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Amino acid cluster formation studied by electrospray ionization mass spectrometry

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The association properties of natural and non-natural amino acids were studied in detail using electrospray ionization mass spectrometry. The results show a highly diverse cluster formation behavior of amino acids. There are differences regarding the degree of clustering (average cluster size), the presence or absence of one or several 'magic' clusters of special stability and the influence of chirality on cluster stability. Cluster formation does not show a good correlation with simple physico-chemical properties (such as solubility), indicating that it is a specific process and not only a simple aggregation during evaporation/ionization. A systematic study of cluster formation of serine derivatives reveals that all functional groups play a prominent role in the binding of the magic octamer. The results support the idea of the zwitterionic character of the octamer. Electrospray ionization of the side-chain acetylated serine shows the formation of a very stable tetramer with a strong preference for homochirality. The results suggest that Ser_8 is made up of two tetramer subunits, held together by hydrogen bonds of the side-chain. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: amino acid; chiral discrimination; cluster; magic number; serine

INTRODUCTION

The investigation of non-covalent complexes is one of the most important fields of mass spectrometry. Specific interactions between biologically relevant molecules have been reported using electrospray[1,2] and other ionization techniques. The self-association behavior of small molecules, such as amino acids[3–14] and nucleosides,[15–17] is at the focus of interest since they represent the building blocks of macromolecules and also the initial step in the evolution of life on Earth.[6,9,13,18]

The formation of non-covalent amino acid clusters upon electrospray ionization (ESI) was first observed by Meng and Fenn.[19] They studied the association properties of arginine, leucine and histidine in detail, and suggested a solubility-dependent aggregation during ionization.[19] The topic, however, became more exciting when Cooks and co-workers observed unusually stable octamer formation in the case of serine.[4,6,7] Further investigations have demonstrated exceptional properties for the octamer: it has a great preference for homochiral cluster formation and undergoes unique chiroselective substitution reactions with a number of compounds, such as other amino acids, sugars, phosphoric acid and transition metal ions.[10,13,18] Substitutions with optically active compounds showed transmission of chiral information, leading to suggestions explaining the origin of homochirality on Earth.[6,13,18]

Several experiments and theoretical calculations have been performed to determine the structure of the serine octamer.[5–9,12,20] A large number of structures have been proposed, but there is no consensus yet. In addition to theoretical calculations, an indirect way to obtain information about the structure of the octamer is to examine the effects of functional group modifications on the clustering process. Such investigations have shown that all functional groups of serine are involved in the formation of the octameric assembly.[9,20] This result is based on the experience that blocking of any of the functional groups leads to a highly decreased cluster formation ability and to the loss of preference for homochirality.[6,9,18,20] In these studies, several serine analogs bearing protecting groups or lacking functional groups, serine-related natural and non-natural amino acids (e.g. cysteine, threonine, homoserine) have been used, among which only a few have shown characteristic clustering properties, but even these showed no chiral preference. However, using a large protecting group (t-Boc) is not very useful in this respect, as steric effects may dominate. In our experiments, we used small protecting groups that may provide a more realistic insight into the role of the hydrogen-bonding network stabilizing the serine octamer.

The aim of the present work was to investigate the cluster formation properties of various amino acids in a representative way, in order to elucidate a possible

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connection between cluster formation abilities and physicochemical properties, such as solubility. All the 20 natural and some non-natural amino acids were subjected to electrospray ionization, followed by tandem mass spectrometric (MS/MS) experiments. The influence of chirality on the association process was also examined. In addition to amino acids, a series of serine-analog compounds were also studied to elucidate the cluster formation properties of this amino acid.

EXPERIMENTAL

Experiments were performed using a Perkin-Elmer SCIEX API-2000 triple-quadrupole mass spectrometer equipped with an ESI source. The operating settings of the mass spectrometer greatly influenced cluster formation. The experimental conditions optimized to study cluster formation were the following: spray voltage 4800 V, nebulizer gas intensity 30 units, curtain gas intensity 20 units, orifice voltage 0 V and O-ring potential 340 V; the heater gas was switched off. Optimized operating conditions for MS/MS experiments were similar to those applied in single MS experiments, except for the orifice voltage (30 V). Solutions were directly infused using a syringe pump at a flow-rate of 10.0 μl min^{-1} and mass spectra were recorded in the positive ion mode.

Amino acids were purchased from Fluka BioChemica (Buchs, Switzerland) and solvents were analytical-grade products from Reanal (Budapest, Hungary). Compounds were used without further purification. Amino acids were dissolved at a concentration of 1×10^{-2} mol l^{-1} using methanol–water (50 : 50, v/v) as solvent. Solutions were acidified with acetic acid at a concentration of 0.1% (v/v).

RESULTS AND DISCUSSION

The cluster formation properties of the amino acids were characterized based on three different parameters: the extent of clustering, relative stability of certain clusters and possible chiral discrimination in the clusters. The extent of cluster ion formation is a prime feature of association. There is, however, no accepted numerical value characterizing this process. Herein, we suggest using the average cluster size (*ACS*) as a quantitative value describing the degree of cluster formation, defined as

$$ACS = \frac{\sum_n nI_n}{\sum_n I_n} \quad (1)$$

where n represents the cluster size (number of monomer units in the cluster) and I_n is the intensity of an n-mer cluster observed in the mass spectrum. In calculating *ACS*, both singly and multiply protonated n-mer clusters were considered, I_n indicating the sum of their abundances. In the case of clusters where overlap with peaks of multiply charged metaclusters may occur, for the sake of simplicity only clusters with the lowest charge states were considered. The relative stability of a certain cluster compared with its neighbors in a homologous series is described by the so-called magic number (*MN*), originally proposed by Cooks and co-workers,[4,6] defined as

$$MN = \frac{2I_n}{I_{n-1} + I_{n+1}} \quad (2)$$

Owing to peak overlapping, in the case of doubly charged clusters the following, modified definition was used: $MN = 2I_n/(I_{n-2} + I_{n+2})$. Chiral preference upon formation of a magic cluster is also of interest, and this was characterized by comparing the magic numbers determined using optically pure and racemic solutions.

Cluster formation depends on a large number of experimental parameters (such as the ionization technique, type of instrument, concentration of solute and solvent used). For example, serine is well known to form an abundant octamer, but the relative intensity of the magic octamer varies over a broad range.[6,10,12] Cluster formation of amino acids can be compared only when they are examined under similar conditions. In the present work, all compounds were examined under the same experimental conditions so the clustering properties of the amino acids were comparable.

The clustering properties of the 20 natural and of some important non-natural amino acids were examined. Degrees of cluster formations (characterized by *ACS*) for optically pure solutions of the amino acids examined are shown in Fig. 1. Mass spectra of the amino acids showed diverse association behavior; based on these clustering properties, they may be tentatively categorized into four different groups as discussed below.

The first group contains amino acids with very low cluster formation ability (*ACS* varies between 1.1 and 2.1). The mass spectrum of L-arginine is a typical example for this group, shown in Fig. 2(a). In the spectrum, only singly protonated dimers and trimers were observed with significant abundance. The other two basic amino acids (lysine and histidine), and also glycine and cystine, showed analogous spectra and a limited degree of association.

A number of amino acids (glutamic acid, aspartic acid, glutamine, asparagine, tyrosine and tryptophan) may be classified in the next group. A typical example is L-glutamic acid, the spectrum of which is shown in Fig. 2(b). The average cluster size in this group varies between about 2.3 and 4.6, but

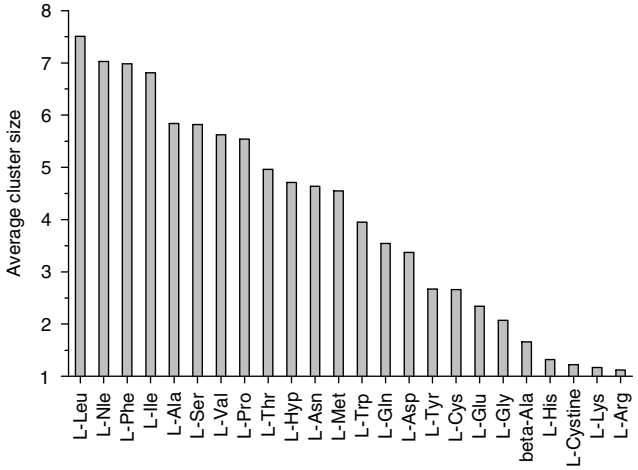

Figure 1. Cluster formation ability of the amino acids examined.

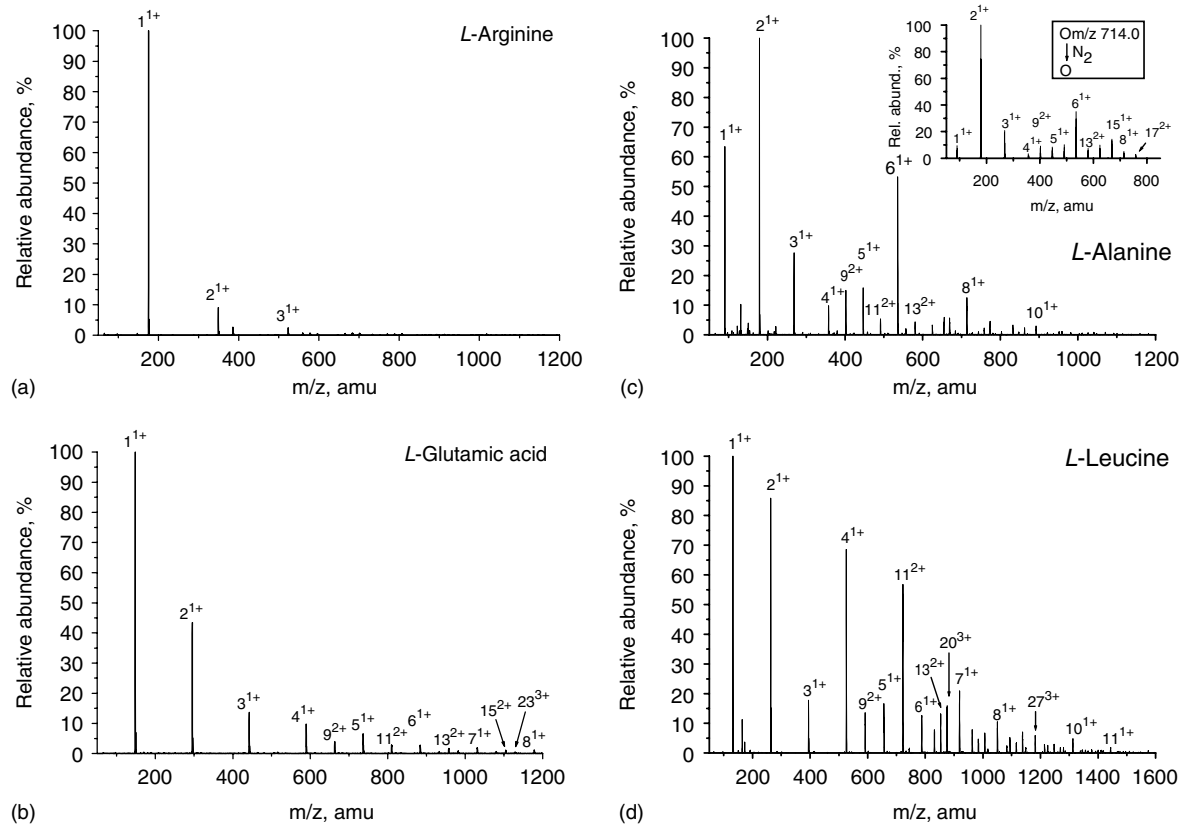

Figure 2. Positive ion ESI mass spectrum of (a) L-arginine, (b) L-glutamic acid, (c) L-alanine and CID spectrum of Ala_8^+ (inset) and (d) L-leucine. Cluster structure $[Aaa_n + mH]^{m+}$ is labeled as n^{m+}.

large clusters (such as Glu_{15}^{2+} and Glu_{23}^{3+}) are also present. All of these amino acids produce characteristic series of multiply charged clusters. The cluster ion abundances, as in the case of glutamic acid (Fig. 2(b)), show a continuous decrease with increasing cluster size and no clusters of particular stability were observed.

The third group of amino acids shows a similarly increased cluster formation ability (*ACS* varies between 2.7 and 5.8), but among the cluster ions produced, one has a prominent abundance and stability. Cysteine, serine and threonine belong to this group, forming 'magic' hexamers, octamers and heptamers, respectively (Tables 1 and 2), as also described in the literature.[6,10,18] We found that alanine also belongs to this group, forming a characteristic hexamer of high stability ($MN = 6.8$), which was not reported previously (Fig. 2(c)). Note that the *MN* of the alanine hexamer is even higher than that of Ser_8^+ (6.8 vs 4.7) under comparable conditions. The unusual stability of the hexamer was also demonstrated in collision-induced dissociation (CID) studies. The CID spectra of larger protonated alanine clusters show preferential formation of the hexamer, as also observed in the case of the octamer shown in the inset in Fig. 2(c). The CID spectrum of Ala_8^+ further reveals the presence of overlapping metaclusters with the general structure $[Ala_{8m} + mH]^{m+} (m = 1, 2, 3)$, indicating the formation of multiply charged metaclusters. Note that the peak corresponding to Ala_{17}^{2+} in the CID spectrum of Ala_8^+ indicates the presence of the triply charged 24-mer metacluster.

The fourth group contains amino acids with an apolar side-chain, which, in our experiments, showed the highest degree of cluster formation, with *ACS* values varying between 5.5 and 7.5. These compounds formed series of multiply charged clusters of particular abundance; proline and valine, for example, produced clusters as large as Pro_{28}^{3+} and Pro_{29}^{3+} with significant abundance. Intense cluster formation was also identified for phenylalanine, which produced a stable doubly charged tridecamer cluster ($[Phe_{13} + 2H]^{2+}$, $MN = 4.0$). Methionine and the three leucine isomers showed very similar spectra (the example of leucine is shown in Fig. 2(d)). These compounds formed characteristic 4^+, 7^+ and 11^{2+} type clusters.

Serine-like chiral selectivity of cluster formation was studied by comparing the mass spectra of optically pure solutions (L) and racemic (DL) mixtures in the case of amino acids with a high preference for clustering. Significant homochiral preference was measured for the protonated alanine hexamer. In this case, the relative abundance of Ala_6^+ in racemic solution decreased to about half of that measured in the optically pure solution. This change is illustrated by the shift of the *MN* value of the hexamer shown in Table 1. Changes in abundance were also observed for other amino acids, showing a preference for either homochirality or heterochirality (Table 1). It is interesting that several amino acids showed different chiral preferences in the formation of special clusters. For instance, leucine, isoleucine, norleucine and valine formed more abundant tetramers from racemic mixtures than they did from optically pure solutions. In contrast, strong homochiral preference was observed in the case of the 11^{2+} clusters of the same amino acids, except norleucine. Similar behavior was observed for

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Table 1. Characteristic clusters and chiral preference observed in cluster formation (L, optically pure; DL, racemic solutions)

Amino acid	*ACS*	Characteristic clusters	*MN* in L	*MN* in DL	Chiral preference
Ala	5.8	6^{+}	6.8	5.6	Homochiral
		8^{+}	4.5	3.5	Homochiral
		10^{+}	3.5	1.0	Homochiral
		15^{2+}	2.4	1.8	Homochiral
Ile	6.8	4^{+}	4.5	6.4	Heterochiral
		6^{+}	0.9	1.7	Heterochiral
		11^{2+}	3.7	2.7	Homochiral
Leu	7.5	4^{+}	3.9	4.9	Heterochiral
		10^{+}	1.4	1.1	Homochiral
		11^{2+}	6.2	4.0	Homochiral
Nle	7.0	4^{+}	4.4	5.9	Heterochiral
		9^{2+}	2.7	0.4	Homochiral
		11^{2+}	0.8	5.3	Heterochiral
		15^{2+}	2.8	0.9	Homochiral
Pro	5.5	4^{+}	1.9	1.0	Homochiral
		5^{+}	0.9	2.0	Heterochiral
		6^{+}	1.5	0.5	Homochiral
		9^{2+}	4.2	2.7	Homochiral
		10^{+}	0.5	1.7	Heterochiral
		13^{2+}	1.7	0.8	Homochiral
		15^{2+}	1.6	2.8	Heterochiral
Thr	5.0	4^{+}	4.0	2.5	Homochiral
		7^{+}	2.6	1.3	Homochiral
		10^{+}	1.3	0.8	Homochiral
Val	5.6	4^{+}	3.4	6.0	Heterochiral
		6^{+}	1.1	2.1	Heterochiral
		11^{2+}	4.3	2.2	Homochiral

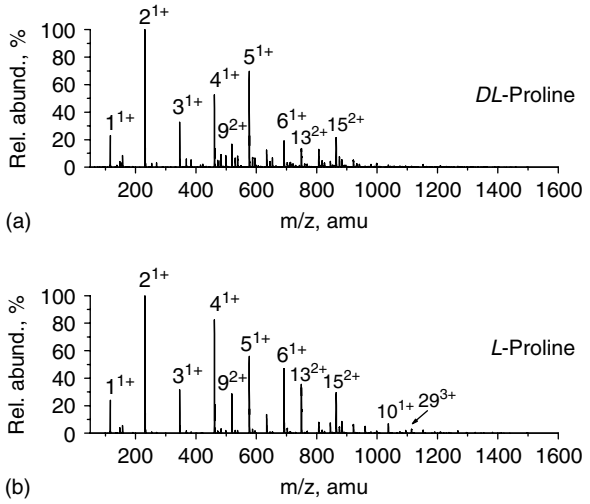

Figure 3. Positive ion ESI mass spectrum of (a) DL-proline and (b) L-proline. Cluster structure $[\mathrm{Aaa}_n + m\mathrm{H}]^{m+}$ is labeled as n^{m+}.

proline. Spectra produced from optically pure and from racemic solutions showed a significant change in the pattern of cluster ion in abundances (Fig. 3(a) and (b)). Note that an analogous, but less pronounced, effect was also observed for serine clusters recently.[21]

The hypothesis that cluster formation is directly related to solubility[19] has been studied in detail. In order to elucidate this connection, the solubilities of the amino acids were measured in acidified methanol–water (50:50, v/v), the same solvent as used to obtain the ESI mass spectra. The solubility, however, did not show any simple correlation with the degree of clustering (neither with *ACS* nor with the clustering behavior described above), which suggests that amino acid aggregation upon ESI is not simply related to solubility. To give an example, proline shows a high degree of clustering in spite of its very high solubility. This suggests that solubility itself cannot be the only driving force of association. The degree of cluster formation was studied as a function of various other physico-chemical properties, such as $pK_a(\mathrm{COOH})$, $pK_a(\mathrm{NH_2})$, pK_a(side-chain), isoelectric point, proton affinity (*PA*), hydrophobicity and calculated residual non-polar surface area.[22] However, none of these parameters showed a good correlation with the degree of clustering. *PA* and non-polar residual side-chain area were found to show partial correlation for some amino acids. Those with a highly basic side-chain (arginine, lysine and histidine), i.e. with high *PA*, show only a very small degree of clustering. It is known that *PA* is inversely related to the stability (dissociation energy) of protonated dimers. A comparison of CH_3OH and CH_3NH_2 may illustrate this point: the *PA* values are 754.3 kJ mol^{-1} for CH_3OH and 899 kJ mol^{-1} for CH_3NH_2,[22] whereas the dissociation energies of the proton-bound dimers are 136 and 106 kJ mol^{-1}, respectively.[22] This may explain the lack of abundant clustering observed for the

Table 2. Serine derivatives examined and their clustering properties[a]

Location of modification	Compound	Abbreviation	*ACS*	Characteristic cluster	*MN* in L	*MN* in DL	Chiral preference
—	L-Serine	L-Ser	5.8	Octamer	4.2	1.7	Homochiral
N-Terminus	*N*-Methyl-L-serine	*N*-Me-L-Ser	4.0	—	—	—	—
C-Terminus	L-Serine methyl ester	L-Ser-OMe	1.4	—	—	—	—
	L-Serine amide	L-Ser-NH_2	1.4	—	—	—	—
	2-Aminopropane-1,3-diol	Serol	1.6	—	—	—	—
Side-chain	L-Homoserine	L-HomoSer	1.6	—	—	—	—
	O-Acetyl-L-serine	L-Ser(Ac)	1.7	Tetramer	9.9	5.0	Homochiral

[a] Dashes indicate no characteristic cluster formation. *ACS*, average cluster size; *MN*, magic number.

highly basic amino acids. The correlation between *PA* and extent of clustering (measured by *ACS*), however, breaks down for other amino acids, suggesting the significance of other factors in addition to *PA*. Another partial correlation was found between the degree of cluster formation and the non-polar residual side-chain areas. All apolar amino acids showed a high degree of cluster formation, which (within this group) correlates well with the non-polar residual side-chain area. This relationship indicates that among the factors governing cluster formation, hydrophobic interactions between the apolar side-chains of the amino acids play a significant role.

The effect of amino acid concentration on the extent of cluster formation upon ESI was examined in a wide range ($1 \times 10^{-5} - 1\ \mathrm{mol\,l^{-1}}$). This shows that the behavior described above is valid over a wide concentration range, although the extent of aggregation depends on concentration. At low concentration the degree of clustering increases and reaches a maximum around $10^{-2}\ \mathrm{mol\,l^{-1}}$. At high concentration, surprisingly, formation of aggregates with a smaller cluster size is favored. A similar result was noticed previously for arginine.[3] The lack of a clear correlation between cluster formation propensity and most simple physico-chemical properties of amino acids suggests that non-covalent cluster formation of amino acids cannot be interpreted as simple aggregation during electrospray. It is, in fact, a specific process, depending on various physico-chemical and structural properties.

Cluster formation of serine derivatives has also been studied. All functional groups were modified using sterically small groups (Fig. 4); in this way, changes observed in cluster formation should primarily reflect changes in the hydrogen-bonding networks, while steric effects are likely to have only a secondary role. As noted before, all modifications on serine result in a decreased propensity for cluster formation and destroy the structure of the magic octamer (Table 2).[6,9,20] This shows that all functional groups are involved in stabilizing the protonated octamer. In our experiments, *N*-methyl-serine showed a significant degree of clustering ($ACS = 4.0$), but no formation of magic clusters (Fig. 5) and no preference for homochirality (Table 2). Amino acids modified at the C-terminus barely produced clusters (Table 2, Fig. 5); only dimer and trimer cluster ions were observed with significant abundance. Modification of the C-terminus blocks the possibility of the formation of salt bridges, so this observation is in accord with the idea that serine clusters are stabilized

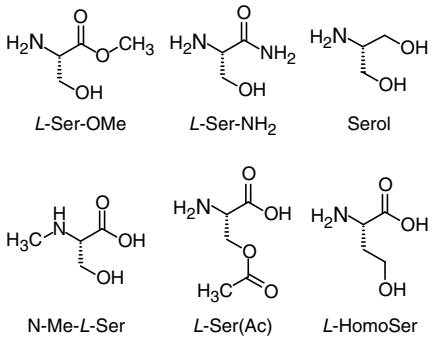

Figure 4. Serine derivatives and related compounds examined.

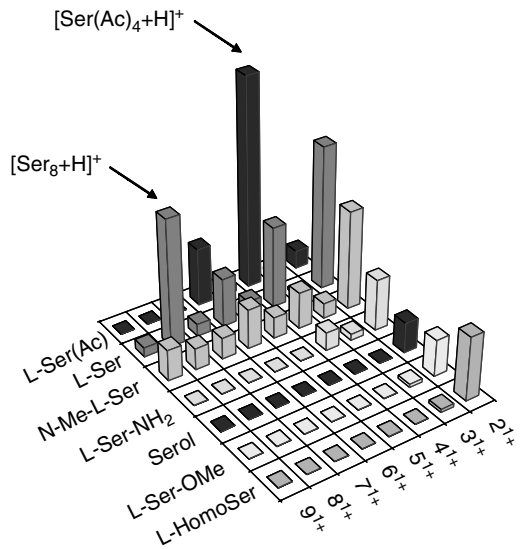

Figure 5. Magic number effects of serine derivatives for the clusters $[M_n + H]^+$, $n = 2-9$. Magic number is shown on the ordinate. Cluster structure $[Aaa_n + mH]^{m+}$ is labeled as n^{m+}.

by salt bridges.[5,9,20,23] Note that a joint theoretical and experimental study has recently demonstrated a preference for a gas-phase salt-bridge structure for a cluster as small as the protonated serine dimer.[24] The side-chain also has a significant influence on the stability of the octamer. Amino acids that produce significant magic clusters (threonine, cysteine, alanine) are closely related to serine. L-Threonine, containing an additional methylene group, gives unusually abundant heptamers and octamers,[4,6,9,10,20] and L-cysteine, containing an SH group instead of the hydroxyl group, similarly to alanine, forms hexamers upon ESI.[6,18] However, a simple enlargement of the side-chain by an additional methylene group (L-homoserine) ruins the formation of the magic octamer (Table 2, Fig. 5).[9]

The side-chain acetylated serine derivative, surprisingly, formed an unusually abundant protonated tetramer (Fig. 6). The magic number of the Ser(Ac) tetramer was about twice than that calculated for the serine octamer under the

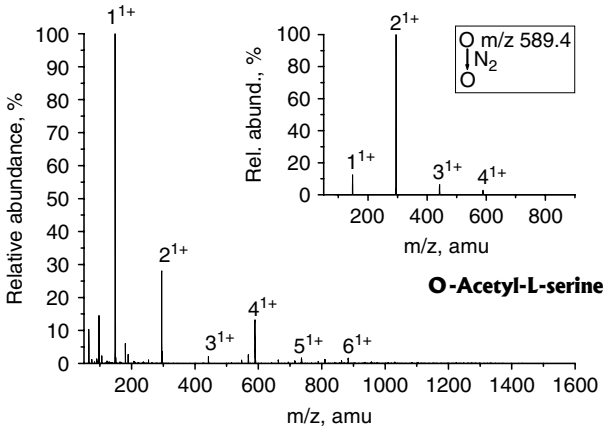

Figure 6. Positive ion ESI mass spectrum of Ser(Ac) and CID spectrum of the protonated Ser(Ac) tetramer (inset). Cluster structure $[Aaa_n + mH]^{m+}$ is labeled as n^{m+}.

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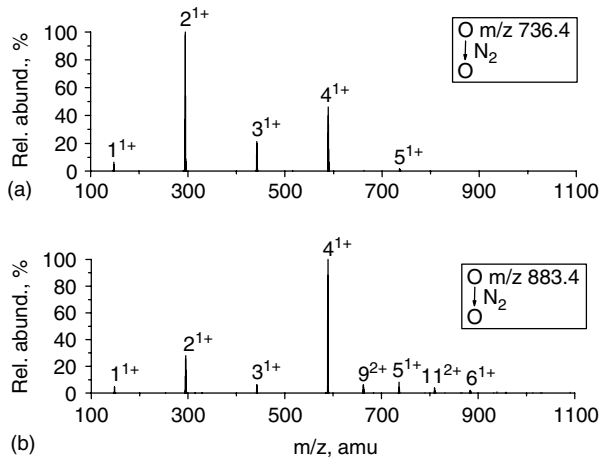

Figure 7. (a) CID spectrum of the protonated Ser(Ac) pentamer and (b) CID spectrum of the protonated Ser(Ac) hexamer. Cluster structure $[Aaa_n + mH]^{m+}$ is labeled as n^{m+}.

same conditions, indicating its exceptional stability (Table 2). Note that the *MN* of Ser_8 reported here is less than that reported elsewhere,[4,6] owing to the different experimental conditions, and especially to the construction of the SCIEX triple-quadrupole mass spectrometer, which is known to form relatively hot ions.[25–27] The tetramer cluster of Ser(Ac) showed a preferred loss of a dimer subunit upon CID (Fig. 6, inset). CID studies of the Ser(Ac) pentamer and hexamer also proved the great stability of the $[Ser(Ac)_4 + H]^+$ cluster (Fig. 7). For instance, in the CID spectrum of the hexamer ion (Fig. 7(b)), very abundant tetramer clusters were observed. Furthermore, the presence of multiply charged clusters in this spectrum reveals the formation of overlapping cluster ion series with a general structure of $[Ser(Ac)_{6m} + mH]^{m+}$ ($m = 1, 2, 3$).

The formation of a magic tetramer cluster is unique because modifications of the hydroxyl group of serine have shown no magic cluster formation reported in the literature so far. Study of the chiral behavior of the tetramer revealed that Ser(Ac) forms more abundant tetramers when an optically pure solution is electrosprayed, compared with the racemic solution. The side-chain acetylated serine, therefore, has a high preference for homochiral tetramer formation (Table 2), similarly to the serine octamer. This is the first time that a derivative of serine has been proved to be able to form magic number clusters and to have a homochiral preference for cluster formation. It is tempting to assign this behavior to a possible structural relationship between the $[Ser(Ac)_4 + H]^+$ and the $[Ser_8 + H]^+$ clusters. Based on these results, a structure can be suggested for the serine octamer in which two tetramers are linked together by H-bonds of the $—CH_2—OH$ side-chains.

CONCLUSIONS

The non-covalent cluster formation properties of amino acids show a very diverse picture. In polar and protic media, amino acids with an apolar side-chain show a very strong cluster formation propensity, whereas the basic ones scarcely form cluster ions upon ESI. Neither solubility nor any simple physico-chemical properties of the amino acids explain the differences in clustering observed. Cluster formation of amino acids is a specific self-association process determined by various factors. Many amino acids produce specific magic clusters, with a preference for homo- or even for heterochirality, the most important ones listed in Table 1.

Study of the clustering properties of serine derivatives showed that the side-chain acetylated compound is able to form stable tetramers with a high homochiral preference. Our results support the idea that the serine octamer is composed of zwitterionic serine monomers, and imply that in its structure two tetramer units are bound together by hydrogen bonds formed between the hydroxyl functional groups in the side-chain.

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