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Hydration of small peptides

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Abstract

The results for the sequential hydration of small peptides (<15 residues) obtained in our group are reviewed and put in perspective with other work published in the literature where appropriate. Our findings are based on hydration equilibrium measurements in a high-pressure drift cell inserted into an electrospray mass spectrometer and on calculations employing molecular mechanics and density functional theory methods. It is found that the ionic functional groups typically present in peptides, the ammonium, guanidinium, and carboxylate groups, are the primary target of water molecules binding to peptides. Whereas the water–guanidinium binding energy is fairly constant at 9 ± 1 kcal/mol, the water binding energy of an ammonium group ranges from 7 to 15 kcal/mol depending on how exposed the ammonium group is. A five-residue peptide containing an ammonium group is in favorable cases large enough to fully self-solvate the charge, but a pentapeptide containing a guanidinium group is too small to efficiently shield the charge of this much larger ionic group. The water–carboxylate interaction amounts to 13 kcal/mol with smaller values for a shielded carboxylate group. Both water bound to water in a second solvation shell and charge remote water molecules on the surface of the peptide are bound by 7–8 kcal/mol. The presence of several ionic groups in multiply charged peptides increases the number of favorable hydration sites, but does not enhance the water–peptide binding energy significantly. Water binding energies measured for the first four water molecules bound to protonated bradykinin do not show the declining trend typically observed for other peptides but are constant at 10 kcal/mol, a result consistent with a molecule containing a salt bridge with several good hydration sites. Questions regarding peptide structural changes as a function of number of solvating water molecules are discussed. Not much is known at present about the effect of individual water molecules on the conformation of peptides and on the stability of peptide zwitterions.

Keywords: Ammonium group; Guanidinium group; Carboxylate group; Water binding site; Water binding energy; Zwitterion; Peptide conformation

1. Introduction

The medium where biological processes occur in living organisms can vary over a wide range of properties from hydrophobic to hydrophilic, from low to high ionic strengths, from acidic to basic conditions, from low-viscosity fluids allowing rapid diffusion to highly viscous diffusion-restricting liquids or gels. However, independent of the exact nature and composition of the environment in the biological system the biomolecule always finds itself surrounded by a condensed phase medium composed of other molecules strongly interacting with the biomolecule. The most abundant molecule in biological systems is water and therefore most biomolecules are to a greater or lesser degree in close contact with liquid water.

One of the most interesting questions regarding the interaction of a biomolecule with its surroundings is whether certain properties such as conformation of the biomolecule is inherent to the biomolecule or whether it is mostly a consequence of its interaction with the medium.

For example, the Alzheimer's Amyloid-β peptide, Aβ-(1–42), was experimentally (by NMR) found to be near fully α-helical in organic solvents [1], but is essentially unstructured in water, although a hydrophobic core appears to be defined (residues 17–21) [2]. Structures obtained by molecular mechanics using an implicit water solvent model agree with these findings [3]. These theoretical structures feature a core of hydrophobic residues and loose loops of hydrophilic residues attempting to shield the hydrophobic core. The pre-

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ferred solvent-free theoretical structures are found to be different from both the Aβ-(1–42) structure in organic solvents and in water [3]. The solvent-free Aβ-(1–42) molecule forms a compact structure with a hydro*philic* core with the hydro*phobic* residues on the surface.

This example nicely demonstrates the significance of solvation and why we would like to understand solvation effects. Our initial approach is to add individual water molecules to the fully desolvated state of a biomolecule and study energetic and structural effects as a function of water addition. We evaluate water binding energies, water binding sites, and structural changes of the biomolecule. For peptides these structural changes may include change of conformation and zwitterion formation.

In this paper we summarize results obtained in our group for a range of small peptides (<15 residues) by drift cell equilibrium measurements of peptide ions in water vapor and by theoretical methods such as molecular mechanics and electronic structure calculations.

2. Methods

The instrumentation used to carry out the hydration experiments has been described previously [4]. Briefly, ions formed by electrospray ionization (ESI) travel through a capillary into the vacuum system and are subsequently guided by an ion funnel through two stages of differential pumping. The ions are then injected into a drift cell containing a precisely measured pressure of water vapor (0.1–2 Torr) at a carefully controlled temperature of 240–360 K. The ions drift through the cell under the influence of a weak uniform electric field (∼5 V/cm at 1 Torr). After the ions exit the cell through a small orifice they are mass analyzed in a quadrupole mass filter and detected.

The amount of water pickup by the ions is measured as an increase in mass. Establishment of an equilibrium is confirmed by verifying that mass spectra recorded at different drift voltages (and therefore at different drift/reaction times) are identical. From the intensity ratio I_A/I_B of two adjacent peaks in the hydration mass spectrum (masses m_A and $m_B = m_A + 18$) the equilibrium constant for adding one water molecule to species A is determined together with the known water vapor pressure. Measuring the equilibrium constant as a function of temperature and applying a van't Hoff type of analysis yields ΔH° and ΔS° for the hydration process. In this paper an index n attached to the symbol of thermodynamic quantities (e.g. ΔH_n°) refers to the number of water molecules bound to the *product* ion $M^{\pm z}\cdot(H_2O)_n$ (see Eq. (1)).

$$M^{\pm z}\cdot(H_2O)_{n-1} + H_2O \overset{\Delta H_n^\circ, \Delta S_n^\circ}{\rightleftarrows} M^{\pm z}\cdot(H_2O)_n \qquad (1)$$

Theoretical methods include molecular mechanics (MM) and density functional theory (DFT) calculations. MM studies are based on the AMBER force field as implemented in the AMBER suite of programs [5]. For the DFT calculations the GAUSSIAN98 software package is used [6], employing the B3LYP functional and basis sets ranging from 6-31G* to 6-311++G** depending on the system.

3. The significance of ionic groups

One of the most interesting questions regarding hydration is that of water binding sites. Can we make a good guess of the preferred water binding sites on the surface of a peptide or protein? Let us have a look at the solution-phase NMR structure of myoglobin [7] shown in Fig. 1, where all of the ionic groups are highlighted in blue (positive) and red (negative). It can be seen that many (if not all) ionic groups are located on the surface of the molecule and that some of them stick out into solution. From this observation we conclude that the solvent, water, interacts particularly favorably with the charged groups of the biomolecule. Hence, we expect that ionic groups on peptides or proteins offer some of the most preferred water binding sites.

Hydration mass spec data obtained in our lab confirm these conclusions [8]. An example is shown in Fig. 2, where charge states +1, +2, and +3 of the peptide neurotensin (ELYENKPRRPYIL) are compared with each other. It is evident that under identical conditions the species with three charged groups picks up more water molecules (∼12 on average) than that with two charged groups (∼6 on average), which in turn picks up more than singly charged neurotensin (∼3 on average). Hence, the charged groups present in a peptide have a dominating effect on hydration.

What are the charged groups in peptides and proteins? Interestingly under physiological conditions (pH 7) there are

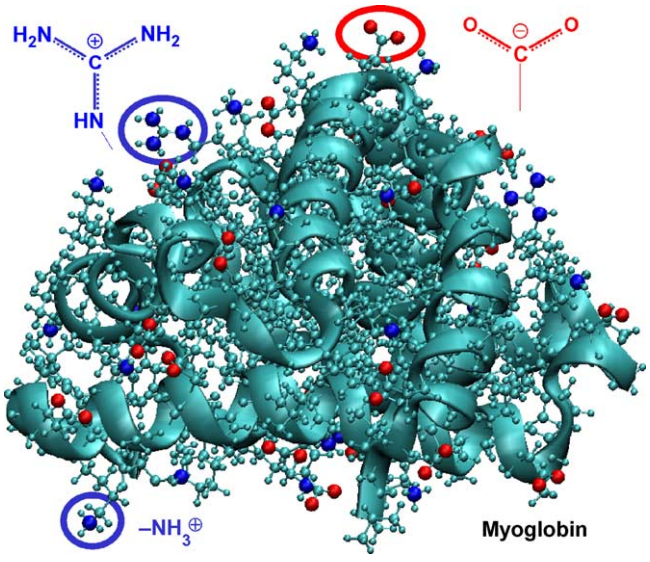


Fig. 1. Solution structure of carbonmonoxy myoglobin (pdb-code 1MYF) determined from NMR distance and chemical shift constraints [7]. The nitrogen atoms of positive ionic groups (ammonium and guanidinium) are shown in blue, the oxygen atoms of negative ionic groups (carboxylate) in red.

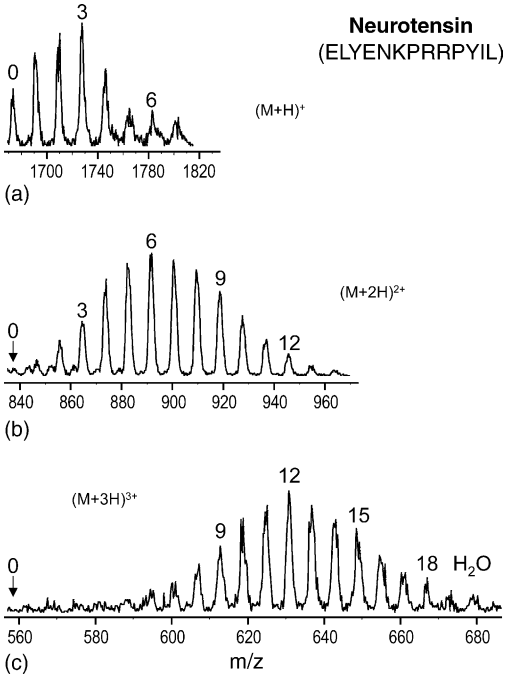


Fig. 2. Three sections of the neurotensin electrospray mass spectrum recorded after the ions were passed through a drift cell filled with 1.3 Torr of water vapor at 260 K. The numbers above the peaks indicate the number n of water molecules included in the species $(M + zH)^{+z}\cdot(H_2O)_n$. Charge state (a) $z = 1$; (b) $z = 2$; and (c) $z = 3$.

only three types of ionic groups inherent to systems composed of the 20 naturally occurring amino acids: the ammonium, guanidinium, and carboxylate groups. Ammonium groups are present at the N-terminus and in lysine (K) side chains, guanidinium groups in arginine (R), and carboxylate groups at the C-terminus and in aspartic (D) and glutamic acid (E) side chains. A fourth ionic group, the imidazole group present in histidine (H), exists under acidic conditions ($pK_a = 6.0$ for histidine side chain). Therefore the major focus of our work was directed towards understanding hydration patterns of these individual ionic groups. In the following sections we will present data for systems with one ionic group including the ammonium group, the guanidinium group, and the carboxylate group, and for systems with several ionic groups including multiply charged peptide ions and salt bridge systems.

4. Hydration of ionic groups

4.1. The ammonium group

Hydration of the ammonium group has extensively been studied by a number of researchers [8–13]. In one of our studies [8,12] we find by theory that three water molecules bind directly to the ammonium group of protonated methylamine, each water molecule forming a hydrogen bond to one of the three hydrogen atoms of the ammonium group. The fourth water molecule binds to one of the first three waters starting a new solvation shell. Hence, in this example we expect three strongly bound water molecules and a fourth water molecule with a substantially smaller binding energy. However, the same theory (B3LYP/6-311++G**) yielding the structural solvation shell pattern indicates that there is a steady drop in the binding energies for water molecules one through four without a noticeable step between three and four: 16.9, 13.9, 11.9, and 9.3 kcal/mol. These $[CH_3NH_3^+\cdot(H_2O)_{n-1}]\cdots H_2O$ binding energies include corrections for zero point energies and basis set superposition errors. The experimentally determined water binding energies of 17.8, 14.6, 12.3, and 10.3 kcal/mol (Table 1) do not follow a solvation shell pattern, either [9,10]. The experimental values for ammonium alkanes with larger alkyl chains are slightly smaller but decrease just as smoothly, e.g. 14.8, 12.1, 9.6, and 7.5 kcal/mol for *n*-decylamine [8]. How can these trends be understood?

It is expected that electrostatic interactions are important in these ionic hydrogen bonds and that the water–ammonium interaction is fairly sensitive to the actual amount of charge present on the ammonium group. A natural bond orbital (NBO) analysis can be carried out to probe the charge distribution theoretically. An NBO analysis for $CH_3NH_3^+$ reveals that more than a third of the positive charge (0.35) is actually located on the methyl group [12]. Hence, the ammonium group carrying a formal charge of +1 possesses in reality just a fraction (0.65) of the positive charge. This fraction is expected to be even smaller for ammonium ions RNH_3^+ with larger alkyl R-groups. Therefore, the water–ammonium interaction is expected to be smaller for a larger R-group in agreement with experimental water binding energies, e.g. 14.8, 17.8, and 19.9 kcal/mol for *n*-decylamine, methylamine, and ammonia, respectively (Table 1) [8–10]. The amount of charge present on the ammonium group is further decreased once a water molecule is bound to it. NBO calculations on the $CH_3NH_3^+\cdot H_2O$ system indicate that 5% of the positive charge sits on H_2O [12]. Hence, the next water molecule approaching this system has less charge available on the ammonium group to interact with, resulting in a smaller binding energy: 14.6 kcal/mol for the second versus 17.8 kcal/mol for the first H_2O bound to $CH_3NH_3^+$. By the time $CH_3NH_3^+$ is solvated by three water molecules 10% of the charge has moved to the solvent water molecules [12].

If charge is converted into energy using Coulomb's law it becomes apparent that the electrostatic interaction between one water molecule and the rest of the system decreases steadily as hydration proceeds from $CH_3NH_3^+$ to $CH_3NH_3^+\cdot(H_2O)_4$. No step between the third and fourth water molecule is observed [12]. Since the decreasing trend of electrostatic interaction nicely reproduces the experimental trend of water binding energies we are confident that the experimental trend is caused by electrostatics.

An interesting point is that water binding energies obtained by AMBER MM-calculations on protonated *n*-decylamine show a distinct solvation shell pattern: three

Table 1
Experimental hydration enthalpies[a] of selected ions

	Compound	z[b]	ΔH_n° (kcal/mol)					Reference
			$n=1$	$n=2$	$n=3$	$n=4$	$n=5$	
$-NH_3^+$	Methylamine[c]	+1	17.8	14.6	12.3	10.3		[9,10]
	n-Decylamine	+1	14.8	12.1	9.6	7.5	6.7	[8]
	Diaminododecane[d]	+2	15.7	15.7	13.4	13.6		[11]
	Acetyllysine[e]	+1	10.6	8.4	8.3	7.2		[12]
	Ac-AAKAA[f]	+1	8.5	7.4				[12]
	Ac-AAAAK[f]	+1	6.9					[12]
$-NHC(NH_2)_2^+$	Arginine	+1	9.0					[16]
	Arginine-OMe[f]	+1	9.2					[16]
	Ac-AARAA[f]	+1	9.5	8.1				[8]
	AARAA-OMe[f]	+1	9.4	8.4	7.6			[8]
$-COO^-$	Capric acid	−1	13.0	10.4	9.5			This work
	Dialanine	−1	11.6	9.4	8.5			[17]
	Aspartic acid	−1	9.2	7.8	7.0			This work
	Dialanine dimer[g]	−1	8.4					[17]

[a] See Eq. (1). Error limits ±0.3 kcal/mol.
[b] Charge state z indicates z-fold protonation ($z>0$) or deprotonation ($z<0$), respectively.
[c] Values are an average of those given in refs. [9,10].
[d] $H_2N(CH_2)_{12}NH_2$.
[e] $H_3CCONHCH(C_4H_8NH_2)COOH$.
[f] A is alanine, K lysine, R arginine. Ac indicates an acetylated N-terminus, OMe a methyl esterified C-terminus.
[g] $[H_2NCH(CH_3)CONHCH(CH_3)COOH]_2$; one unit deprotonated, one unit neutral.

strongly bound water molecules in a first solvation shell (13.9, 12.6, and 11.4 kcal/mol) and weakly bound water molecules in the second solvation shell (<8 kcal/mol) [8,12]. This effect is a consequence of the fact that atomic charges are kept constant in MM-calculations independent on conformation and independent on whether a ligand is bound or not. In contrast, in electronic structure calculations including DFT the charge distribution is naturally adjusted as the geometry is optimized allowing for the correct computation of electrostatic interactions.

In contrast to alkylamines, peptide ammonium groups can be tied up in intramolecular hydrogen bonds with electron-rich functional groups. The carbonyl oxygen atoms of amides carry a relatively large negative charge and are therefore particularly good solvating agents for positive charges. Protonated acetyllysine is a potential candidate for a miniature model of a peptide since it contains both an ammonium and an amide group with the potential of forming a hydrogen bond between the backbone acetyl oxygen and the side chain ammonium group. Molecular modeling results obtained for this system indicate that the $C{=}O\cdots H_3N^+$ interaction is not only possible but preferred in this case (Fig. 3). An NBO analysis of protonated acetyllysine in the $C{=}O\cdots H_3N^+$ hydrogen-bound conformation indicates that part of the positive charge, formally residing on the ammonium group, migrates over to the amide [12]. With relatively little positive charge density left on the ammonium group it is therefore not surprising that the experimental water binding energies for acetyllysine are rather small, 10.6 for the first and 8.4 kcal/mol for the second water molecule (Table 1) [12].

In larger peptides, with each residue providing at least one amide carbonyl oxygen, ammonium groups can be fully self-solvated if sterically possible. MM-calculations on the pentapeptide Ac-AAAAK ("Ac" denotes N-terminal acetylation, "A" is alanine) indicate that all three hydrogen atoms of the ammonium group are indeed engaged in intramolecular hydrogen bonds with three backbone carbonyl groups (Fig. 4b), in a similar fashion as in the previously reported structures of the larger, helical peptides Ac-A_xK, $x=15$–20 [14,15]. Simulations of the singly hydrated Ac-AAAAK system show the preferred water binding site in a charge remote location near the C-terminus [12]. MM-simulations on the Ac-AAKAA isomer, however, indicate that the ammonium group cannot be fully self-solvated in this case (Fig. 4a). Only two carbonyl oxygen atoms are within reach of the ammonium group to form hydrogen bonds without twisting the molecule into energetically unreasonable conformations. The first water molecule of hydration adding to Ac-AAKAA is found to bind directly to the charge thereby filling the first

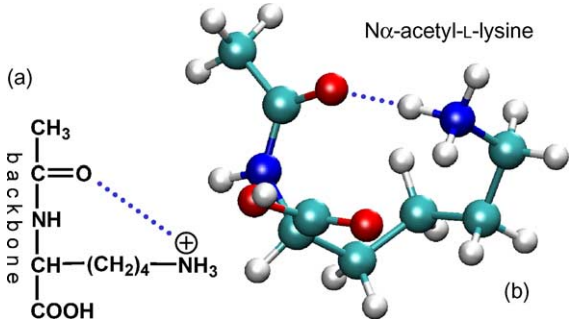


Fig. 3. (a) Line formula and (b) molecular mechanics structure of the miniature model peptide *N*α-acetyllysine indicating self-solvation of the lysine ammonium group by a "backbone" carbonyl group (blue dotted line).

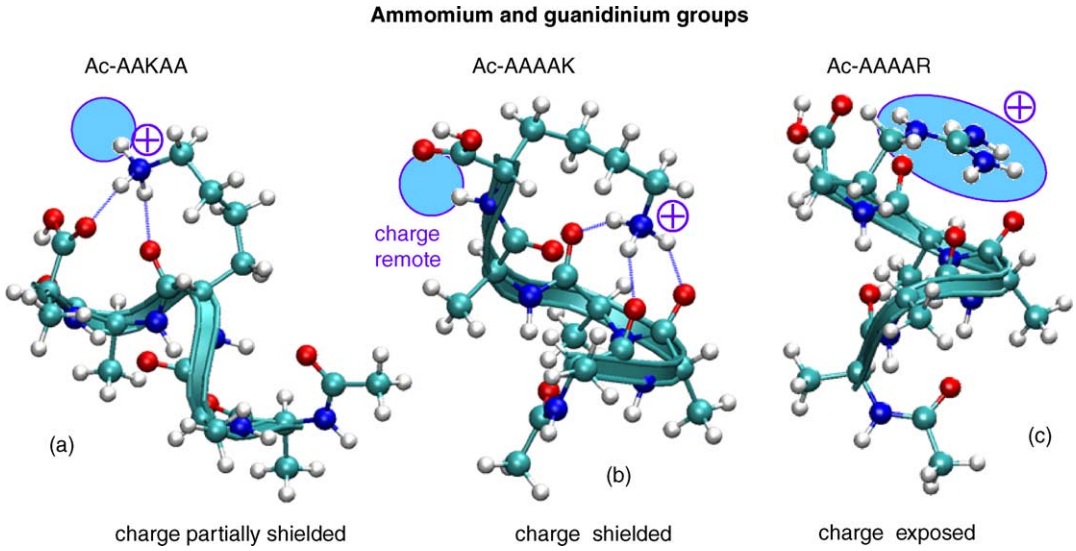


Fig. 4. Molecular mechanics structures of the acetylated pentapeptides: (a) Ac-AAKAA containing a partially self-solvated ammonium group; (b) Ac-AAAAK containing a fully self-solvated ammonium group; and (c) Ac-AAAAR containing an exposed guanidinium group. The preferred hydration site(s) are indicated as the blue shaded area.

solvation shell of the charged site according to AMBER [12]. Experimental water binding energies for the two isomers are 6.9 for Ac-AAAAK and 8.5 kcal/mol for Ac-AAKAA, respectively (Table 1) [12]. This result is what is expected on the basis of the AMBER simulations: a weakly bound charge remote water molecule in the case of Ac-AAAAK and a more strongly bound water directly interacting with the ammonium group for Ac-AAKAA.

In summary, the $RNH_3^+ \cdots H_2O$ binding energy is strongly dependent on how exposed the ammonium group is. The water binding energy of a fully exposed ammonium ion is ∼15 kcal/mol (*n*-decylamine) and slightly larger for very small systems (∼18 kcal/mol for methylamine). Values are smaller for ammonium groups that are not fully exposed as in the case of adding the second water molecule to *n*-decylamine (12 kcal/mol) or in the case of acetyllysine with an intramolecular hydrogen bond to the ammonium group (10 kcal/mol). Water binding energies for more buried ammonium groups with only one solvation site available are ∼9 kcal/mol (first H_2O in Ac-AAKAA, third H_2O in *n*-decylamine). Water adding to water in a second solvation shell is bound by ∼8 kcal/mol (fourth H_2O in *n*-decylamine). Charge remote water addition results in a binding energy of ∼7 kcal/mol (Ac-AAAAK).

4.2. The guanidinium group

The water binding energy of the guanidinium group is of the order of 9 kcal/mol [8]. Values measured for protonated arginine and arginine methyl ester are 9.0 and 9.2 kcal/mol, respectively (Table 1) [16]. These values are substantially smaller than those measured for ammonium systems of comparable size, 14.8 kcal/mol for both protonated *n*-decylamine and protonated dialanine [8]. This effect can be rationalized as reflecting the difference in size and therefore charge density of the two ionic groups: the large guanidinium group with an extensively delocalized charge leading to a weak electrostatic interaction with water versus the small ammonium group with a highly localized charge leading to a strong water interaction.

However, the large size of the guanidinium group allows more water molecules into the first solvation shell. A comparison of model structures of the two pentapeptides Ac-AAAAK and Ac-AAAAR indicates that while the charge in Ac-AAAAK is fully self-solvated this is not the case for Ac-AAAAR (Fig. 4b and c). The Ac-AAAAR model suggests that water molecules are able to bind directly to the guanidinium group in contrast to Ac-AAAAK where the model indicates charge-remote water addition. Experimental water binding energies of a series of arginine containing pentapeptides are all in the range of 9–10 kcal/mol [8], in close agreement with arginine itself (Table 1). For ammonium ions, on the other hand, a very large decrease in the water binding energy is found with increasing system size and increasing ability to shield the charge from 14.8 to 12.3 to 10.5 to 6.9 kcal/mol for the protonated molecules AA, AAA, AAAAA, and Ac-AAAAK [8,12]. Hence, experiment supports the theoretical finding that pentapeptides are not large enough to fully self-solvate and shield the charge of the large guanidinium group.

For the guanidinium as for the ammonium group the first water molecule is the most strongly bound. Values for the water–guanidinium binding energy are 9–10 kcal/mol for the first, 8–9 kcal/mol for the second, and 7–8 kcal/mol for the third water molecule (see, e.g. Ac-AARAA, Table 1) [8].

In summary, the water–guanidinium interaction amounts to ∼9 kcal/mol. For the second and third water molecule the

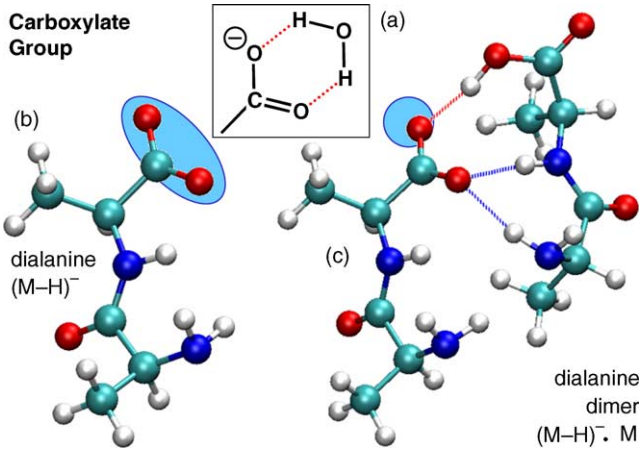


Fig. 5. Hydration sites of carboxylate containing species: (a) preferred geometry of one water molecule binding to the fully exposed carboxylate group of deprotonated dialanine; (b) molecular mechanics structures of deprotonated dialanine $(AA - H)^-$; and (c) of deprotonated dialanine dimer $(AA - H)^-{\cdot}AA$. The preferred water binding site(s) are indicated as the blue shaded area.

binding energy drops to a value that is indistinguishable from water binding in a charge-remote location (∼7 kcal/mol).

4.3. The carboxylate group

The water binding energy measured for deprotonated capric acid, $CH_3(CH_2)_8COO^-$, is 13.0 kcal/mol, 12% smaller than for the ammonium ion $CH_3(CH_2)_9NH_3^+$ of comparable size (Table 1). Similarly, water is less strongly bound to deprotonated dialanine (11.6 kcal/mol) than to protonated dialanine (14.8 kcal/mol) [8,13,17]. As in the examples of the previous sections the difference can be explained in terms of electrostatics with the water dipole binding more strongly to the highly concentrated positive charge of the ammonium ion compared to the more diffuse negative charge of the carboxylate ion.

As for the ammonium and guanidinium groups water binding energies decrease with increasing degree of hydration: 9.4 and 8.5 kcal/mol for the second and third water molecule binding to deprotonated dialanine (Table 1) [17]. AMBER indicates that four water molecules are able to bind in the first solvation shell, two water molecules to each carboxylate oxygen atom [17]. The four water molecules form a ring structure, where each water molecule forms (in addition to the interaction with the caboxylate) a donating and an accepting hydrogen bond with its two water neighbors.

Theory (AMBER and B3LYP/6-31G*) indicates that the water molecule of the singly hydrated species interacts simultaneously with both carboxylate oxygen atoms (O_C) yielding a six member cyclic arrangement (Fig. 5a) with $O_C \cdots H–O$ angles of <145° (B3LYP/6–31G*) [17]. This geometry with a relatively large deviation from linearity is not ideal for hydrogen bonds. However, structures with just one ideal linear hydrogen bond are calculated to be less favorable by several kilocalories per mole [17].

Carboxylate groups in peptides are expected to be self-solvated by good hydrogen bond donors such as amide hydrogen atoms if sterically feasible. One condition is obviously that the covalently bound "spacer" separating the amide from the carboxylate, –CO–NH–(spacer)–COO^-, is large enough to allow optimum alignment of –NH– with respect to $–COO^-$. In deprotonated dialanine where the spacer is just one carbon atom, an ethylidene group (Fig. 5b), the interaction between the amide and the carboxylate is limited to a poor hydrogen bond (1.86 Å, 120°, optimum B3LYP/6–31G* geometry). In the dialanine dimer $(AA–H)^-{\cdot}AA$, however, the carboxylate of one unit $(AA–H)^-$ is able to interact very favorably with the amide of the other unit AA (1.89 Å, 167°) according to theory (Fig. 5c) [17].

The reasons for studying dialanine are obvious: AA is a very simple peptide yielding data relatively easy to interpret and representing a molecule small enough for thorough theoretical modeling. The reason for studying the dialanine dimer is perhaps less obvious. However, the mass spectrum obtained by electrospraying dialanine in the negative ion mode (in an attempt to study $[AA–H]^-$) shows exclusively the dimer $(AA–H)^-{\cdot}AA$ [17]. Hence, the interesting question arises how solvation of $(AA–H)^-$ by AA (leading to the dimer) competes with solvation by water.

The dimer $(AA–H)^-{\cdot}AA$ can be dissociated into the monomer $(AA–H)^-$ in front of the drift cell by high energy collisions (100 eV lab) with the drift cell buffer gas (H_2O). Ions entering the cell quickly lose their excess energy and rapidly establish a thermal equilibrium with the buffer gas. Hydrating the $(AA–H)^-$ monomer in equilibrium with 1.3 Torr of water vapor at 260 K indicates that five water molecules are bound to the peptide on average. Experiments carried out under identical conditions with the dimer $(AA–H)^-{\cdot}AA$ show that the average species in this case is a singly hydrated $(AA–H)^-{\cdot}AA{\cdot}H_2O$ cluster. Hence, the monomer binds water molecules much more readily than the dimer. The data suggest that one neutral AA unit has the "solvating power" of four water molecules, i.e. solvation of the $(AA–H)^-$ anion is accomplished about equally well by either a neutral AA unit or four water molecules.

A glance at the model structures obtained theoretically (Fig. 5) shows that the carboxylate group in $(AA–H)^-$ is hardly stabilized by any interactions with the rest of the molecule. The negative charge is fully exposed and available for intermolecular interaction. In the $(AA–H)^-{\cdot}AA$ dimer, on the other hand, the situation is very different. In this case there is a strong interaction between the $(AA–H)^-$ carboxylate group and the neutral AA peptide, resulting in a well-shielded negative charge. Hence, on the basis of the model structures it is expected that the monomer adds water molecules much more readily than the dimer in agreement with experiment. Model structures of the $(AA–H)^-{\cdot}(H_2O)_4$ system indicate that a similar degree of charge solvation is achieved as in $(AA–H)^-{\cdot}AA$ explaining the experimental observation that $(AA–H)^-$ adds four more water molecules on average than $(AA–H)^-{\cdot}AA$ [17].

In summary, the water–carboxylate binding energy is 12–13 kcal/mol. Values are smaller for a partially self-solvated carboxylate group and drop to 8 kcal/mol when the charge is well shielded (dialanine dimer).

5. Molecules with several ionic groups

For molecules carrying more than one ionic group the question arises whether each individual ionic group is hydrated following the findings outlined above for the ammonium, guanidinium, and carboxylate groups or whether there are additional effects due to the presence of the additional charges on the molecule.

Two cases have to be differentiated: molecules with several charges of the same sign (leading to multiply charged ions) and molecules with charges of opposite sign (potentially leading to salt-bridge structures).

5.1. Multiply charged ions

Hydration of doubly charged diammonium alkane systems has been studied by Kebarle and coworkers [11]. Results for $H_3N(CH_2)_{12}NH_3^{2+}$ are summarized in Table 1 along with our findings of the singly protonated *n*-decylamine system [8]. It is evident that the binding energy of the first water molecule is only slightly larger for $H_3N(CH_2)_{12}NH_3^{2+}$ than for $H_3C(CH_2)_9NH_3^+$. The rather small difference between the two values is most likely due to slightly different charge densities on the ammonium groups in the two cases: in $H_3C(CH_2)_9NH_3^+$ part of the charge is delocalized over 10 carbon atoms, in $H_3N(CH_2)_{12}NH_3^{2+}$ only over 6 carbon atoms per charge. The situation is very different for very small ions such as atomic ions where addition of an extra charge has a tremendous effect on the water binding energy. The water binding energy of the doubly charged Ca^{2+} ion (with a radius of 1.00 Å [18]) is ∼51 kcal/mol [19], twice that of the singly charged Na^+ ion (∼25 kcal/mol, 0.99 Å radius) [20,21]. Not surprisingly, water bound to these small ions interacts simultaneously with all the charge present on the ion. This is not so in larger systems such as peptides where two positive ionic groups, driven by Coulomb repulsion, are generally separated from each other by a distance much longer than the size of the water molecule. In such systems the water molecule is either bound to one charge or the other, but not simultaneously to both.

The second water molecule is the one that benefits from the presence of the second charge in $H_3N(CH_2)_{12}NH_3^{2+}$ compared to $H_3C(CH_2)_9NH_3^+$ (Table 1). It is bound equally strongly than the first water, whereas in $H_3C(CH_2)_9NH_3^+$ there is an 18% drop from the first to the second water. The third water in $H_3N(CH_2)_{12}NH_3^{2+}$ sharing an ammonium group with another water molecule binds similarly strongly as the second water in $H_3C(CH_2)_9NH_3^+$ [8,11].

Whereas the patterns summarized by Eqs. (2) and (3) are obvious for the simple mono-/diammonium alkane system (Table 1), the situation is expected to be less clear cut for peptides.

Table 2
Experimental hydration enthalpies[a] (in kcal/mol) of neurotensin (ELYENKPRRPYIL)

	$z=1$		$z=2$		$z=3$	
	n	$-\Delta H_n^\circ$	n	$-\Delta H_n^\circ$	n	$-\Delta H_n^\circ$
$0<n/z\leq 1$	1	9.2	1	10.3	1	15
			2	8.9	2	12
					3	9.5
$1<n/z\leq 2$	2	9.8	3	9.6	4	9.3
			4	9.4	5	9.4
					6	9.8
$2<n/z\leq 3$	3	9	5	8.5	7	8.8
			6	8	8	10
					9	9
$3<n/z\leq 4$	4	9	7	9	10	9

[a] z indicates the charge state, n the number of water molecules (see Eq. (1)). Error limits ±0.3 for fractional and ±1 kcal/mol for integer values.

$$\Delta H_n^{\circ(\mathrm{mono})} \cong \Delta H_{2n}^{\circ(\mathrm{di})} \quad (n = 1, 2) \tag{2}$$

$$\Delta H_n^{\circ(\mathrm{di})} \cong \Delta H_{n+1}^{\circ(\mathrm{di})} \quad (n = 1, 3) \tag{3}$$

The overlap of several other effects (self-solvation, multi-dentate peptide–water interaction, peptide conformational adjustments) might obscure an obvious trend in the water binding energies. For instance, the water binding energies for neurotensin (charge states +1, +2, and +3) given in Table 2 do not unambiguously follow the $H_3C(CH_2)_9NH_3^+/H_3N(CH_2)_{12}NH_3^{2+}$ trend [8]. However, data such as those shown in Fig. 2 are overall consistent with the ammonium alkane trend indicating that the number of water molecules adding to the peptide neurotensin under given experimental conditions is strongly related to the number of ionic groups present in the peptide. If we focus on the peaks of largest mass in each of the mass spectra in Fig. 2, we conclude that up to six or seven water molecules add to charge state +1 and about twice and three times as many to charge states +2 and +3, respectively. In other words, the data suggest that the degree of hydration is directly proportional to the number of charges present on the peptide. This result holds for virtually every peptide (5–15 residues) we have measured: for each charge six or seven water molecules are added (260 K and 1.3 Torr of water vapor) [8].

How can we rationalize the neurotensin data of Table 2? Based on the insight gained from Fig. 2 and the $H_3C(CH_2)_9NH_3^+/H_3N(CH_2)_{12}NH_3^{2+}$ system it makes sense to compare ΔH_n° of charge state +1 with ΔH_{2n}° and ΔH_{3n}° of charge states +2 and +3, respectively, and to check for pair or triple wise patterns within charge states +2 and +3, respectively. In other words, we would like to explore the validity of Eqs. (4)–(6) (in analogy to Eqs. (2) and (3)).

$$\Delta H_n^{\circ(z=1)} \cong \Delta H_{2n}^{\circ(z=2)} \cong \Delta H_{3n}^{\circ(z=3)} \quad (n = 1\text{–}3) \tag{4}$$

$$\Delta H_n^{\circ(z=2)} \cong \Delta H_{n+1}^{\circ(z=2)} \quad (n = 1, 3, 5) \tag{5}$$

$$\Delta H_n^{\circ(z=3)} \cong \Delta H_{n+1}^{\circ(z=3)} \cong \Delta H_{n+2}^{\circ(z=3)} \quad (n = 1, 4, 7) \tag{6}$$

With the exception of $\Delta H_1^{\circ(z=3)}$ (and perhaps $\Delta H_2^{\circ(z=3)}$) the values in Table 2 are fairly constant with a possible slight drop with increasing n. Hence, Eqs. (4)–(6) are readily satisfied for nearly all values of n. For instance, for the data set "$1 < n/z \leq 2$" Eqs. (4)–(6) lead to $-9.8 \cong -9.4 \cong -9.8$ kcal/mol, $-9.6 \cong -9.4$ kcal/mol, and $-9.3 \cong -9.4 \cong -9.8$ kcal/mol.

The only clear outlier not following the general trend is $\Delta H_1^{\circ(z=3)}$ with a significantly more negative value of -15 kcal/mol. Thus, we have to address the question why there is a rather strong charge state dependence for the ΔH_1°-values from -9.2 to -10.3 to -15 kcal/mol, a distinct deviation from the $H_3C(CH_2)_9NH_3^+/H_3N(CH_2)_{12}NH_3^{2+}$ behavior. In the following we will show that there are at least two distinct differences between peptide charge states and the mono-/diammonium alkane system: the degree of charge exposure and the nature of the charged groups. While the charged groups in $H_3C(CH_2)_9NH_3^+$ and $H_3N(CH_2)_{12}NH_3^{2+}$ are exclusively fully exposed ammonium groups, the charged groups in neurotensin are not necessarily all of the same type and the charge exposure is very likely charge state dependent. The charge in a singly charged peptide of sufficient size tends to be buried in the interior of the molecule, well self-solvated and difficult to hydrate. In high charge states, on the other hand, Coulomb repulsion leads to fairly well exposed ionic groups potentially offering very favorable hydration sites. The most likely sites of initial protonation in neurotensin are the two arginine side chains. The measured binding energies of 9.3 and 10.2 kcal/mol for $z = 1$ and 2 are consistent with this expectation. In charge state +3, however, the third charge sits most likely on the lysine side chain yielding an ammonium ion. The relatively large value of 15 kcal/mol for $-\Delta H_1^{\circ(z=3)}$ is consistent with a water molecule bound to a fairly exposed ammonium group. Since the peptide has three charges it will most likely be extended leading to an exposed ammonium group consistent with the observed hydration energy. It should be emphasized that the value of 15 kcal/mol is not unusually large (see *n*-decylamine in Table 1) and there is no indication that it is enhanced by the presence of the other two charges.

In summary, the number of favorable hydration sites in a peptide with charge z is proportional to z indicating that multiply charged peptides are hydrated like z singly charged peptides. This concept leads to a set of relationships between the various hydration enthalpies outlined in Eqs. (4)–(6). However, although these relationships hold in general, they are not expected to hold rigorously, because the type of peptide–water interaction (type of ionic group, degree of charge exposure) is different for each value of n and z.

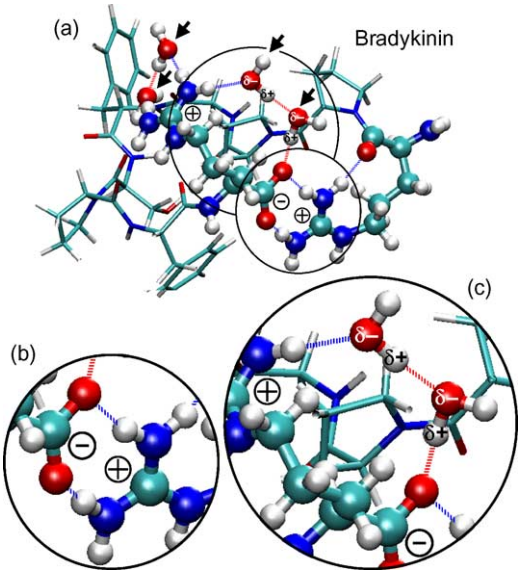


Fig. 6. (a) Molecular mechanics structure of protonated bradykinin hydrated with four water molecules. The arrows indicate the position of the water molecules. The regions inside the two circles are blown up in the panels below. Blow-up of the (b) arg^1 guanidinium–arg^9 carboxylate salt bridge and (c) solvent-separated ion pair of the arg^9 guanidinium–arg^9 carboxylate salt bridge.

5.2. Salt bridges

Ionic groups of opposite sign attract each other. Molecules containing both cationic and anionic groups can form intramolecular ionic bonds or salt bridges. Bradykinin (BK) is a nonapeptide (RPPGFSPFR) with the potential of forming a stable salt bridge structure in the gas phase, as it contains two arginine residues and an acidic C-terminus. There is a host of experimental data available that suggests that the $(BK + H)^+$ gas-phase structure includes three ionic groups, two guanidinium and one carboxylate group [22–27]. Molecular modeling of this system indicates that the charged groups closely interact with each other, forming a $(+)\cdots(-)\cdots(+)$ salt bridge system [28]. Fig. 6 shows a $(BK + H)^+$ salt bridge structure obtained by molecular modeling (Barran et al., unpublished results) with the salt bridge between the arg^1 guanidinium and the C-terminal arg^9 carboxylate high-lighted in panel b. A second salt bridge is present between the arg^9 guanidinium group and the carboxylate. However, the model shown in Fig. 6 also contains four water molecules and two of them have inserted into the arg^9 guanidinium–carboxylate salt bridge (Fig. 6c) forming a solvent-separated ion pair. The water molecules with their dipoles tucked in nicely between the two ionic groups, $(-)\cdots H_2O\cdots H_2O\cdots(+)$, appear to be sitting in perfect hydration sites. However, the enthalpy change for hydrating the salt bridge (Scheme 1) is not necessarily larger than average because the electrostatically fa-

$$(–)\cdots(+) \;+\; 2\,H_2O \;\rightarrow\; (–)\cdots H_2O\cdots H_2O\cdots(+)$$

Scheme 1.

Table 3
Experimental hydration enthalpies and entropies[a] of protonated bradykinin

n	$-\Delta H_n^\circ$ (kcal mol^{-1})	$-\Delta S_n^\circ$ (cal mol^{-1} K^{-1})
1	10.7 (0.3)	26 (1)
2	10.1 (0.3)	25 (1)
3	10.1 (0.3)	26 (1)
4	10 (1)	27 (4)

[a] See Eq. (1). Error limits given in parentheses.

vored salt bridge $(-)\cdots(+)$ has to be broken, resulting in an increase of the guanidinium H to carboxylate O distance from 1.8 to 4.9 Å.

Experimental hydration enthalpies for the first two water molecules are not unusually high (-10.4 ± 0.3 kcal/mol), but the values stay unusually constant at 10 kcal/mol up to the fourth water molecule (Table 3) [8]. Generally, hydration enthalpies drop to values of -8 or -9 kcal/mol for singly charged peptides by the time four water molecules are added (see Tables 1 and 2). This exceptional trend of constant ΔH_n°-values for $(BK + H)^+$ indicates that there are many good hydration sites, a situation expected for a salt-bridge structure with three ionic groups. Fig. 6 shows that all four water molecules in $(BK + H)^+ \cdot (H_2O)_4$ are directly bound to one of the ionic groups. The peptide BK is too small to fully self-solvate three charged groups, but since it is large enough to efficiently shield the charge of just one guanidinium group [28], the data presented here provides additional support for the hypothesis that gas-phase $(BK + H)^+$ is a salt-bridge structure, at least when it is partially hydrated.

In summary, salt bridges offer perfect hydration sites. However, water insertion into the salt bridge comes at the expense of separating the salt bridge ion pair. As a consequence, the water–salt bridge binding energy appears to be of the same order as the water–cation or water–anion binding energy. Hence, for salt bridges as for multiply charged ions the presence of more than one charge does not enhance the water binding energy compared to a singly charged ion.

6. Structural changes induced by hydration

At this point we understand the first steps of hydration of peptides fairly well with respect to water binding sites and energies. However, one of the most interesting issues still to be addressed is that of peptide structural changes as a function of water addition. Structural changes expected to occur include change of conformation and proton transfer (zwitterion formation). Little is known about the mechanism of these structural changes and about the role of individual water molecules. Relevant questions are how the relative energies of the various isomers change as a function of hydration and how the barriers between the potential minima change.

These challenging questions are difficult to address experimentally. In the following sections we will report on advances made in our group by showing first results of work in progress and suggesting future experiments with new instrumentation more suitable for these kinds of studies.

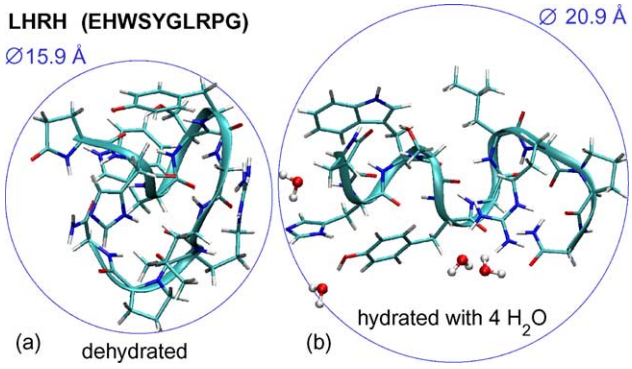


Fig. 7. Molecular mechanics structures of protonated human luteinizing hormone releasing hormone (LHRH): (a) dehydrated and (b) hydrated with four water molecules. The diameters of the smallest encompassing spheres are 15.9 and 20.9 Å, respectively.

6.1. Change of conformation

The kind of question we would like to address here is how the Alzheimer's Amyloid-β peptide (see Section 1) ends up in its preferred solution-phase structure, where the hydrophobic parts are buried in the interior, as individual water molecules are added to the preferred gas-phase structure, where the hydrophobic residues are on the surface [3]. Is this major structural rearrangement occurring in one step at a critical number of water molecules or is it occurring in many little steps, one step with each additional water molecule? How high are the barriers? Can we kinetically trap gas-phase structures in solution as they are being hydrated and, vice versa, can we trap solution-phase structures in the gas phase as they are being dehydrated?

We do not know the answers to these interesting questions yet. But we would like to use preliminary results obtained for the peptide luteinizing hormone releasing hormone (LHRH) to demonstrate how we intend to address those questions. Important properties of the decapeptide LHRH (EHWSYGLRPG) include the presence of one arginine (arg^8) and the absence of any carboxyl and amino groups. The N-terminus is blocked by forming an amide with the glu^1 side chain, the C-terminus by forming an $-CONH_2$ amide. Theoretical work indicates that the gas-phase structure of protonated LHRH is very compact (Fig. 7a), in contrast to the structure of LHRH hydrated by four water molecules (Fig. 7b) which is much more extended in close agreement with the structure expected in aqueous solution (Barran et al., unpublished results; see also ref. [29]). The smallest spheres encompassing the two structures have diameters of 15.9 Å for the dehydrated and 20.9 Å for the four-fold hydrated molecule, respectively. This dramatic increase in size due to the addition of just four water molecules should easily be detected by an ion mobility based cross section measurement [16]. However, our present nano-ESI experimental setup does not

allow measurement of cross sections of hydrated species [4]. Two fundamental modifications of the instrumentation are required.

First, we have to be able to form hydrated species before they enter the drift cell. This can readily be achieved by transferring incompletely desolvated ions from the electrospray ion source into the mass spectrometer. Several research groups have demonstrated that this is possible [30,31]. In fact, in general mass spectrometrists go to great lengths to ensure complete desolvation by passing the ions through a series of dehydration stages (counter-flow of dry nitrogen, heated capillary, etc.). The second instrumental modification includes insertion of a mass filter in front of the drift cell. With the ability to mass select in front and after the drift cell we will be able to determine whether the ions entering the cell have the same mass as those exiting. If this is not the case, i.e. if water molecules dissociate from the hydrated ions as they drift through the cell, the cell temperature can be decreased until the lifetime of the hydrated species is long enough to do the experiment.

In summary, not much is known yet about the effect of individual water molecules on the peptide conformation. Preliminary theoretical results on the peptide LHRH indicate that the conformation of the peptide hydrated by a small number of water molecules (less than one H_2O per two residues) is more similar to the solution-phase structure than to the fully desolvated structure. Ion mobility experiments should be able to verify this result.

6.2. Zwitterion formation

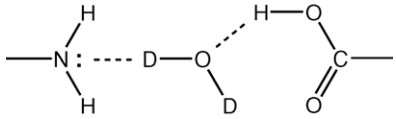
The amino acid glycine is known to be a zwitterion in aqueous solution, but it is also known that it is not a zwitterion in the gas phase [32–36]. Hence, at some point during the hydration process the carboxylic proton transfers to the amino group. Theory indicates that the gas-phase zwitterion of glycine is not even a minimum on the potential energy surface and converts without barrier to the neutral form [37]. However, zwitterionic gly·$(H_2O)_2$ is believed to be stable based on theory but still less stable by 12 kcal/mol than the neutral ground state [37]. Recent photoelectron spectroscopy data indicate that glycine–water clusters, gly·$(H_2O)_n$, with $n < 5$ are neutral whereas clusters with $n \geq 5$ are zwitterionic in their most stable geometry [38]. These results together indicate that the glycine zwitterion structure is stabilized on average by 4 kcal/mol per water molecule added.

Peptides, like amino acids, are known to form zwitterions in water with every amine and guanidine protonated and every carboxyl group deprotonated (at pH 7). In the gas phase the situation is not so clear. For instance for the protonated pentapeptide AARAA several structural isomers are potentially possible. It is reasonable to assume that all low-energy isomers of $(AARAA + H)^+$ include a protonated arginine side chain, but it is not obvious whether a backbone with neutral termini ($H_2N \cdots COOH$) or a zwitterionic backbone ($H_3N^+ \cdots COO^-$) is energetically more favorable.

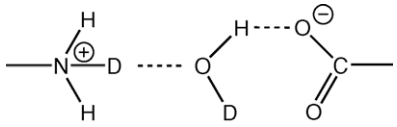
We got interested in this particular peptide because gas-phase H/D-exchange experiments with D_2O show rapid exchange for at least three hydrogen atoms for $(AARAA + H)^+$, whereas the corresponding molecules with one or the other terminus blocked, Ac-AARAA and AARAA-OMe, respectively, show basically no exchange [39]. Neither the $CH_3CONH \cdots COOH$ backbone of protonated Ac-AARAA nor $H_2N \cdots COOCH_3$ of protonated AARAA-OMe can form a low-energy zwitterion because either the basic N-terminus or the acidic C-terminus is blocked. Only $(AARAA + H)^+$ with free termini, showing extensive H/D-exchange, could have a $H_3N^+ \cdots COO^-$ backbone. Therefore based on the H/D-exchange result it is tempting to conclude that $(AARAA + H)^+$ is zwitterionic. However, calculations indicate that zwitterion (or salt bridge) structures of $(AARAA + H)^+$ are very compact in disagreement with ion mobility experiments which yield relatively large cross sections for the $(AARAA + H)^+$ ion [39]. Calculated charge solvation structures, on the other hand, are less compact and agree with the experimental cross section. An extensive search of the $(AARAA + H)^+$ potential energy surface on the B3LYP/6–31 + G(d,p) level of theory confirms that the species with neutral termini is 4.8 kcal/mol more stable than the salt bridge species [39].

However, for the hydrated species, $(AARAA + H)^+ \cdot H_2O$, the salt bridge structure is calculated to be only 1.8 kcal/mol less stable than the charge solvation structure, yielding a 3 kcal/mol stabilization of the zwitterion upon addition of one water molecule compared to the non-zwitterion form [39]. This number compares favorably with the 4 kcal/mol value obtained for the stabilization of the glycine zwitterion and could indicate a more general trend which could be used to estimate the number of water molecules required to make the zwitterion of a given species the most stable structure. In the case of $(AARAA + H)^+$ addition of two water molecules is expected to generate a stable zwitterion based on this trend. However, no calculations have been carried out on the $(AARAA + H)^+ \cdot (H_2O)_2$ system and clearly more research is needed to establish general rules.

The extensive theory done on the $(AARAA + H)^+ + H_2O$ system provided an explanation of the gas-phase H/D-exchange mechanism [39]. Low energy structures were found where the water molecule inserted between the N- and C-terminus (Scheme 2), a configuration ideally set up for H/D-exchange via the "relay"-mechanism [40]. The hydrated salt bridge structure resulting from relay H/D-exchange (Scheme 3) is calculated to be nearly isoenergetic with the starting charge solvation structure [39]. The transition state between these two structures was characterized yielding es-



Scheme 2.



Scheme 3.

sentially no barrier after zero point energy corrections [39]. Hence, theory was able to explain the fact that the three hydrogen atoms of the N- and C-termini underwent facile H/D-exchange. Theory also explained why neither Ac-AARAA nor AARAA-OMe are able to undergo H/D-exchange because Ac-AARAA is missing the basic amino group and AARAA-OMe the acidic hydrogen required in Scheme 2. This example shows that gas-phase H/D-exchange data are difficult to interpret and require an in-depth analysis.

In summary, zwitterions are far less stable in the gas phase than in aqueous solution. Many amino acids and small peptides that are known to be zwitterions in solution are not zwitterions when deprived of solvent stabilization. However, first results indicate that a relatively small number of water molecules is required to stabilize zwitterions and salt bridges relative to corresponding structures with neutral acidic and basic groups.

7. Entropy

Equilibrium experiments yield not only energetic but also entropic information (Eq. (1)). Fig. 8 shows all hydration entropy values measured in our group as a function of the corresponding ΔH°-value [8,12,17]. The plot includes ΔS_n°-data for all values of n, for positive and negative, and for singly and multiply charges ions. It can be seen that all ΔS°-values are negative (ranging from -10 to -40 cal mol^{-1} K^{-1}), because the decrease of entropy due to the loss of translational and rotational degrees of freedom upon binding a water molecule is only partly compensated by the gain of vibrational entropy.

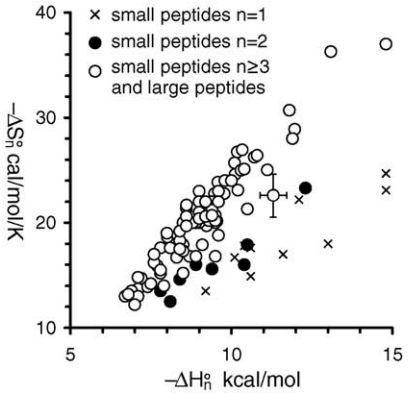


Fig. 8. Entropy–enthalpy plot of all the peptide hydration data (see Eq. (1)) collected in our lab. The ×-symbols and solid dots indicate data obtained for adding the first and second water molecule, respectively, to small molecules (100–200 amu). The open circles represent all the other data. The error bars shown for one data point are typical for most data included here.

The data shown in Fig. 8 reveal that values for (ΔS°, ΔH°)-pairs are not randomly scattered, but cover a triangular range with the corner points (10, 5), (20, 15), and (40 cal mol^{-1} K^{-1}, 15 kcal mol^{-1}). Entropy-enthalpy pairs with small $\Delta S^\circ/\Delta H^\circ$-ratio are found to be predominantly ΔS_1° and ΔH_1°-values of amino acids, dipeptides, and other small molecules (100–200 amu), shown as ×-symbols in Fig. 8. A search for (ΔS_2°, ΔH_2°)-pairs of the same set of molecules (100–200 amu) yields data (solid dots in Fig. 8) which are scattered between the ×-symbols and the open circles. The open circles represent all of the data obtained for peptides with mass >200 amu and ($\Delta S_{n>2}^\circ$, $\Delta H_{n>2}^\circ$)-data of small molecules.

The data within each set, the ×'es, the dots, and the circles (see Fig. 8), show a distinct correlation between entropy and enthalpy. The × set of data with the smallest $\Delta S^\circ/\Delta H^\circ$-ratios indicates that binding the first water molecule to small molecules yields relatively floppy hydrates (small loss of entropy compared to the corresponding change of enthalpy). $\Delta S^\circ/\Delta H^\circ$-ratios are somewhat larger for adding the second H_2O to small molecules (solid dots), but the resulting hydrates are still more floppy than the majority of hydrates (open circles). A correlation between entropy and enthalpy is expected at least to some degree because a tightly bound water molecule gives rise to both a large binding energy and a large loss of entropy.

In summary, the change of entropy upon binding one water molecule to a naked or partially hydrated peptide ion, $M^{\pm z}\cdot(H_2O)_{n-1}$, is in the range from -10 to -40 cal mol^{-1} K^{-1}. For a given n and within a family of molecules ΔS°-values strongly correlate with the corresponding ΔH°-values. Small molecules with a mass of 100–200 amu and peptides with a mass of >200 amu each comprise a distinct family of molecules.

8. Conclusions

We have studied the first steps of hydration of peptides (<15 residues). It was found that ionic groups offer particularly good hydration sites which lead to a thorough study of the ionic groups typically present in peptides: the ammonium, guanidinium, and carboxylate group. We have studied the effect of combining several ionic groups on the same molecule, the effect of multiply charged ions and salt bridge effects. All of these studies lead to a good understanding of peptide hydration with respect to water binding sites and binding energies. The loss of entropy associated with hydration is well characterized, too. There is a brief summary at the end of each section in the paper highlighting the major findings of the section.

Issues that still need to be addressed in the future include hydration beyond the first steps and peptide structural changes as a function of hydration including change of con-

formation and zwitterion formation. However, first results indicate that the most interesting structural changes of peptides might occur during the very first steps of hydration.

Acknowledgments

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