The effect of association with sulfate on the electrophoretic mobility of (poly-)arginine and (poly-)lysine

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Abstract

Domains rich in cationic amino acids are ubiquitous in peptides with ability to cross cell membranes, which is likely related to the binding of such polypeptides to anionic groups on the membrane surface. In order to shed more light on these interactions we investigated specific interactions between basic amino acids and oligopeptides thereof and anions by means of electrophoretic experiments and molecular dynamics simulations. To this end, we measured the electrophoretic mobilities of arginine, lysine, tetraarginine, and tetralysine in sodium chloride and sodium sulfate electrolytes as a function of ionic strength. The mobility was found to be consistently lower in sodium sulfate than in sodium chloride at the same ionic strength.

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The decrease in mobility in sodium sulfate was greater for tetraarginine than for tetralysine and was larger for tetrapeptides compared to corresponding free amino acids. On the basis of molecular dynamics simulations and Bjerrum theory we rationalize these results in terms of enhanced association between the amino acid side chains and sulfate. Simulations also predict a greater affinity of sulfate to the guanidinium side chain groups of arginine than to the ammonium groups of lysine, since the planar guanidinium geometry allows simultaneous strong hydrogen bonding to two sulfate oxygens. We show that the sulfate binding to arginine, but not to lysine, is cooperative. These result are consistent with the greater decrease in the mobility of arginine compared to that of lysine upon adding sulfate salt. The non-specific mobility retardation by sulfate is ascribed to its electrostatic interaction with the cationic amino acid side chain groups.

Introduction

Peptides that have the ability to cross the cellular membrane and are used to deliver specific molecules into the cell are often rich in cationic amino acids, especially arginine.[1,2] The cationic guanidinium side chain group of arginine can effectively interact with various moieties present in the membrane, in particular with the negatively charged head groups, which may facilitate such transport.[2] The monovalent guanidinium cation is composed of three amino groups bonded to a single central carbon atom, see Figure 1. The ion is quasi-aromatic,[3] because of conjugations between the lone pairs of the nitrogens and the empty p-orbital of the carbon, and its geometry is planar.[4,5] Thus, the guanidinium ion is capable of acting as a hydrogen bond donor in the plane of the ion only. Because of its positive charge and extensive electron delocalization the guanidinium ion is not an efficient hydrogen bond acceptor, therefore the “faces” of the guanidinium ion are poorly hydrated.[5] The multifaceted guanidinium motif is of general importance in biochemistry. In addition to it being found in the side chain of the protein-forming amino acid arginine, guanidinium salts are also commonly used as protein denaturants. To a certain extent, the guanidinium ion has a structure and geometry similar to that of urea (diaminomethanal), which is another com-

mon denaturant.[6]

Molecular simulations and neutron scattering experiments have shown that there is an unusually large degree of ion association in guanidinium sulfate solutions.[7] This is due to the formation of network-like structures where the guanidinium ion bridges sulfates by forming pairs of hydrogen bonds between sulfate oxygens and guanidinium hydrogens. Such association is made particularly favorable by the distance between the hydrogens in the guanidinium cation, which closely matches the distance between the sulfate oxygens so that two hydrogen bonds are possible between each guanidinium-sulfate pair.[7,8] In addition, it has been demonstrated that guanidinium sulfate does not show the strong denaturing effect of other guanidinium salts.[9] This has been ascribed to the strong guanidinium-sulfate association in the bulk solution competing with the binding of guanidinium to the protein that causes denaturation.[7] Strong ion association is also consistent with osmotic and transport properties of guanidinium sulfate solutions - the mean activity coefficient reaches much lower values and the viscosity much higher values for high concentration than is typical for sulfates with monovalent cations.[10] The guanidinium motif also has specific affinity for other oxyions and carboxylates and it is common in synthetic complexing agents for anions.[8] Self-assembled monolayers of surfactants with guanidinium head groups specifically binds phosphate residues of nucleotide phosphates, which is explained by dual hydrogen bonding to the oxygens of the phosphate residues.[11] It is also remarkable that arginine is commonly found in anion binding sites in proteins, the binding to which is inhibited by arginine specific reagents.[12]

Given the strong affinity of guanidinium to sulfate, it is interesting to ask whether sulfate would be more strongly adsorbed on polyarginine due to ion pairing with the side chain groups than simple electrostatics would suggest. Such an affinity to specific anions may have direct biochemical relevance. As mentioned above, polypeptides capable of crossing membranes frequently have domains that are rich in cationic amino acids.[1,2] Such membrane penetration does not appear to be dependent on any particular secondary or tertiary structure of the cation-rich domain and even very simple peptides such as nonaarginine[13] and heptaarginine[14] are capable of crossing cell membranes. High affinity of the peptide to heparin sulfate proteoglycans, which contain highly anionic

sulfonated glycosaminoglucan moieties, appears to be required for membrane crossing in living cells, however.[13,15] Furthermore, arginine residues are more efficient in promoting the ability of peptides to cross membrane than lysine residues, which have been ascribed to a stronger affinity of arginine than of lysine to anionic groups.[16]

Excess adsorption of ions on polypeptides can be detected in electrophoresis experiments as an abnormally large decrease in electrophoretic mobility in the presence of sulfate salts. For a sufficiently strong adsorption of polyvalent ions, even a reversal in the sign of the electrophoretic mobility is possible.[17–19] For solutions containing ions of valency higher than one, the interpretation of electrophoresis experiments in terms of specific interactions is rather complicated. This it due to the fact that screening in such solutions tend not to be well described by the Debye-Hückel limiting law, which constitutes the foundation of the theoretical framework in which most electrophoresis experiments are interpreted.[20] Due to ion-pairing, and other less intuitive types of ion-ion correlations, screening by polyvalent ions is typically more effective than predicted by a linear treatment of the electrolyte.[21–24] As sulfate is divalent, deviations from Debye-Hückel theory can be expected and need to be accounted for when its specific binding is investigated.

Di- and polyarginine have been shown to share a salient feature predicted by simulations for bulk guanidinium salts, namely that the guanidinium moieties form cation-cation contact ion pairs in a manner analogous to aromatic stacking.[25–28] Guanidinium-guanidinium pairing is suppressed in the sulfate solutions due to competition with the formation of cation-anion network-like structure.[7] In the peptides, stacking is seen as transient close contacts between the arginine side chains. It is not a priori obvious that the balance between guanidinium self-stacking and the formation of cation-anion networks with sulfate is the same in the peptides as in bulk guanidinium sulfate solution. In particular, the situation for the peptide side chains is sterically completely different from that in the bulk solution.

The aim of the present study is to show by means of molecular dynamics (MD) simulations and electrophoretic measurements that the specific association between sulfate and arginine side chains enhances the electrostatic screening of arginine and polyarginine and discuss the conse-

quences thereof. The sulfate anion may be regarded as a prototypical tetrahedral oxyion and may, therefore, serve also as a proxy for understanding more complicated systems. To this end, we carried out capillary electrophoresis of polyarginine in the presence of sodium sulfate, as well as MD simulations of the same system. For comparison, we have also carried out analogous experiments and simulations for polylysine. Polylysine does not show any side chain pairing,[28] nor do bulk solutions of ammonium sulfate exhibit any signs of association beyond what can be expected from the ionic valencies; the activity coefficients are similar to those of the alkali metal sulfates.[29] As a reference, experiments were also carried out in sodium chloride, where neither specific binding nor a strong non-Debye-Hückel behavior should occur. To investigate the effect of the direct interaction between the side chain groups and sulfate, we have also carried out experiments and simulations for arginine and lysine as free amino acids. For these species, there is clearly no possibility of intramolecular association between side chains. For sufficiently low amino acid concentrations, the characteristics of the pairing with the counter ions can, therefore, be investigated in isolation from effects due to side chain pairing and network formation with sulfate.

Experimental Method

Chemicals

Sodium hydroxide, p.a., sodium chloride, p.a., sodium sulfate, p.a., sodium hydroxide, p.a., and thiourea, p.a., were purchased from Lachema (Czech Republic). L-arginine, 99,5 %, was delivered by Fluka (Switzerland). D,L-lysine, 98 %, was obtained from Sigma (USA). Tetraarginine, trifluoroacetate salt, and tetralysine, acetate salt, were purchased from Bachem (Switzerland). Methanol, HPLC grade, was purchased from Merck (Germany). Bis-tris buffer (bis[2-hydroxyethyl]amino-tris[hydroxymethyl]methane), 98 %, and hydrochloric acid, 36.5-38 % Molecular Biology Tested, was obtained from Sigma (USA). Background electrolytes and samples were prepared using deionized water produced by a Milli-Q system, Millipore (USA).

Instrumentation

A 7100 CE instrument, Agilent Technologies (Germany), was used for all experiments. A fused silica capillary, 75 μm id, coated with hydrophilic polymer (CEP) was purchased from Agilent Technologies (Germany) and cut to 80.0 cm total length (71.5 cm to the detection window). Prior to the first use, the capillary was flushed for 20 min with methanol and for 10 min with deionized water using a pressure of 100 kPa. The diode array UV detector was operated at a wavelength of 200 nm. The temperature was kept at 25 °C by air cooling.

Mobility measurements

A procedure derived from the electro-osmotic flow (EOF) mobility measurement method from ref. 30 was employed to determine electrophoretic mobilities of the amino acids and tetrapeptides. As the aim of this study was to investigate the influence of the background electrolyte (BGE) anion, it was essential to keep the BGE composition as simple as possible. Thus, no buffer was used, except for test measurements aimed at assessing the effect of variations in pH in the unbuffered solutions. Under such conditions, it was found necessary to use a capillary with deactivated inner walls. This setup would under the standard experimental protocol result in long migration times, during which stable conditions would be difficult to keep. Therefore, a protocol intended to minimize the time in the capillary was employed.

Before each run, the capillary was flushed for 5 min with methanol, 5 min with deionized water and 2 min with BGE using a pressure of 100 kPa. A zone of 2 mmol/l aqueous solution of the studied analyte with 0.1 g/l thiourea was injected into the capillary by a pressure of 5 kPa for 3 s. The zone was then pushed into the capillary by the application of 5 kPa of pressure for 2 min in order to avoid the loss of the thiourea zone in case of reversed EOF. Subsequently, a voltage of +10 kV was applied for 10 min. During this period, the thiourea zone was mobilized only by EOF while the motion of the analyte zone resulted from the combination of EOF and electrophoretic mobility. Then a zone of aqueous solution of 0.1 g/l thiourea was injected with a pressure of 5 kPa for 3 s. Finally, all the zones in the capillary were mobilized by the application of 5 kPa of pressure

and the UV detector recorded the zones passing through the detection window. At the beginning of this pressure mobilization, the registration of the UV signal was started. The electrophoretic mobility, μ, of each analyte was calculated according to[31]

$$\mu = \frac{(t_2 - t_1) l_d}{t_3 t_U} \frac{l_c}{U} \tag{1}$$

where t_1 stands for the time needed for the analyte zone to be pushed through the detection window, t_2 and t_3 by analogy for the times of the first and the second thiourea zone, respectively, l_c stands for the total capillary length, l_d for the length to the detection window, U for the voltage applied and t_U for the duration of the voltage application.

The mobility of arginine and lysine was measured for 10, 20, 50, 70, 100, and 120 mM ionic strength in sodium chloride and in sodium sulfate BGE. For tetraarginine and tetralysine mobility measurements was made for 2, 5, 10, 50, 70, 100, 120, and 150 mM ionic strengths in the same BGEs. To investigate the effect of omitting buffer, the mobility of each analyte in each BGE was measured at 50 mM ionic strength in the presence of 10 mM Bis-Tris buffer, adjusted to pH 6.5 with hydrochloric acid and sodium hydroxide.

Simulation Method

Molecular dynamics simulations were carried out using the AMBER 10 program package.[32] For the amino acids and peptides the polarizable version of the force field parm99 was used.[33] For sodium the polarizable Smith-Dang parameters[34] were used and for sulfate we adopted the model described in ref. 35, with the polarizability on each oxygen reduced to 1.0 Å^3 in order to avoid the so-called polarization catastrophe.[36] The polarizable POL3 water model was employed.[37] The principal reason for using a more sophisticated (and costly) polarizable force field is that we found that sodium sulfate tends to artificially form clusters reminiscent of crystallites in SPC/E water even for concentrations well below the experimental solubility. In POL3 water no such artifacts occur. The fact that inclusion of polarization improves the description of aqueous sulfate solutions

will be discussed in detail in a forthcoming technical paper.

The oligo-peptides considered were N-acetyl-tetra-lysinium-C-methylamide, N-acetyl-deca-lysinium-C-methylamide, N-acetyl-tetra-arginium-C-methylamide, and N-acetyl-deca-arginium-C-methylamide, referred to respectively as tetralysine, decalysine, tetraarginine and decaarginine. For the tetrapeptides, the simulation boxes contained, in addition to the oligopeptide, 2300 water molecules, 12 sulfate ions, and 20 sodium ions. For the decapeptides the simulation box contained 5925 water molecules and 31 sulfate ions, and 52 sodium ions for polylysine and 6138 water molecules, 32 sulfate ions, and 54 sodium ions for polyarginine. This yields an overall sulfate concentration of 0.29 m and a sodium sulfate concentration of 0.24 m in all systems. The extra sulfates are the counter ions to the peptide. For comparison, simulations of the terminated amino acids N^{α}-acetyl-lysinium-C-methylamide and N^{α}-acetyl-arginium-C-methylamide, referred to below as lysine and arginine, were also carried out. The simulation contained one amino acid molecule, 575 water molecules, 3 sulfate ions, and 5 sodium ions, which corresponds to the same ion concentrations as in the oligo-peptide systems. Note that in the experiment non-terminated amino acids and peptides were used. In order to estimate the effect of the terminal groups (acetyl and methylamide), simulations of tetraarginine and tetralysine without these were also carried out. Here, the C-termini were deprotonated and the N-termini were protonated, as is the appropriate state for neutral pH. In every other respect, the simulation conditions were identical to those for the terminated tetrapeptides.

Simulations of the oligo-peptides started out in a stretched out conformation with the side chains extending in the plane of the backbone in an alternating (all trans) fashion. After 0.5 ns of equilibration the trajectories were propagated for 20 ns for the decapeptides, 40 ns for the tetrapeptides, and 100 ns for the single amino acids. This was sufficient to converge the ion distribution around the peptides.[28] During the simulations, which were unlikely to be long enough to fully explore the conformational space of the peptides, these acquired random coil configuration.

Simulation Results

The radial distribution functions of ions around the side chain groups of the single amino acids and oligo-peptides are shown in Figure 2. The results for the unterminated tetrapeptides were not significantly different from those for the terminated ones and are not shown. For the arginine and the polyarginines the first sulfate peak is located at about 4.3 Å. The peak has a broad shoulder towards larger distances. The rather narrow "top" of the peak corresponds to the configuration with a sulfate hydrogen bonding to two guanidinium hydrogens, while the shoulder corresponds to looser association with one or no hydrogen bonds. There is a weak second peak at around 7 Å that can be assigned to solvent-separated ion pairs. For the tetra- and decapeptides this peak is considerably more pronounced than for the free amino acids. Note that there is a region where the radial distribution function for the tetra- and decapeptides seems to level of at a value above one. This is due to overlap between the ionic atmospheres of the side chain groups that creates a region of elevated counter ion concentration around the peptides. For tetraarginine the transition from this plateau to the true limiting value of one occurs between 9 and 10 Å, as can be seen in Figure 2. For decaarginine it occurs between about 16 and 20 Å and is not shown. For lysine and peptides composed thereof, the first peak in the sulfate side chain group distribution function is located at about 4.0 Å. The second peak is located at about 6 Å and is quite pronounced for the free amino acid, as well as the peptides. The height of the first peak of the sulfate-amino acid side chain group distribution function is higher for arginine than for lysine containing species. Moreover, it is the same for deca- and tetralysine as for lysine, while it is significantly increased for the tetraarginine, and to an even greater extent for decaarginine, compared to arginine. The simulations thus show that there is a specific affinity between sulfate and the guanidinium residues of arginine and that this affinity increases with increasing chain length, which suggests a considerable degree of cooperativity.

The sodium-side chain group radial distribution function does not exhibit appreciable peaks for any distance for any of the amino acids or peptides, as expected for the distribution function between like-charged species. The small peaks at about 5 Å may be ascribed to ion pairing between

sodium and sulfate ions associated to the amino acid side chains. The height of this peak is very similar for arginine and lysine. For the oligo-peptides, the cation-side chain group radial distribution function displays an extended region where it is below the limiting value. This corresponds to the region where there is enrichment of counter ions, as discussed above. This depletion is larger, and varies more with the length of the peptides, for lysine than for arginine. As the charge of the peptide has to be neutralized one way or another, i.e., either by enrichment of counter ions or depletion of co-ions in its vicinity, this is commensurate with stronger sulfate binding to arginine than to lysine.

In order to relate the simulations to the electrophoretic mobilities, the total charge $Q(s)$ within a distance s from any atom of the peptides and amino acids was calculated. Note that if s were to coincide with the distance to the slip-plane, $Q(s)$ would be the electrophoretic charge of the molecule. In Figure 3, $Q(s)$ is shown for a range of values of s between 3 and 6 Å. As the exact location of the slip-plane in the experiments is unknown, this information is not sufficient to quantitatively predict the electrophoretic mobilities. A quantitative prediction could in principle be obtained from a theory that uses the radial distribution function as input, such as that presented in ref. 38. This would, however, require very high quality data (in terms of low statistical noise) and the results would still be sensitive to the assumptions made about the hydrodynamic interactions. Note also that the concentration in the simulations, necessary to obtain statistically relevant data within reasonable computer time, is significantly higher than the maximum concentration that could be studied experimentally. (The increased conductivity at high ionic strength gives rise to increased heating of the capillary, which becomes problematic at ionic strengths above few hundred mM.) For these reasons, the present simulations can only aid in the interpretation of features of the experimental data that show at most a weak concentration dependence. As shown below, the difference between sulfate binding to arginine versus lysine is a feature that fulfills this condition.

Unequivocal, albeit qualitative, predictions can thus be made from the information presented in Figure 3. The mobility of lysine and deca-lysine should be less affected by the presence of sulfate than that of arginine and decaarginine. Also, the mobility of polyarginine should be reduced

much more than the mobility of arginine whereas lysine and polylysine should have their mobility reduced to a similar degree. These conclusions are not strongly dependent on the exact value of *s*. For very large values of *s*, corresponding to a distant slip plane, it follows from Figure 3 that the mobility of decaarginine would actually be negative. The conventional wisdom regarding the location of the slip-plane is that it is located just outside the first hydration layer,[39,40] which is certainly closer than the 5.5 Å where $Q(s)$ of decaarginine turns negative. Reversal of the electrophoretic mobility would thus most likely not be realized in experiments on the present system. Given that the rate of decline of $Q(s)$ with increasing *s* appears sensitive to the length of the peptide reversal of the electrophoretic mobility cannot, however, be excluded for longer polyarginines.

Experimental results

The primary experimental results are shown in Figure 4. The mobilities of the investigated species in sodium sulfate BGE are consistently lower than those in sodium chloride for a given ionic strength. The difference is especially pronounced for the tetrapeptides, but for the free amino acids it is also considerable. For the free amino acids, the mobility decreases more steeply with concentration in sodium sulfate than in sodium chloride. For the tetrapeptides, the difference in mobility in the two electrolytes is almost constant over the concentration range considered. This near-constant shift is greater for tetraarginine than for tetralysine by about 20 %. For the free amino acids the relative difference in the mobility of arginine and lysine in different salts is harder to quantify because the magnitude of the difference is smaller in relation to the experimental error. It appears, however, that the mobility of arginine is slightly more reduced than that of lysine. In the presence of buffer, the mobilities were consistently lower by up to about 15 % (not shown). Nevertheless, the relative mobilities of (tetra-)arginine and (tetra-)lysine in sodium chloride or sodium sulfate remained practically unchanged by the presence of buffer.

In order to aid the comparison between (tetra-)arginine and (tetra-)lysine, the ratios of the electrophoretic mobilities in sodium sulfate to those in sodium chloride are shown in Figure 5.

This type of comparison is useful because the differences in mobility due to variations in limiting mobility and non-specific ionic strength effects partly cancel. For both the free amino acids and the peptides, the mobility ratio is smaller for arginine than for lysine. Moreover, the difference is significantly larger for the tetrapeptides than for the free amino acids. This is in good qualitative agreement with the expectation raised by the simulation results that the difference in ion adsorption between arginine and lysine is greater for longer peptides.

The ratio of the mobilities in sodium chloride and in sodium sulfate should extrapolate to unity at infinite dilution because the limiting mobility should not depend on the choice of BGE. While this seems to be the case for the free amino acids, the ratio for the tetrapeptides is rather far from one even at the lowest concentration considered. For the present experimental data for the tetrapeptides to be consistent with the correct limiting behavior, the mobility must thus vary very quickly with BGE concentration for low concentrations. The mobility ratio as a function of concentration does indeed curve upward with decreasing concentration in the lower end of the concentration range considered.

Discussion

Electrophoretic measurements and MD simulations provide a consistent picture of the interactions of aqueous sulfate ions with (poly-)arginine and (poly-)lysine. The electrophoretic mobility of the amino acids and tetrapeptides was found to be consistently lower in the presence of sulfate compared to chloride at the same ionic strength. A straightforward explanation for this is that the positively charged side chains form ion pairs (and possibly ion triplets, etc. in the case of peptides) with sulfate and that it is actually the mobility of such associated species that is measured in the electrophoresis experiments. As the analyte mobility is influenced by the mobilities of the BGE ions, moderate variation in electrophoretic mobility with the choice of BGE is not in itself evidence of association.[20] However, the electrophoretic mobility of multiply charged anionic analytes has been found to be systematically lower than what can be accounted for by this effect.[41]

The decrease in mobility observed here for the tetrapeptides in the presence of sulfate are larger that those reported in ref. 41. Thus, the difference in mobility of sulfate and chloride ions is hardly a viable explanation in this case. Strong decrease in and even reversal of electrophoretic mobility of peptides, which was ascribed to ion-pairing, has also been reported in the presence of a polyanionic buffer additive.[17]

For the explanation in terms of ion-pairing to be consistent with the seemingly anomalous behavior of the mobilities of the tetrapeptides, the ion pairing must be strong enough to be almost complete even for the lowest concentration for which the mobility was measured, which corresponds to a sulfate concentrations of only 0.7 mM. That the same qualitative behavior is seen in both tetraarginine and tetralysine, although it is quantitatively more pronounced the former species, indicates that much of the association is due to a non-specific mechanism, presumably electrostatics. If the interpretation in terms of ion-pairing is correct, then peptide-sulfate association would also imply that the osmotic properties of a peptide solution would be modified by the presence of a sulfate. The osmotic pressure of a mixture of tetralysine or tetraarginine and sodium sulfate should be smaller than the sum of the osmotic pressures exerted by each of the components in isolation. Although measurements of the osmotic pressure may be experimentally difficult for the rather low concentrations considered here, this in principle offers an independent way of testing the validity of the ion pairing mechanism.

In the simple, but remarkably successful framework of Bjerrum theory,[21,22] ion pairing is treated as a chemical equilibrium between "free" ions and ion-pairs. The latter are regarded as ideal solutes while the former are treated as a Debye-Hückel electrolyte. The equilibrium constant for association in a model electrolyte, where the anion and the cation are regarded as hard spheres of equal diameter a and with charge of equal magnitude ze_0 but opposite signs embedded in a dielectric continuum with relative permittivity ε, is determined by the strength of the electrostatic interaction as[21,22]

$$K = 4\pi \int_a^d r^2 dr e^{\frac{z^2 l_B}{r}}. \tag{2}$$

The Bjerrum length, l_B, is given by

$$l_B = \frac{e_0^2}{4\pi\varepsilon\varepsilon_0 k_b T}, \tag{3}$$

and interpreted as the distance between unit charges where the interaction energy is equal to the thermal energy, $k_B T$. In water at room temperature l_B is around 7 Å. The upper cut-off of the integral, d, is to some extent arbitrary, but as the largest contribution to the value of the integral typically comes from the region close to ion-ion contact, K should depend only weakly on d. The value suggested by Bjerrum is, i.e. $d = z^2 l_B/2$, is adopted here.[21,22]

If the differences between the model envisioned by Bjerrum and the present situation are taken into account, this framework can be adopted to treat the association between sulfate and the peptides. In a solution of low ionic strength the tetrapeptides should behave as quadravalent ions. (For higher ionic strength this need not be the case, if the typical distance between the charged side chain groups is greater than the Debye-Hückel screening length they should rather act as independently screened monovalent ions.) The effective radius of the peptide is subject to considerable uncertainty, but we can bracket it by the radius of the charged side chain groups and the radius of gyration of the peptide. The possibility of more highly associated species than pairs must be allowed for, which can be done by postulating sequential association according to

$$\mathrm{P}^{n+} + \mathrm{I}^{m-} \rightarrow \mathrm{PI}^{(n-m)+} \tag{4}$$

$$\mathrm{PI}^{(n-m)+} + \mathrm{I}^{m-} \rightarrow \mathrm{PI}_2^{(n-2m)+} \tag{5}$$

etc., where P denotes “peptide” and I denotes “ion”. Each association step has an equilibrium constant K_n. If the assumptions of Bjerrum theory are adopted but the constraint that the electrolyte must be symmetric is relaxed, the values of these equilibrium constants are given by

$$K_n = 4\pi \int_a^d r^2 dr e^{\frac{(z_p-(n-1)z_i)z_i l_B}{r}}. \tag{6}$$

where z_p is the valency of the peptide and z_i is the valency of the counter ion. The size parameter

a here carries the interpretation of a distance of closest approach between ions and peptides.

In Table 1, association constants for two values of *a*, 4.0 and 7.0 Å, are shown. These values can be expected to bracket the "realistic" value. The Bjerrum length is assumed to be 7.15 Å, which is appropriate for water, with a dielectric constant of 78.36,[29] at 25°C. The association constants depend in a highly non-linear way on both the valencies and *a*, the latter being a poorly known quantity. Nevertheless, the first association constant for a tetrapeptide with sulfate is so large for both limiting values of *a* that virtually complete association is predicted even for the lowest concentration considered. Thus, the Bjerrum picture is consistent with the ion pairing hypothesis presented above, regardless of the exact choice of parameters. The values of the association constants also suggest that the second association step in sulfate and the first in chloride should be strongly shifted to the right, Eq. (5). Also subsequent association steps in chloride may be non-negligible. For the free amino acids Bjerrum theory predicts no association with chloride counter ions. With sulfate counter ions, weak pairing is predicted. This is consistent with the observations that the mobility ratio decreases over the experimental range and seems to extrapolate properly to one, see Figure 5.

Table 1: Bjerrum theory association constants in units of M^{-1}. z_p is the charge on the (poly)peptide and z_i is the charge on the anion (sulfate or chloride)

		$a = 4.0$ Å	$a = 7.0$ Å
$z_p = 4, z_i = 2$	K_1	8.1×10^4	3.0×10^3
	K_2	2.6×10^2	1.0×10^2
$z_p = 4, z_i = 1$	K_1	2.6×10^2	1.0×10^2
	K_2	64	25
	K_3	10	4.0×10^{-1}
	K_4	0	0
$z_p = 1, z_i = 2$	K_1	10	4.0×10^{-1}
$z_p = 1, z_i = 1$	K_1	0	0

As the size of the lysine side chain groups is slightly smaller than that of arginine, a straightforward application of Bjerrum theory would result in the conclusion that the mobility of lysine should decrease more in the presence of sulfate than that of arginine. This is contrary to the experimental results. In light of the simulation results, this can be explained by an additional affinity

between sulfate and arginine that is not due to simple electrostatics. For this reason Bjerrum theory is likely to predict too low association constants for arginine relative to lysine.

Ion-pairing has been suggested as a mechanism for penetration of polycationic peptides into membranes.[42,43] Strong ion-pairing, either due to specific or purely electrostatic interactions, with anionic substrates offers a mechanism by which highly charged peptides can enter the hydrophobic environment of the cell membrane. The present results may thus shed new light on the reason for the marked difference between arginine and lysine also in this context. The simulations show that binding of sulfate to polyarginine, but not to polylysine, is cooperative. This may explain why replacement of lysine by arginine in the membrane-penetrating peptide Penetratin enhances penetration, whereas the opposite replacement diminishes it.[16] The counter-intuitive feature that membrane penetrating peptides contain several cationic residues in close proximity[1] may, in light of the preceding discussion, be at least partially explained by the fact that the high concentration of charge promotes ion-pairing for electrostatic reasons.

Conclusions

The interaction between sulfate ions and (poly-)arginine and (poly-)lysine in water was investigated using electrophoretic measurements and MD simulations. We have found experimentally that in aqueous sulfate solutions the electrophoretic mobility of arginine and tetraarginine decreases more strongly with salt concentration than that of lysine and tetralysine. This difference is, however, relatively modest compared to the non-specific decrease in the mobility seen for both arginine and lysine in the presence of sulfate. For polyarginine and polylysine both the non-specific decrease in mobility and the difference between the two peptides was larger than for the free amino acids. While the difference in mobilities between arginine and lysine was explained using MD simulations in terms of specific interactions between sulfate and the guanidinium motif of the arginine side chain group, the non-specific effect of sulfate was rationalized in terms of ion pairing for purely electrostatic reasons. The observed specific anionic affinity of polyarginine compared to

polylysine may have implications for cell membrane penetration by cationic peptides.[2,13,15,16,42,43]

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Figure 1. Structure of the guanidinium cation. Blue is nitrogen, cyan is carbon, and white is hydrogen.

Figure 2. Radial distribution functions and cumulative numbers for the sulfate and sodium ions and the amino acid side chain group, taken as the guanidinium carbon and the ammonium nitrogen for arginine (sub-figure a) and lysine (sub-figure b), respectively. Red color denotes the single amino acids, blue the tetrapeptides and black the decapeptides. The dashed lines are the cumulative numbers of ions corresponding to the distribution functions. The radial distribution functions are normalized using the average concentration of each species in the whole box.

Figure 3. The total amount of charge within s of any atom of a peptide in sodium sulfate solution, including the charge of the peptide itself, normalized by the charge of the peptide. Panel a is for (tetra-, deca-)arginine and panel b is for (tetra-, deca-)lysine. As in Figure 2, red color denotes the single amino acids, blue the tetrapeptides and black the decapeptides. The error bars show the mean absolute difference in $Q(s)/Q$ between the first half of the simulation and the full simulation.

Figure 4. Measured electrophoretic mobility of amino acids (panel a) and tetrapeptides (panel b) as a function of ionic strength. Blue color denotes (tetra-)arginine and red color denotes (tetra-)lysine. The dashed and full lines signify that the background electrolyte is sodium chloride and sodium sulfate, respectively.

Figure 5. Ratio between the measured mobility in sodium sulfate and that in sodium chloride as a function of ionic strength. Red color denotes amino acids and blue color denotes tetrapeptides. The $\times$ symbols are for (tetra-)lysine and the $+$ symbols are for (tetra-)arginine.

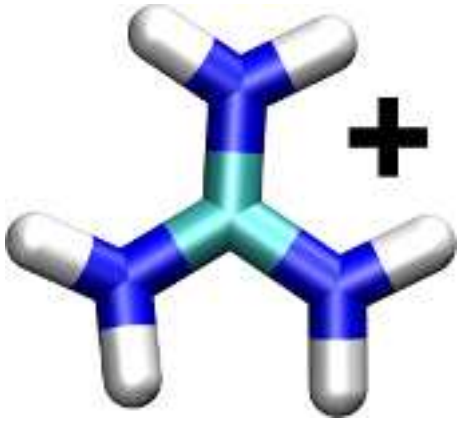

Figure 1

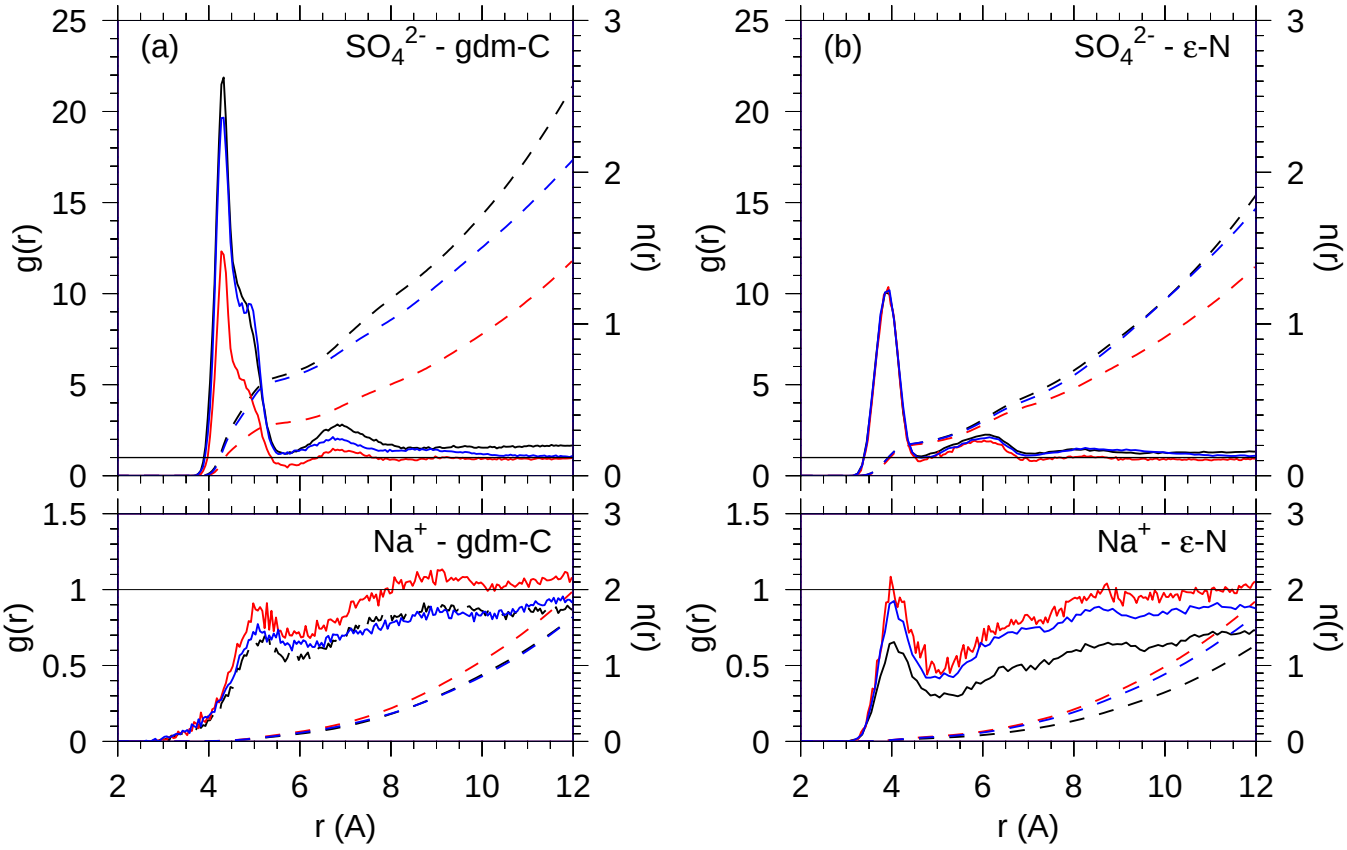

Figure 2

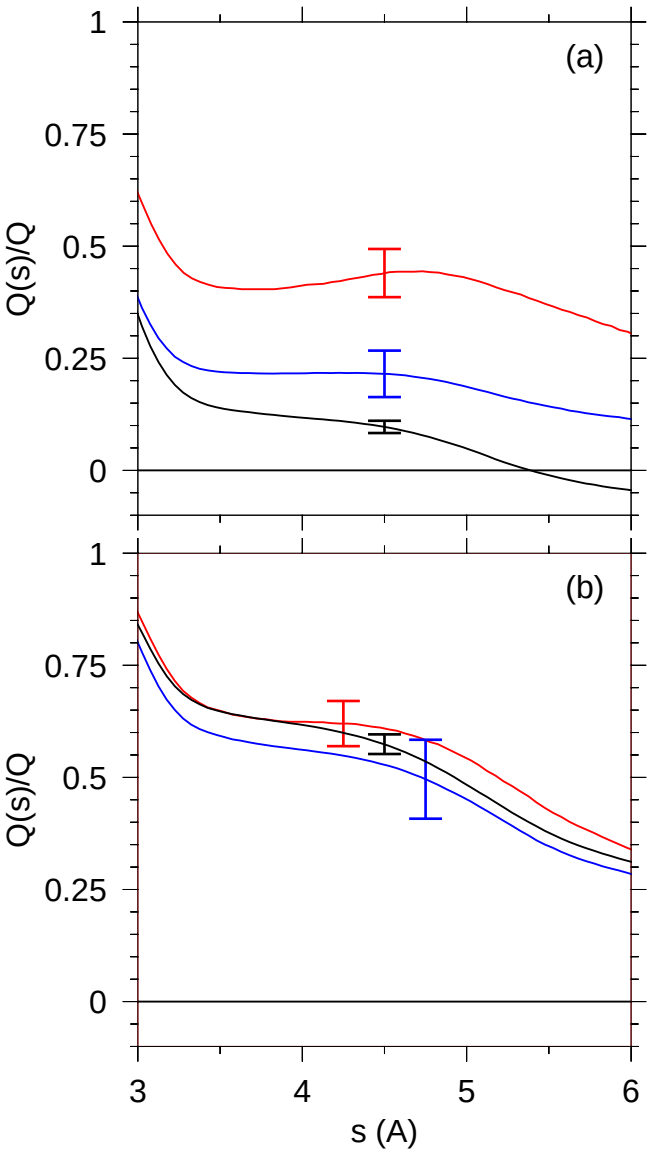

Figure 3

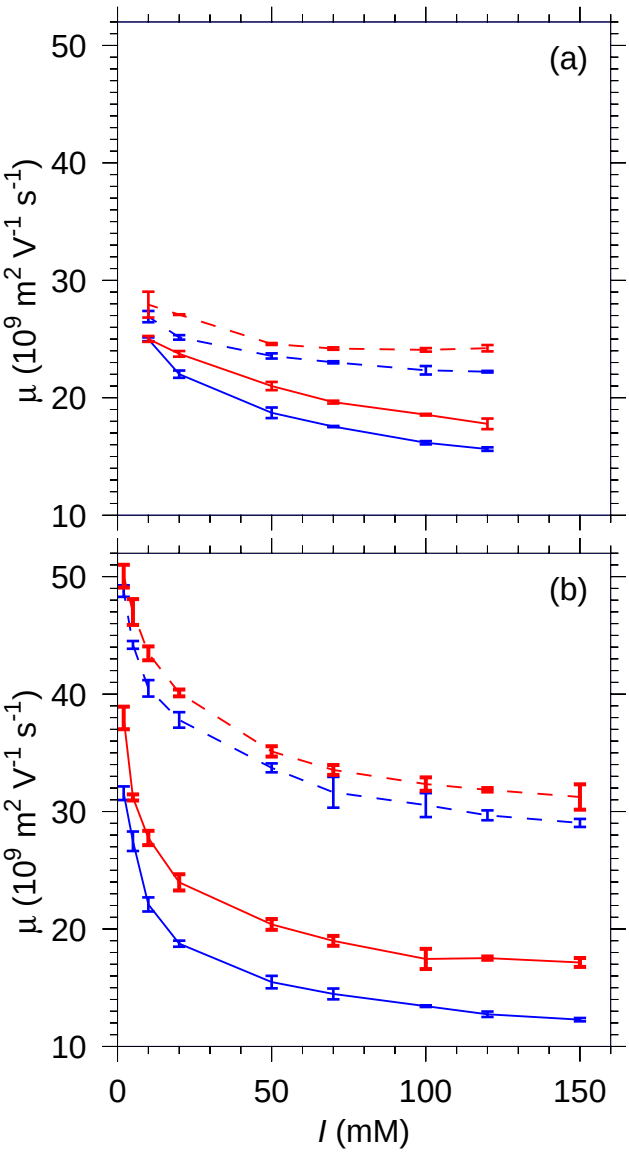

Figure 4

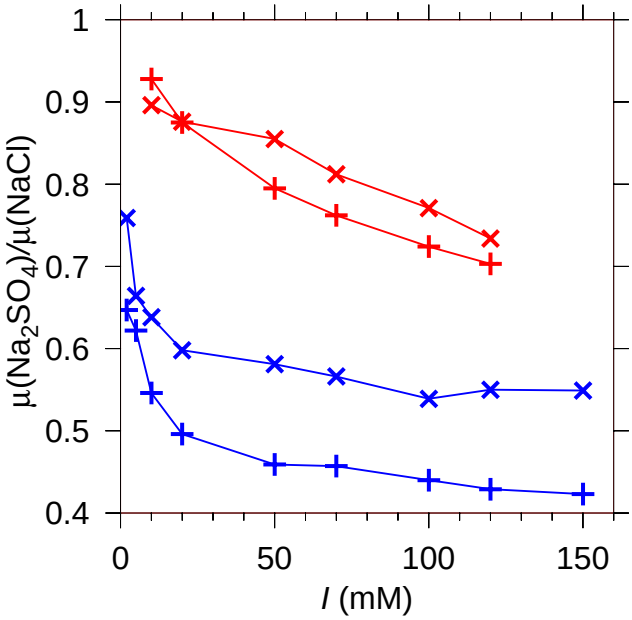

Figure 5