mechanism. As a consequence, solution phase properties of the octamer become important, particularly in relation to interface effects present on the surface of the charged droplet. In contrast, other clusters of serine, including the B form of the octamer, are probably generated by the charge residue mechanism and may have no connection to condensed phase phenomena.

Keywords: Chirality; Cluster; Electrospray; Threonine; Alanine

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1. Introduction

Since the discovery of the unusual serine octamer by Cooks et al. [1] this cluster has drawn the interest of many groups [2–5]. When sampled from a variety of different sources, the serine octamer exhibits several intriguing properties. First, it is an unusually abundant cluster, exhibiting intensities far exceeding those clusters which surround it. Second, the unusual abundance is connected to the enantiomeric composition, with the homochiral or enantiopure form of the cluster leading to the most intense octamer peaks. Third, the degree of unusual abundance is also strongly dependent on the precise source conditions [6]. In fact, under appropriate gentle sampling conditions, the serine octamer is virtually the only species observed [1,7]. Finally, the serine octamer is one of few known examples where spontaneous resolution occurs in an abiotic chemical system, leading to connections with origin of life studies [8].

Despite the application of various experimental and theoretical techniques, there are a variety of questions about the serine octamer which remain unresolved. For example, a consensus for the exact structure of the octamer has not been reached. Part of the difficulty relates to the existence of two forms of the octamer. H/D exchange experiments have demonstrated the presence of two serine octamer structures (called the A and B forms) under certain conditions [9,10]. The relationship between the A and B forms has not been explained. In addition, no reason for the absence of magic octamers among other closely related amino acids has been forthcoming. For example, threonine, which differs by the addition of a single methyl group, does not have the same propensity as serine to form octamers [8,11]. In addition, recent studies employing NMR and IR spectroscopy suggest that the serine octamer does not exist in bulk solution, raising the question as to whether the octamer is purely a gas phase phenomenon [12].

The majority of experiments examining serine clusters have utilized electrospray ionization (ESI) or sonic spray ionization (SSI) coupled with mass spectrometry (although recent results utilizing sublimation may be tangentially related) [13]. Therefore the mechanism by which ions are obtained from charged droplets during the ESI process is of vital interest in relation to the origin of the serine octamer itself. Unfortunately, the mechanism by which ESI generates ions is not universally accepted. SSI has been studied even less. Nevertheless, there are two frequently employed ideas: charge residue and ion evaporation.

Ion evaporation is thought to occur in small, highly charged droplets which emit nearly desolvated ions [14–16]. The solva-

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tion properties of the ion therefore play an important role in ion evaporation because the ion must be removed from solvent [17], which is always an energetically unfavorable process. This "desolvation penalty" can be reduced by the addition of hydrophobic groups [18] or by surrogate solvation with noncovalent adducts [19], increasing the surface activity and observed ion abundances for these ions relative to fully solvated ions. If a cluster (as opposed to a molecule) is generated by ion evaporation, then the cluster *must exist* in the droplet prior to ionization. As a consequence, the solution phase properties of the cluster will determine (in part) whether observation of that cluster is favored or not. For a system such as the serine octamer, where multiple structures are possible, the properties of each structure will influence its suitability for ion evaporation. Most recent work supports the ion evaporation mechanism as the likely source for small ions; therefore, it is not likely to generate large molecular clusters [20].

The charge residue mechanism generates ions by sequential Coulomb explosions in conjunction with solvent evaporation, eventually removing all of the solvent and leaving "residue" charges on the molecule(s) of interest [21,22]. Support for the charge residue model comes primarily from work done on proteins and other large molecules. Clusters generated by charge residue *do not necessarily exist* in solution, but rather may be formed in the final steps of desolvation if the interactions between the monomers are strong and the initial concentration of monomers is high. A similar process can lead to the observation of anomalous protein adduct complexes which do not exist in solution [23]. Therefore, clusters generated by charge residue are not likely to exhibit any strong connections to solution phase properties and are likely to be large and multiply charged.

Herein we present results which suggest that the serine octamer is generated by a different mechanism than other serine clusters which are observed in ESI-MS experiments. Under appropriate conditions, spectra can be obtained where the octamer is virtually the only peak present. If the capillary temperature is then raised, an additional distribution of very large clusters at high *m/z* is obtained. We postulate that the A form of the serine octamer is generated by ion evaporation while the B form and most (if not all) remaining clusters are formed directly or indirectly by the charge residue mechanism. Additionally, experimental results for clusters of threonine, alanine, and valine are compared with those of serine.

2. Experimental

All mass spectra were obtained using a Finnigan LTQ linear ion trap mass spectrometer (Thermo Electron, San Jose, CA) equipped with a standard electrospray ionization source with no modification. Specific ion optic parameters were as follows: capillary voltage 16 V, spray voltage 4 kV, and the tube lens voltage was 90 V or −35 V as indicated in the spectra. The capillary temperature was 120 °C or 215 °C as indicated in the spectra. L-Serine, L-alanine, L-valine, and L-threonine (Fluka, ultra purity of ≥99.5%) were purchased from Sigma–Aldrich (St. Louis, MO) and were used as received. Acetonitrile (EMD Chemicals Inc., Gibbstown, NJ), NANOpure DIamond (Macalaster Bicknell Co., New Haven, CT) purified water and acetic acid (EMD Chemicals Inc., Gibbstown, NJ) were used for all solutions. Amino acid solutions were 0.01 M in 49.95/49.95/0.1 water/acetonitrile/acetic acid (v/v/v).

The aqueous solvation energies were calculated using the Spartan 06 (Wavefunction Inc., Irvine, CA) implementation of the SM5.4 model of Cramer and co-workers [24]. Structures were taken from Ref. [2].

3. Results and discussion

A typical ESI-MS spectrum for a 0.01 M solution of serine is shown in Fig. 1a. Although the serine octamer is the most abundant peak, it is clear that other clusters are present in significant abundance, including larger doubly and triply charged clusters. Similar spectra have been obtained on various instruments with different source configurations. Collisional heating introduced by an additional 60 V accelerating potential at the exit of the source region of the instrument leads to the spectrum shown in Fig. 1b. Notably, the protonated dimer is much more intense, accompanied by reduced intensity for the remaining clusters. The relative intensity of the octamer to the remaining clusters has not increased, suggesting that it is not unusually stable once in the gas phase. If it were, then presumably the other clusters would dissociate at lower energies than the octamer, enhancing its abundance relative to the other clusters. This is not observed to happen. Therefore, it is not possible to preferentially retain serine octamer by heating and dissociating the other clusters in the gas phase. Furthermore, since the unusual abundance of the serine octamer is not due to exceptional gas phase stability, the results suggest that the origin of its unusual abundance must occur prior to introduction into the gas phase.

However, it is possible to obtain spectra in which the serine octamer is the dominant species [1]. The data shown in Fig. 1c was obtained under much gentler source conditions. In the LTQ, this can be achieved by lowering the tube lens voltage substantially below levels that would typically be used for analytical analysis. Reducing the magnitude of the fields in the tube lens allows ions to be transmitted with little or no collisional heating, but at the cost of several orders of magnitude in ion count. In the absence of collisional heating, it is possible for charged droplets to penetrate further into the mass spectrometer. This favors the formation of species generated directly from droplets (i.e., by ion evaporation). The droplets themselves are subsequently lost by colliding with an offset skimmer. As seen in Fig. 1c, the serine octamer is virtually the only species present under these very mild conditions. Nearly identical results were obtained previously on a home built instrument with a capillary/ion funnel interface [7]. By reducing the DC drop between consecutive elements in the source region of this instrument, a spectrum dominated by serine octamer was produced (also at the cost of several orders of magnitude in ion count).

These results suggest that the octamer is formed by a mechanism which does not rely on rigorous droplet evaporation or collisional heating and differs from that which yields other serine clusters. Further support for this notion is provided in Fig. 1d. This spectrum was acquired under identical conditions

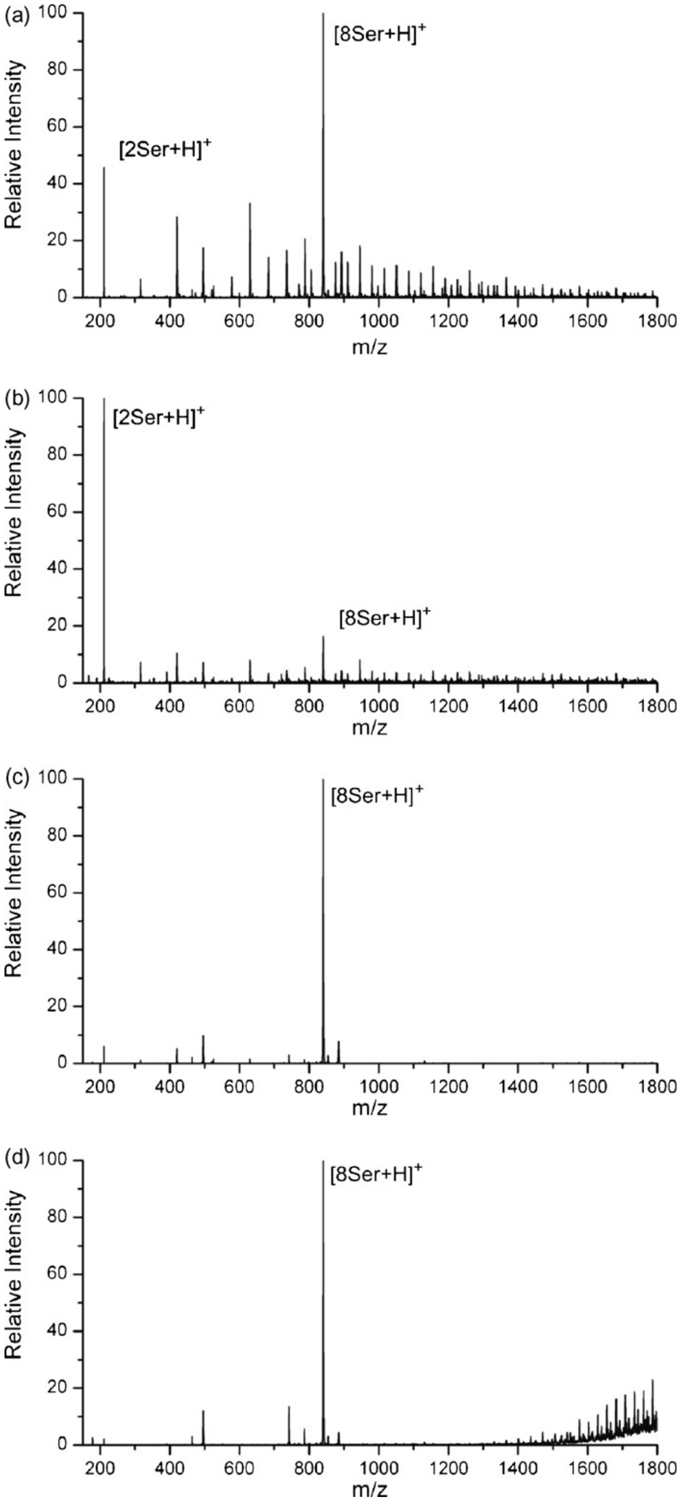

Fig. 1. (a) Typical ESI-MS spectrum of serine clusters, although the octamer is a magic number cluster, other clusters are present in significant intensity. (b) Spectrum of serine clusters with additional collisional heating applied at the exit of the source. The relative intensity of the octamer and all large clusters is reduced. (c) ESI-MS spectrum of serine clusters acquired under gentle conditions. The serine octamer is virtually the only cluster observed. (d) This spectrum was obtained under identical conditions to (c) except that the capillary temperature was increased by ∼95 °C. The cluster distribution on the right represents quadruply charged clusters with 60 or more serines.

to that shown in Fig. 1c, with the exception of increased capillary temperature from 120 °C to 215 °C. The increased capillary temperature leads to the re-emergence of other clusters, but only at the high end of the *m*/*z* range. Close examination of these clusters reveals a dominant distribution with peaks separated by ∼26 *m*/*z*, indicating the presence of quadruply charged clusters containing approximately 60 serines or more. The serine octamer is still present as the dominant peak, but there is an obvious separation between two populations of clusters. This separation is strongly suggestive that the two cluster populations are formed by different mechanisms.

Based on the data shown in Fig. 1, we postulate that the serine octamer is formed by ion evaporation. As mentioned in the introduction, small molecules (or clusters) are the best candidates for ion evaporation. The serine octamer is the same size as a small peptide and is therefore a viable candidate for ion evaporation. The fact that no other small clusters of serine are generated by ion evaporation suggests that the serine octamer must have special properties that distinguish it from other small clusters of serine. This issue is addressed in terms of solvation properties at the surface of charged droplets further below. The remaining serine clusters observed in Fig. 1d are much larger in size, approaching the size of proteins. Therefore, it is much more likely that these clusters are formed by the charge residue mechanism. Under typical source conditions, heating of these larger clusters would lead to break-up and eventually yield the full cluster distribution observed in Fig. 1a. Thus the “typical” full spectrum is a concatenation of clusters generated by ion evaporation and charge residue, with the ultimate size of the charge residue clusters being dictated by the amount of collisional heating applied in the source region of the instrument.

The proposed break-up mechanism explains how the A and B forms of the serine octamer could be created. The results are most consistent with the A form being generated directly by ion evaporation and the B form being made by fragmentation of larger charge residue clusters. In order to test this assertion, the very different properties of the A and B forms of the serine octamer can be used to identify which population is present in the current experiments. The B form has no preference for homochirality, and is formed in equal abundance from racemic and enantiopure solutions [8]. Furthermore, the B form is less stable, preventing characterization by ion mobility or isolation in an ion trap [2,5]. The A form has a strong homochiral preference, and it is much more abundant when sampled from an enantiopure solution [1,2,28]. We have confirmed that the serine octamer in Fig. 1c is principally the A form by measuring the chiral selectivity of this cluster. The octamer/dimer ratio drops from 20:1 to 1:1 when the solution is changed from enantiopure to racemic, demonstrating that this octamer has a strong preference for homochirality. Similarly, we generated serine octamer via successive dissociations of larger clusters to test the chiral selectivity of an octamer generated by fragmentation in the gas phase. The resulting octamer to dimer ratios for two different starting clusters from enantiopure and racemic solutions were (3:1, 2:1) for one cluster and (10:1, 9:1) for the other. Each pair of numbers should be considered independently. The differences between each pair are within the experimental uncertainty, meaning that serine octamers generated by dissociation of larger clusters have no preference for homochirality. This result is in agreement with what is known about the B form and simultaneously confirms that serine octamers and other small serine clusters can be generated by the break-up of larger clusters.

Previously, the enhanced presence of serine octamer under gentle source conditions had been interpreted as evidence for generation via the charge residue mechanism [9]. Although this was a reasonable conclusion at the time (i.e., if a gentle source equates with increased octamer, then the octamer is formed by a gentle mechanism), genesis by ion evaporation is in better agreement with the present results; namely, a gentle source leads to enhanced octamer abundance by obscuring the presence of other larger clusters. In fact, later studies showed that extremely large clusters of serine are generated by gentle sonic spray ionization [3]. Assuming that the serine octamer is generated by ion evaporation, there must be barriers that prevent the assembly of magic octamers for the remaining amino acids or for other small clusters of serine itself.

3.1. Related amino acids

Rather than examining all amino acids, we will focus on several that are closely related to serine. Threonine is the most obvious choice because it differs from serine by one additional methyl group. Experimental results for threonine are shown in Fig. 2. Under typical analytical conditions, threonine does not exhibit the same degree of clustering as serine (Fig. 2a). Threonine monomer and dimer dominate the spectrum while the threonine octamer is present only as a very small peak. Under the same gentle source conditions described above, somewhat larger clusters are observed. Even numbered clusters including the dimer and tetramer dominate the spectrum. The threonine octamer is more abundant under these conditions, though not the dominant peak as the serine octamer is in Fig. 1c. It is likely that all of these small threonine clusters are formed by ion evaporation, including the threonine octamer. Therefore, the addition of a single methyl group (which slightly enhances hydrophobicity relative to serine itself) enables clusters smaller than octamers to be generated by ion evaporation. Increasing the capillary temperature again reveals the presence of larger clusters in substantial abundance (Fig. 2c). These clusters are probably formed by the charge residue mechanism because they are very large and well separated from the smaller clusters.

An additional piece of evidence from an unlikely source supports the notion that very gentle source conditions favor ion evaporation in these experiments. In Fig. 2b, a contaminant peak (*) emerges exhibiting substantial relative intensity. This peak actually corresponds to the protonated threonine adduct of dioctyl phthalate (DOP), a common plasticizer with largely hydrophobic character. The relative hydrophobicity of a molecule can substantially influence its apparent relative concentration in ion evaporation experiments, with more hydrophobic molecules appearing to be more abundant [18]. Although DOP is present only in trace concentrations, its high hydrophobicity greatly enhances its relative abundance. This would only be expected with conditions that favor ion evaporation.

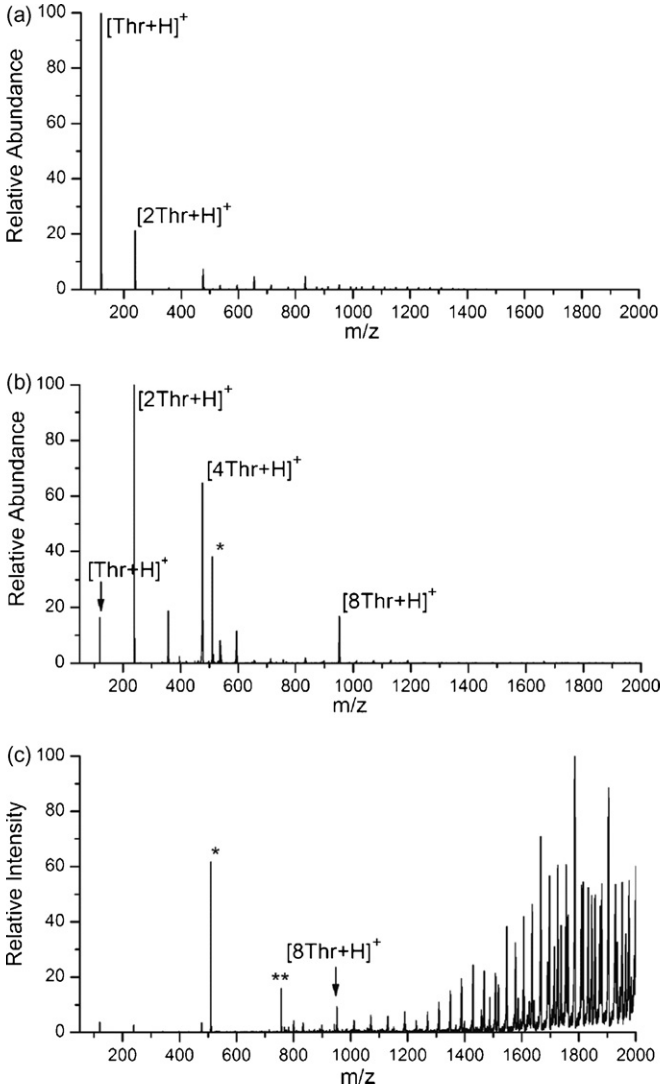

Fig. 2. (a) ESI-MS spectrum of threonine clusters. No magic octamer is observed. (b) ESI-MS spectrum of threonine clusters acquired under gentle conditions. A magic threonine octamer is present under these conditions in addition to other clusters. (c) Heating from the capillary again reveals the presence of a second cluster distribution at higher *m*/*z*. *Chemical noise [DOP + Thr + H]$^+$ and **adduct of threonine with unknown polymer.

If ion evaporation is a viable mechanism for generating small molecular clusters, then the solvation properties of the side chains may play an important role as seen above with threonine. In order to investigate this possibility further, we examined clusters of alanine. Alanine is the smallest hydrophobic amino acid and differs from serine by the substitution of H for OH. The results for alanine clusters are shown in Fig. 3. Under typical conditions, alanine does not cluster well (Fig. 3a). Under mild sampling conditions, the dimer becomes the largest pure cluster, and a small amount of magic pentamer is observed. Also present is a DOP + alanine cluster similar to the situation with threonine described above. Increasing the capillary temperature reveals larger clusters of many sizes which do not separate into well distinguished groups. The intensities of the smaller clusters

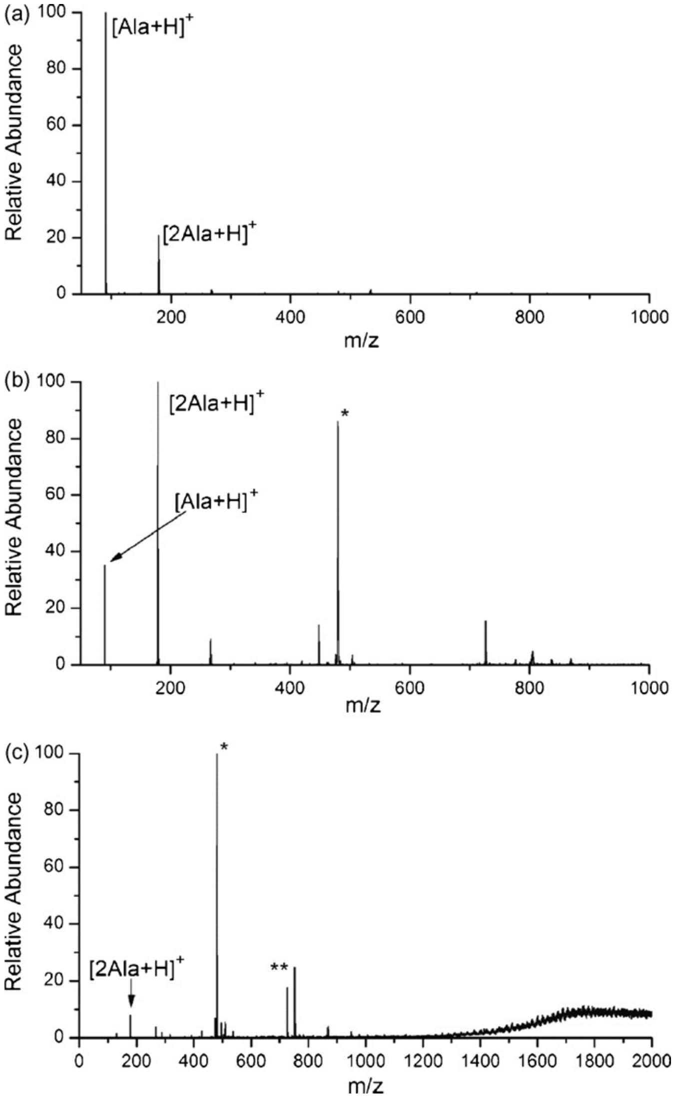

Fig. 3. (a) ESI-MS spectrum of alanine clusters. Very little clustering is observed. (b) ESI-MS spectrum of alanine clusters acquired under gentle conditions. The dimer dominates the spectrum. (c) Heating from the capillary again reveals the presence of a second cluster distribution at higher *m/z*. *Chemical noise [DOP + Ala + H]$^+$ and **adduct of alanine with unidentified polymer.

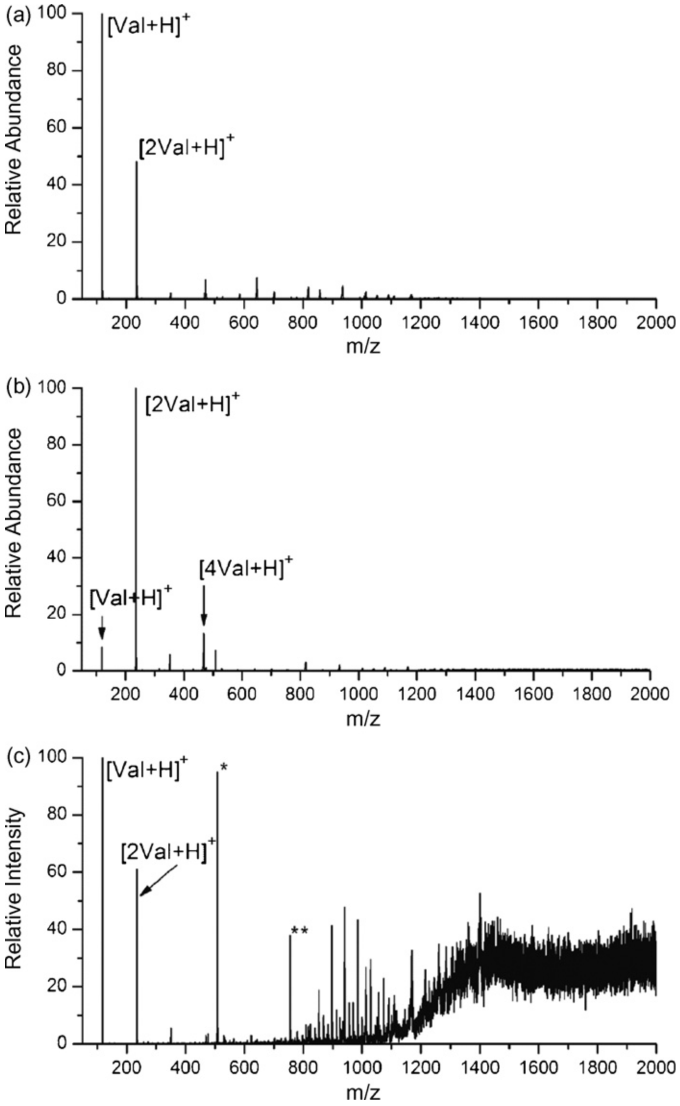

Fig. 4. (a) ESI-MS spectrum of valine clusters. The monomer is the base peak. (b) ESI-MS spectrum of valine clusters acquired under gentle conditions. The dimer dominates the spectrum. (c) Heating from the capillary again reveals the presence of a second cluster distribution at higher *m/z* and causes some dissociation of the weakly bound dimer. *Chemical noise [DOP + Val + H]$^+$ and **adduct of valine with unidentified polymer.

are diminished while contaminate peaks dominate the spectrum. Nevertheless, there are two cluster populations which are clearly present again suggesting that two mechanisms are at work. The absence of any alanine octamer is noted. In fact, the general trend favoring the formation of smaller clusters with increasing hydrophobic character from serine to threonine is continued with alanine.

This trend continues further with valine (see Fig. 4). Valine is more hydrophobic than alanine, which should lead to the observation of even smaller clusters. As shown in Fig. 4a, valine does not form abundant clusters with the spectrum being dominated by monomer and dimer peaks. Under very mild conditions, the dimer becomes very dominant, suggesting that this small unit is the most favorable product of the ion evaporation process. Mild thermal heating of the clusters again reveals a second distribution at much higher *m/z* values which presumably correspond to very large clusters of valine (Fig. 4c). No octamer of valine is formed, although there is a small amount of tetramer observed under mild conditions. The trends between side chain solvation and observed cluster distributions are continued as expected with valine.

4. Structure of the serine octamer

The data above suggests that solvation effects are likely to play an important role in the formation of clusters generated by ion evaporation. Can solvation effects similarly shed any light on the structure of the serine octamer? The lowest energy homochiral structure proposed [2] for the serine octamer is cube-like with a zwitterionic core as shown in Fig. 5. The cross section for this structure is also in agreement with measured values.

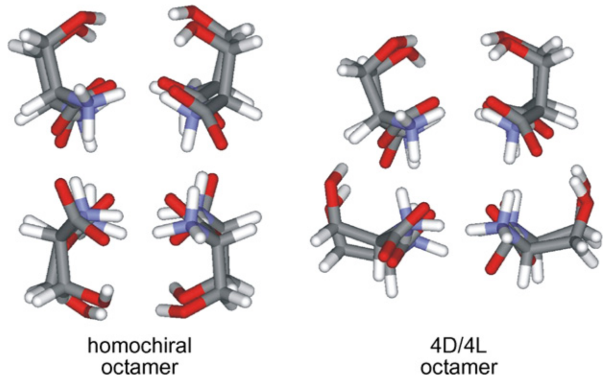

Fig. 5. Two previously proposed [2] potential structures for the serine octamer that are nearly degenerate in energy and collision cross section. The structures can only be discriminated on the basis of solvation energy as described in the text.

However, several issues related to this structure have not been explained. For example, in Fig. 5 a similar structure is shown which is composed of 4D and 4L serines. This structure has a zwitterionic core and is nearly identical in energy and collision cross section to the homochiral structure [2]. Despite the apparent similarities, 4D/4L structures are distinctly *disfavored* in the experimental data. Statistically, 4D/4L structures should be the most abundant species when the clusters are generated from a racemic solution. Experimentally, isotopic labeling studies have shown that these structures are not generated in any significant abundance [1,2]. Given that energetically the two structures are nearly identical, there must be another important factor which hinders formation of the 4D/4L cluster. Examination of the solvation properties of these structures offers a rational explanation for these observations.

The solvation energies for the two structures in Fig. 5 are −774 kJ/mol (homochiral) and −891 kJ/mol (4D/4L). The difference in solvation is therefore 117 kJ/mol, which is a substantial amount of energy. If cluster assembly in a racemic solution were controlled entirely by a stochastic process, then homochiral octamers would represent 0.78% of all octamers while 4D/4L octamers would represent 27.3%. For the specific structures in Fig. 5, energetic constraints would also favor formation of the 4D/4L structure in solution. Therefore homochiral octamers are predicted to be a minor species in bulk solution. However, the relative abundances could be much closer at the interfacial region on the surface of a charged droplet where the homochiral structure could be energetically favored in a partially solvated state. If both structures formed on the surface of a charged electrospray droplet, the solvation properties would drive the 4D/4L octamer away from the surface and into the center of the droplet while the homochiral octamer would preferentially remain on the surface. This situation would favor ionization (and subsequent detection by MS) of the homochiral structure. Therefore, it is possible (and virtually certain) that 4D/4L clusters exist in solution, but observing them in the mass spectrum is disfavored due to differential solvation effects relative to the homochiral octamers.

In related research, recent experiments have demonstrated that serine octamers can be generated in substantial abundance by sublimation from the solid state [25]. Clearly, this cannot occur by ion evaporation; however, relative solvation may still play an important role in a slightly different manner. It has already been shown that the gas phase properties of the serine octamer do not lead to its unusual abundance (Fig. 1b), suggesting that the mechanism of formation may be important once again. Essentially, solvation energy is a measure of the strength of the interactions between a molecule and the surrounding environment. In the sublimation experiments, no solvent is present, but other molecules of serine serve effectively the same purpose. Thus, the homochiral octamer could still be preferentially formed because it is effectively "less sticky". Furthermore, cluster formation will occur on a surface, providing a parallel to the surface dominated process of ion evaporation.

These observations support the homochiral structure shown in Fig. 5 for the A form of the octamer. This structure is in agreement with all experimental data including collision cross section [2,26], IR spectroscopy [27], and H/D exchange kinetics [9,10]. This structure is the lowest energy structure at the highest levels of theory which have been utilized on octamers of serine [4]. In the present work, we have postulated that the solvation properties of this structure may be used to explain the absence of a 4D/4L analog. In addition, the present results suggest that the B form of the octamer may not possess a particular structure, rather it may be a combination of various forms generated by dissociation from larger clusters. The same may be true for most of the other serine clusters which we speculate are formed by charge residue (either directly or indirectly through dissociation) and therefore may represent ensembles of structures. This explanation would be consistent with the observation that most serine clusters exhibit no preference towards chirality [28].

5. Conclusions

The interesting properties of the serine octamer can be rationalized by its formation via ion evaporation. Specifically, the magic homochiral A form is likely generated by this pathway. Furthermore, the above experimental data are more general in nature and strongly suggest that two mechanisms are responsible for the formation of most clusters of amino acids generated by electrospray. Ion evaporation can directly generate small clusters, as in the case of the serine octamer. In addition, clusters of all sizes can be generated by the charge residue mechanism, although in this case large clusters near the size of proteins are initially formed which can then be dissociated into smaller clusters of all sizes. We propose this break-up mechanism for the formation of the B form of the serine octamer. When ion evaporation occurs, the solvation properties of the monomers will influence the observed cluster sizes and the solvation properties of individual clusters can be used to evaluate and compare potential structures.

Finally, generation of the serine octamer by ion evaporation implies that it must exist in solution under appropriate conditions, in contrast to recent claims [12]. The discrepancy can be easily explained by the insensitivity of the implemented solution phase techniques and incorrect assumptions concerning the amount of serine octamer that would be expected to be found. In addition, if clustering occurs primarily at the interface between solution and the gas phase (which may be the likely scenario), then techniques monitoring the bulk solvent may not be appropriate. In this regard, ESI may serve as a magnifier by tremendously increasing the amount of interfacial area present by converting bulk solution into millions of droplets.

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