# Overcharging in Biological Systems: Reversal of Electrophoretic Mobility of Aqueous Polyaspartate by Multivalent Cations

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(Received 18 January 2012; published 3 May 2012)

Charge reversal as an extreme case of charge compensation is directly observed by capillary electrophoresis for a negatively charged peptide in aqueous solutions of trivalent cations. Atomistic and coarse-grained simulations provide molecular interpretation of this effect showing that it is largely of electrostatic origin with a minor contribution of chemical specificity of the salt ions.

DOI: 10.1103/PhysRevLett.108.186101 PACS numbers: 82.45.−h, 34.20.Gj, 82.20.Wt, 83.10.Rs

Many properties of peptides and proteins, such as their solubility and tendency to form complexes, depend to a large extent on the amount of positively and negatively charged amino acid residues at their surfaces. These residues are titratable, therefore, the overall charge of a protein is *p*H dependent. An important point on the titration curve is the point of zero (overall) charge, which typically marks minimal solubility of the protein in water, and which is customarily established as the isoelectric point at which the electrophoretic mobility of the protein changes sign [1]. This is rigorous if no other charges are present in the system. However, neither *in vivo* nor *in vitro* proteins are dissolved in pure water but in electrolyte solutions, the ions of which to a certain extent compensate the charges on the protein surface. This is reflected in the electrophoretic mobility, which makes electrophoresis a suitable method to probe the extent of the charge compensation [2].

In many instances, particularly if only monovalent ions are present in the solution, the point of zero charge and the isoelectric point coincide rather well. However, if multivalent salt ions are present, this is no more necessarily true due to their tendency to pair strongly with oppositely charged groups at the protein surface [3]. Correlation between counterions is also established as a possible mechanism for ion adsorption [4–6], though the magnitude of this effect is moderate for aqueous systems [7]. An extreme case of charge compensation by multivalent ions is overcharging. Within this effect, which is also called charge inversion, the charge of a particle is overcompensated by the adjacent salt ions such that it effectively changes sign. This has been observed repeatedly in colloidal systems involving charged surfaces or flexible polyelectrolytes with direct consequences for particle association and phase properties [8–12]. Only very recently, overcharging was inferred also for proteins from the anomalous phase behavior and zeta potentials of albumins [13,14] and from changes in selectivity of a porin channel in the presence of multivalent metal cations [15]. Here, we present an unequivocal case of charge inversion for a much smaller system than previously studied, namely, a short polypeptide composed of negatively charged amino acids (aspartates), upon moving from aqueous solutions of monovalent to trivalent metal acetates (Ac). This is a biologically relevant system simple enough that quantitatively accurate modeling can be performed. The effect is directly detected as a reversal in electrophoretic mobility close to neutral conditions (at $p\mathrm{H} = 5.5$, i.e., far from the point of zero charge of polyaspartate), as well as overcharging in atomistic molecular dynamics (MD) and coarse-grained simulations.

Capillary electrophoresis experiments were carried out on a 7100 CE system (Agilent Technologies, Waldbronn, Germany) using a 75 $\mu$m i.d. fused-silica capillary coated with hydrophilic capillary electrophoresis polymer purchased from Agilent Technologies. The capillary was cut to 80.0 cm length (71.5 cm to the detection window). The diode-array detector was operated at 200 nm. Temperature was maintained at 25 °C using air cooling. A standard method for electroosmotic flow measurement [16] was modified for peptides, as described in our previous work [17]. Migration of the peptide (2 mM tetra-aspartate) was driven by a voltage of $\pm 10$ kV applied for 10 min. At these conditions Joule heating was negligible. Mobilities were calculated from the distance between zones of the peptide and of a neutral marker (thiourea). Running electrolytes were prepared from acetic acid solutions of appropriate ionic strength that were titrated by the corresponding hydroxides to *p*H 5.5. This value of *p*H, which keeps under control potential water hydrolysis by the employed multivalent cations, is well maintained (within few percent)

 

throughout the experiment. Tetra-aspartate was purchased from Bachem (Bubendorf, Switzerland) and all other chemicals were obtained from Sigma (St. Louis, MO, USA).

At the same time, we performed 100 ns MD simulations (after 1 ns of equilibration using time step of 1 fs) of tetra-aspartate (described using the parm99SB force field [18]) in 1 M aqueous solutions of sodium [19], potassium [20], calcium [21], magnesium [21], and lanthanum [22] chlorides (as the simplest counterions). The unit cell contained a single tetra-aspartate, 40 metal cations, and the corresponding number of chloride anions [23] to achieve overal neutrality, and 2172 SPC/E [24] water molecules. Electronic polarization effects were accounted for by introducing an electronic dielectric continuum, which is practically realized by rescaling all solute charges by $1/\sqrt{\varepsilon_e}$, where $\varepsilon_e = 1.78$ is the electronic part of the dielectric constant of water [25]. Periodic boundary conditions were employed with long range electrostatic interactions beyond a cutoff of 9 Å accounted for using the particle-mesh Ewald procedure [26]. The Berendsen thermostat and barostat with temperature of 300 K and pressure of 1 atm were used [27]. The SHAKE algorithm [28] was employed to constrain all bonds containing hydrogen atoms. MD simulations were performed using the AMBER 11 program package [29].

We also employed a simple coarse-grained model, wherein all ions and charged sites were represented as charged hard spheres of radius 2 Å and the appropriate valency. The solvent was represented as a dielectric continuum such that the Bjerrum length was 7.1 Å. The peptide was modeled as five anionic and one cationic spherical site constrained to move on the surface of a sphere of radius 7.2 Å, which was penetrable to electrolyte ions. The spherical simulation cell was concentric with the peptide sphere with radius of at least seven Debye lengths. The peptide-ion total correlation functions $h_i(r) = g_i(r) - 1$, where $r$ is radial distance and $i$ is an index for ion species, was calculated using the canonical Metropolis Monte Carlo (MC) method [30]. The electrophoretic mobility, $\mu$, was estimated according to [31]

$$\mu = \mu_0 + \frac{2e_0}{3\eta}\sum_i z_i c_i \int_0^\infty r dr h_i(r), \qquad (1)$$

where $z_i$ and $c_i$ denote ion valency and molar concentration, $e_0$ the unit charge, $\eta$ is the solvent viscosity, and $\mu_0$ the limiting value of the mobility at zero salt concentration. Further details about the coarse-grained model are presented in our previous study [32].

The results from electrophoretic measurements are summarized in Fig. 1. The top panel shows electrophoretic mobilities of tetra-aspartate in 0–150 mM aqueous solutions of NaAc, KAc, $MgAc_2$, $CaAc_2$, and $LaAc_3$. We see immediately that the electrophoretic mobility of the negatively charged peptide changes qualitatively with the valency of the metal cation, while the effect of the chemical specificity of an ion of a given valency is much weaker. Monovalent ions only moderately lower the peptide mobility with the effect being slightly larger in NaAc than KAc. This is consistent with the higher affinity of $Na^+$ over $K^+$ to the carboxylate groups on the peptide [33]. The influence of divalent cations is significantly stronger with the electrophoretic mobility of tetra-aspartate approaching close to zero for the highest concentrations studied. Also, the chemical specificity between $Ca^{2+}$ and $Mg^{2+}$ is larger than for monovalent cations and is in accord with the significantly higher affinity of calcium over magnesium for the $COO^-$ group [34]. Nevertheless, it is clear from Fig. 1(a) that chemically specific interactions are secondary compared to the electrostatic effects of the ion valency. This may be partially due to the relatively weak sensitivity of electrophoresis to specific ions effects and it is in accord with our previous study of monovalent and divalent anions interacting with cationic polypeptides (with no overcharging observed) [17,32]. In the present study, the electrostatic effects become particularly pronounced in the case of the trivalent lanthanum cations, which overcompensate the charge of the peptide already at the lowest ionic strength measured. As a result, reversal of electrophoretic mobility as a clear sign of overcharging is observed in all the present measurements involving $La(Ac)_3$ solutions. Also, the nearly constant values of mobility above 50 mM suggest that carboxyl groups of the peptide become saturated by lanthanum cations.

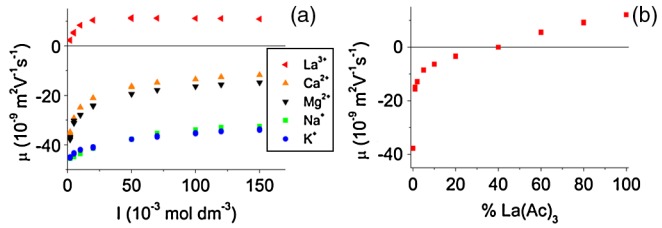


FIG. 1 (color online). Measured electrophoretic mobilities of 2 mM tetra-aspartate in (a) $Na^+$ (green), $K^+$ (blue), $Ca^{2+}$ (orange), $Mg^{2+}$ (black), and $La^{3+}$ (red) acetate solutions as a function of salt ionic strength and (b) mixed solutions of varying normal fractions of $La(Ac)_3$ and NaAc with a total ionic strength of 50 mM.

In order to more clearly demonstrate the crossover from negative to positive mobilities of tetra-aspartate we performed additional experiments at a fixed total salt ionic strength of 50 mM, where we gradually increased the amount of lanthanum and decreased the amount of sodium [Fig. 1(b)]. Charge reversal occurs at about 40% normal fraction of $La^{3+}$ above which the tetra-aspartate polyanion effectively behaves as being positively charged. Note that this corresponds to a higher $La^{3+}$ concentration than that necessary for the crossover in pure $La(Ac)_3$ solution. This is due to the electrostatic screening effect in the mixed solution which, at the same $La^{3+}$ concentration, has a higher ionic strength than the pure solution.

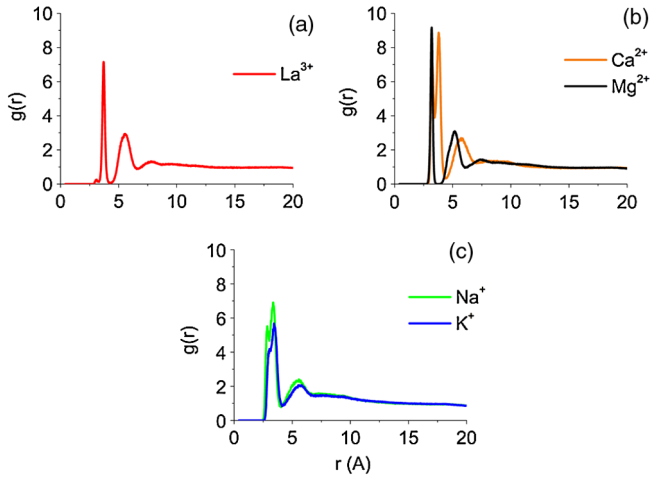


FIG. 2 (color online). Radial distribution functions of the carboxylic carbon atoms of tetra-aspartate and (a) $La^{3+}$ (red), (b) $Ca^{2+}$ (orange) and $Mg^{2+}$ (black), and (c) $Na^{+}$ (green) and $K^{+}$ (blue).

The present measurements of electrophoretic mobility provide direct evidence of charge inversion; however, they do not supply detailed information about the underlying molecular mechanisms. This is provided by atomistic MD simulations from which equilibrium distributions of ions around the peptide can be extracted. Figure 2 provides radial distribution functions between the carbon of the carboxylic groups of tetra-aspartate and the individual metal cations. All the curves exhibit two distinct peaks, first at 4 Å and a secondary one at around 6 Å. The first peak, which corresponds to contact ion pairing between the carboxylic groups of tetra-aspartate and the metal cations, is sizable in all cases (note that the exact height of this peak is very hard to converge for magnesium with a very tight

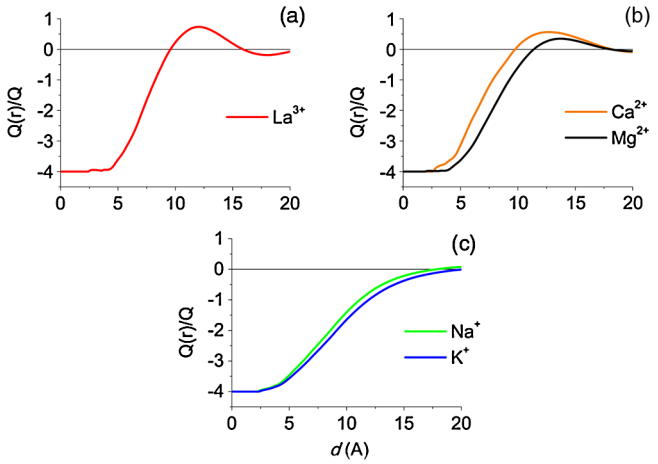


FIG. 3 (color online). The total charge (i.e., the charge of the peptide and that from the solution) within distance from the surface of the tetra-aspartate in aqueous chloride solutions of (a) $La^{3+}$ (red), (b) $Ca^{2+}$ (orange) and $Mg^{2+}$ (black), and (c) $Na^{+}$ (green) and $K^{+}$ (blue).

first solvent shell [35]). This peak is in most cases composed of two subpeaks corresponding to mono- and bi-dentate geometries of the contact ion pairs [36]. The first and also the second peak (corresponding to solvent-separated ion pairs) is more pronounced for $Na^{+}$ than $K^{+}$ and, in general, for multivalent than monovalent ions.

The radial distribution functions (Fig. 2) can be integrated to a given distance from the peptide which, together with the charge on tetra-aspartate, provides the total distance-dependent charge of the solvated peptide, as depicted in Fig. 3. Sodium and potasium gradually compensate the peptide charge such that it almost monotonically converges to zero (with a negligible overcompensation around 20 Å), practically excluding the possibility of overcharging. In contrast, for divalent and trivalent cations the curves of the total charge becomes nonmonotonic with a crossover from negative to positive charges around 10 Å from the peptide surface (i.e., after the second solvent shell around the peptide, where the largest contribution to the observed effect comes from). Such a crossover is a necessary but not sufficient condition for charge reversal to be observed in the electrophoretic mobility measurement. For this, the charge reversal has to be sufficiently large and has to occur within the shear surface, the exact position of which is unknown.

In addition, direct comparison between electrophoretic measurements and atomistic MD simulations is complicated by a mismatch in salt concentrations. In simulations, we employ molar salt concentrations in order to obtain well-converged data, which is beyond what is technically feasible in the experiment. To enable a more direct comparison we performed also coarse-grained MC simulations, which can be easily converged at experimental concentrations. The resulting mobilities as a function of ionic strength are shown in Fig. 4, assuming a limiting mobility $\mu_0$ of around $-55 \times 10^{-9}$ $m^2\,V^{-1}\,s^{-1}$, i.e., similar in magnitude to previous estimates for cationic tetrapeptides

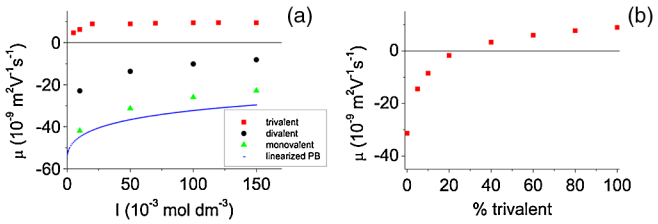


FIG. 4 (color online). Changes in electrophoretic mobility due to electrolyte ions calculated from the coarse-grained model for model tetra-aspartate in (a) pure electrolytes of varying ionic strength and cation valency and (b) mixed solutions of varying normal fractions of trivalent and monovalent cations with a total ionic strength of 50 mM. The data for monovalent and divalent cations are reproduced from our previous simulations [32]. Results from linearized Poisson-Bolzmann theory are also presented in (a).

[32]. The experimental results [Fig. 1(a)] are very well reproduced for monovalent, divalent, as well as trivalent ions [Fig. 4(a)]. Notably, the charge reversal and the almost constant experimental mobility in $La(Ac)_3$ solutions above ionic strength 20 mM is recovered by the coarse-grained model. In contrast, linearized Poisson-Boltzmann theory [37] underestimates the effect of salt already for monovalent ions at the studied concentrations [Fig. 4(a)] and becomes completely unusable for multivalent salts.

Also, the general features of the experimental mobility variation in the mixed NaAc-$La(Ac)_3$ solutions [Fig. 1(b)] are captured [Fig. 4(b)] by the coarse-grained model. The broad agreement between the experimental data and those from the coarse-grained model suggest that electrostatic interactions are dominant in shaping the observed trends in compensation of the peptide charge leading eventually to overcharging for trivalent ions. The fact that some ion specificity could be detected for the peptide mobility in the electrophoresis experiment, as well as in atomistic simulations of distributions of ions around the peptide, indicates, however, that such effects would have to be included in a fully quantitative model of the electrophoretic mobility. Nevertheless, the present combination of electrophoresis experiments with atomistic and coarse-grained simulations clearly demonstrates charge reversal for an anionic peptide in aqueous solutions containing trivalent cations and points to its molecular origin.

We thank Frank Schreiber for stimulating discussions. Support from the Czech Science Foundation (Grant No. 203/08/0114) is gratefully acknowledged. P. J. thanks the Academy of Sciences for support. P. C. thanks the Czech Ministry of Education (Grant No. MSM0021620857) and the Grant Agency of Charles University (Grant No. SVV 2012-265201 and UNCE No. 2012/44).

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