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Molecular crowding has no effect on the dilution thermodynamics of the biologically relevant cation mixtures

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Abstract. The ionic composition of intracellular space is rigorously maintained in the expense of high-energy expenditure. It has been recently postulated that the cytoplasmic ionic composition is optimized so the energy cost of the fluctuations of calcium ion concentration is minimized. Specifically, thermodynamic arguments have been produced to show that the presence of potassium ions at concentrations higher than 100 mM reduce extend of the energy dissipation required for the dilution of calcium cations. No such effect has been measured when sodium ions were present in the solution or when the other divalent cation magnesium was diluted. The experimental observation has been interpreted as the indication of the formation of ionic clusters composed of calcium, chloride and potassium. In order to test the possibility that such clusters may be preserved in biological space, the thermodynamics of ionic mixtures dilution in solutions containing albumins and model lipid bilayers have been measured. Obtained thermograms clearly demonstrate that the energetics of calcium/potassium mixture is qualitatively different from calcium/sodium mixture indicating that the presence of the biologically relevant quantities of proteins and membrane hydrophilic surfaces do not interfere with the properties of the intracellular aqueous phase.

Key words: Thermodynamics of ions — Molecular crowding — Membrane crowding — Isothermal titration calorimetry — Calcium intracellular signaling

Introduction

Ionic homeostasis inside cell is a critical element of proper progression of all its vital functions. The ionic composition of intracellular aqueous phase affects solubility and aggregation state of all molecules filling the cell volume (Hey et al. 2005; Kunz 2010; Huang et al. 2015). Ions also participate in many metabolic processes as necessary cofactors (Murgia et al. 2009; Spitzer and Poolman 2009). Their electrochemical gradients are necessary for the energy and information transformations (Bortner and Cidlowski 2004; Ivannikov et al. 2010). The spatio-temporal distribution of ions is facilitated by an intricate system of fluxes between various compartments driven by dedicated channels, pumps and chelating agents (Boldyrev 1993; Yu 2001; Decrock et al. 2011; Lee et al. 2011; Eisenberg 2013; Chen et al. 2014). Whereas the passive ion flux in the cell is governed by electrochemical gradients, the active transport is associated with energy dissipation. Nearly 70% of adenosine triphosphate (ATP) in some cells is used to maintain the ionic homeostasis (Ivannikov et al. 2010). There are four main cations present in the cytoplasm: potassium, sodium, magnesium and calcium. The averaged concentrations of monovalent potassium (about 140 mM) and sodium (about 4 mM) as well as divalent magnesium (3 mM) are constant in time. Calcium is an unusual cation, which concentration fluctuates between 10^{-10} M and 10^{-4} M, serving as a second messenger in intracellular signaling cascades (Landolfi et al. 1998; Michelangeli et al. 2005; Ivannikov et al. 2010). Such large concentration changes require expenditure of large quantities of metabolic energy reaching 17% of the entire energy dissipated by cells (Eisner et al. 2004; Case et al. 2007; Ivannikov et al. 2010). Recently, we

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have demonstrated that the thermodynamics of the dilution of calcium ions in water is greatly affected by the concentration and type of monovalent cation (Kaczynski et al. 2014). This finding provides the thermodynamic argument for the specific ionic composition of the intracellular space. It has been demonstrated that the thermodynamics of calcium dissolution depends on the combination of ion-water and ion-ion interactions (Landolfi et al. 1998; Arias-Moreno et al. 2010; Liu et al. 2010; Yang et al. 2010). Whereas the dissolution energy of calcium is independent on the sodium chloride concentration, and do not differ significantly from that in water, the effect of the potassium chloride is dramatic. When the potassium quantity rises above the concentration of 100 mM the dilution energy of calcium is dramatically reduced. No such effect is observed for magnesium, the other important divalent cation present in the cytoplasm (Kaczynski et al. 2014). In order to explain the effect the formation of multi-ionic clusters have been postulated (Kaczynski et al. 2014). Such multicomponent ionic clusters would require the sufficient level of hydration, which might be not available in densely packed intracellular space (Spitzer and Poolman 2005; Spitzer and Poolman 2009; Leontidis et al. 2014). In addition, the large quantity of, frequently charged, water-soluble proteins and membrane surfaces might affect organization, dynamics and thermodynamics of ions in the intracellular aqueous phase (Collins 1997; Collins et al. 2007; Ben-Yaakov et al. 2009). Various mixtures of cations have been investigated in order to evaluate the effect of two most numerous components of crowded intracellular environment, hydrophilic proteins and lipid surfaces, on the hydration thermodynamics. For that purpose the water-soluble proteins were modeled with albumins and membrane surfaces were modeled with unilamellar liposomes, both entities in biologically relevant concentrations 50 mg/ml and 7 mg/ml, respectively (Miyoshi and Sugimoto 2008).

Materials and Methods

Materials

Bovine serum albumin (BSA ≥ 98.0%) and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer were obtained from Sigma-Aldrich (Poznan, Poland), calcium chloride, propylene glycol and sodium chloride were purchased from Chempur (Piekary Slaskie, Poland) and potassium chloride was obtained from POCH (Gliwice, Poland), EggPC 90G from Lipoid GmbH (Ludwigshafen, Germany). All chemicals were of analytical grade and used without further purification. 50 mM HEPES buffer (pH = 7.4) was prepared using fresh deionized water (conductivity < 2 μS). All solution containing mixtures of salts were tested for the presence of precipitation using the dynamic light scattering technique. In all cases no particulates larger than 2 nm were detected.

Liposomes preparation

EggPC at concentration 45% (w/w) was dissolved in propylene glycol and incubated for 24 h at 60°C. The addition of 50 mM HEPES buffer was followed by further incubation for 24 h at 60°C to ensure the complete lipid hydration. Next, lipid suspension was extruded 5 times through polycarbonate membrane filters (Whatman, UK). Liposomes were diluted with buffer so that the final concentration of lipid was 7 mg/ml. Size of liposomes was measured using Dynamic Light Scattering (DLS) technique (NanoSizer, Malvern GB). In order to measure the effect of the osmotic pressure difference, caused by high salt concentration, on the liposomes size distribution, the DLS experiment was performed at the end of each titration. No significant change was observed (results not shown).

Isothermal titration calorimetry (ITC) measurements

Sample degassing for 30 minutes preceded each titration experiment. Calorimetric measurements were performed using Thermal Activity Monitor TAM III, equipped with isothermal titration unit (TA Instruments, New Castle, USA). Measurement cell was filled with 800 μl of BSA buffered solution (50 mg/ml), liposomes buffered solution (7 mg/ml) or buffer solution alone. Solutions from syringe were added into cell in a series of 10 μl injections. Each experiment was preceded with a 1 μl injection, which was neglected in the subsequent analysis. The duration of each injection was 10 s and the delay time between injections 1500 s. Experiments were carried out at 25.0°C. In the experiment buffered solutions of $CaCl_2$, KCl, NaCl or their mixtures were titrated into the cell. Concentration of each salt in the mixture was 1.5 M. This ensures that the final concentration of salts in the cell reaches the physiological values. Concentration of BSA and lipids in measurement cell were also selected based on physiological values (Chtterjea and Shinde 2012). Heat of the dilution was calculated from thermograms as an area under peaks expressed in Joules. Positive or negative heat flow indicated an exothermic or endothermic process, respectively. All experiment presented in the paper were carried in triplicates using different preparations of proteins or liposomes. Control experiments were performed in duplicates.

Results and Discussion

The ionic composition of the cell interior is tightly controlled and meticulously maintained despite high-energy

cost. Whereas the concentrations of most intracellular free ions are invariant in time, the concentration of calcium varies widely by orders of magnitudes facilitating the information transfer through cytoplasm (Cheng and Lederer 2008). These fluctuations, in addition to the maintenance of trans-membrane gradients, increase the amount of energy required to sustain the intracellular ionic homeostasis (Ivannikov et al. 2010). The energy required for the sequential release and removal of calcium ions from cytoplasm depends on the difference of respective energetic states. The energetics of calcium ions in the cytoplasmic aqueous phase is affected, as demonstrated elsewhere, by its ionic composition (Kaczynski et al. 2014). Specifically, the type of monovalent cation present in the solution changes the dilution energetics not only quantitatively but also qualitatively, specifically the dilution of calcium mixed with potassium is an exothermic process but, when potassium is replaced with sodium, the dilution becomes endothermic. Based on this observation it has been postulated that ions in aqueous phase form ionic clusters, which may dramatically change the thermodynamic properties of the solution. Ionic clusters are attractive concepts, which offers straightforward explanation, albeit not confirmed by other experimental evidences, of differences between dilution thermodynamics of various ionic mixtures (Kaczynski et al. 2014). Data presented previously have been carried out using biologically relevant concentration of ions but the experimental model has not accounted for the crowding effect produced by high concentrations of proteins and membranes. The cell volume is a maze of aqueous compartments packed with water-soluble proteins (proteins occupy about 40% of cell volume) bordered by variety of membrane surfaces (Chandler 2005; Chtterjea and Shinde 2012). In such an environment the existence of intracellular "bulk" water is unlikely and the effect of various surfaces on distribution of ions is likely. The possible effect of macromolecular and membrane crowding on the structure and dynamics of aqueous phase leads to the model, which treats the "aqueous phase" in cell as a structured mixture of water molecules, ions and small hydrophilic compounds (Spitzer and Poolman 2005, 2009; Leontidis et al. 2014). Despite the crowded conditions and low water activity the cell interior is still capable to provide conditions for the propagation of intracellular signals especially those based on fluctuations of calcium concentration (Taheri-Araghi and Ha 2010; Szekely et al. 2011; Thurley et al. 2012; Wakai and Fissore 2013; Swietach et al. 2015). Calcium is continuously released and reloaded into intracellular stores during the signaling events (Joseph et al. 2014). The calcium signal can last from seconds to hours therefore the intracellular aqueous compartments with elevated calcium concentration should not affect the hydrophilic-hydrophobic balance, which maintain the stability of concentrated intracellular suspension of proteins and membranes (Thurley et al. 2012; Joseph et al. 2014). We have previously demonstrated that the dissolution thermodynamics of mixtures of calcium and potassium chlorides indicate that the two cations combined with chloride ions may form stable clusters, which do not disintegrate upon dilution (Kaczynski et al. 2014). It has also been also observed that the thermodynamics of ionic mixture dilution is mainly controlled by the potassium concentration regardless on the quantity of calcium present in the

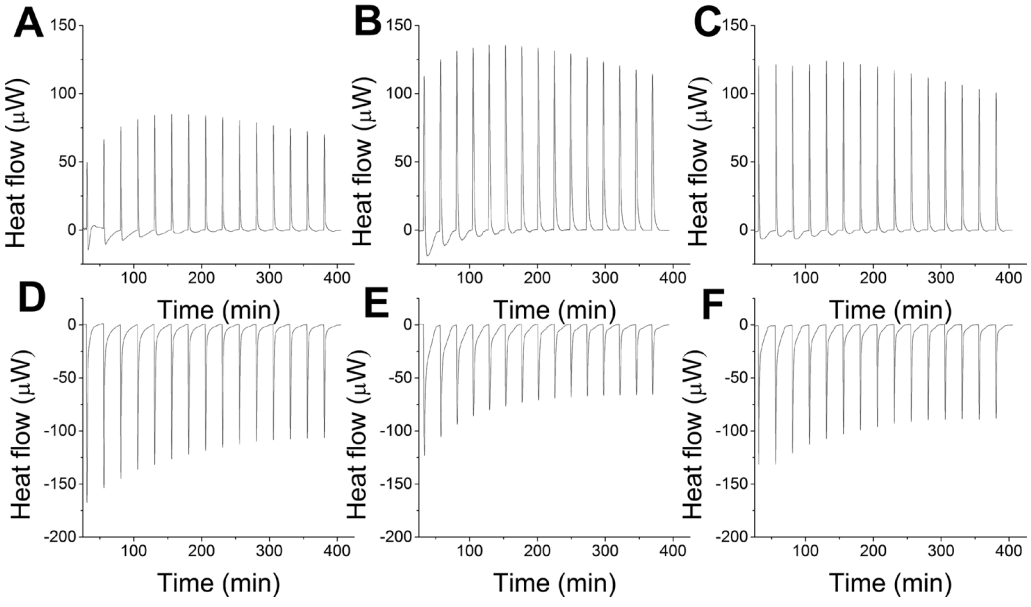

Figure 1. Thermograms of 50 mg/ml albumin solution (**A** and **D**), 7 mg/ml lipid vesicles suspension (**B** and **E**) and buffer (**C** and **F**), titrated with KCl : CaCl2 (1:1) mixture (**A**, **B**, **C**) and NaCl : CaCl2 (1:1) mixture (**D**, **E**, **F**).

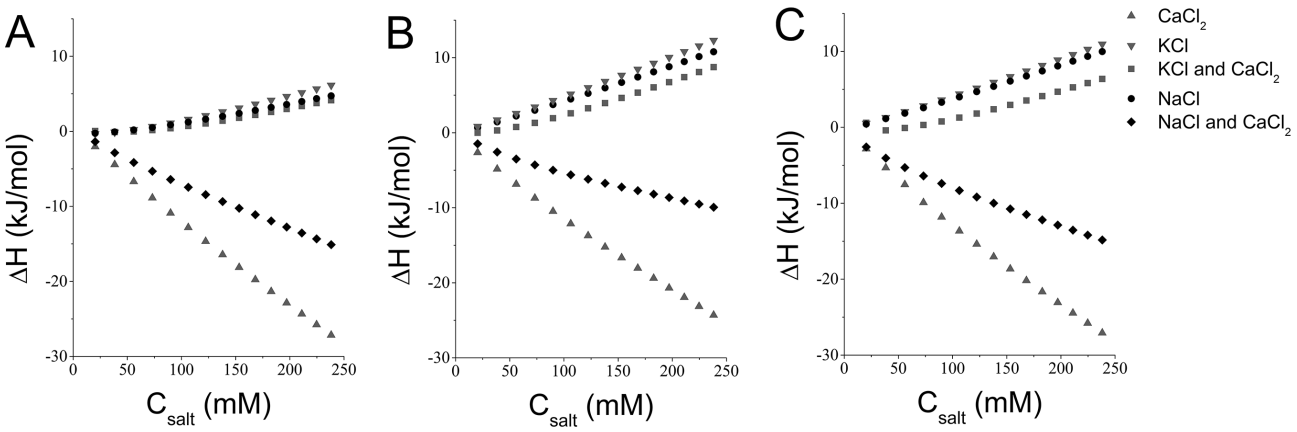

Figure 2. Cumulative enthalpy changes (ΔH) calculated from the dilution experiments carried out for selected salt solutions as a function of the salt concentration (C_{salt}) in the reaction cell in the presence of BSA (**A**), liposomes (**B**) and buffer alone (**C**). The dilution of KCl : $CaCl_2$ (1:1) mixture, NaCl : $CaCl_2$ (1:1) mixture.

mixture what substantiate the assumption of the presence of ionic clusters. However, the crowded cell interior may interfere with dilution thermodynamic by altering local ionic composition. Surfaces of membranes and/or proteins may alter distribution of ions in the adjacent aqueous phases (Pollack 2003; Zhang and Cremer 2006; Porasso and Cascales 2009; Parsegian and Zemb 2011; Parsons et al. 2011; Przybylo et al. 2014). In order to determine the effect of water-soluble proteins and neutral membranes on the dilution thermodynamics, the calorimetric experiment has been performed at the presence of biologically relevant concentrated protein solution and lipid vesicles suspension. The concentrated solution of albumin (50 mg/ml) was used as a model of intracellular aqueous space crowded with proteins, whereas concentrated suspension of lipid vesicles (7 mg/ml) was used to model the effect of membrane surfaces on ions dissolved in the aqueous phase. Figure 1 shows thermograms of two salts mixtures NaCl : $CaCl_2$ (1:1) and KCl : $CaCl_2$ (1:1) titrated into buffer or concentrated albumin or membrane solutions.

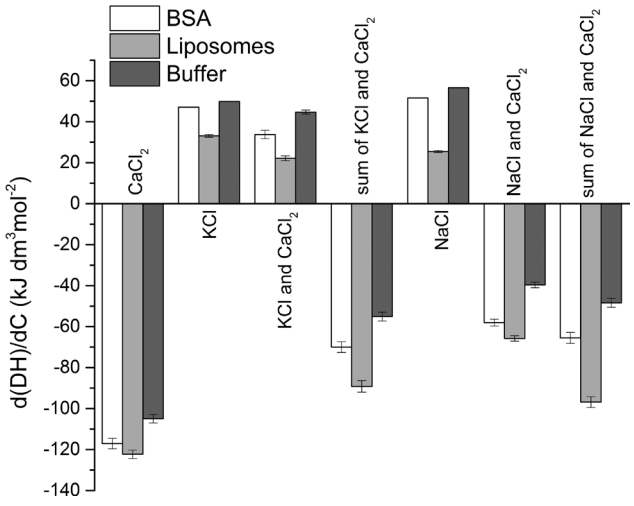

Figure 3. The slopes of the linear dependence of the cumulative enthalpies (d(ΔH/dC) on ions concentration as determined in the dilution experiments 50 mg/ml albumin solution and 7 mg/ml liposome suspension. The calculated values of the slopes for ionic mixtures, expressed as a sum of enthalpies obtained in the dilution experiments performed for each salt alone, are presented for comparison.

Panel C and F on Figure 1 show that when the buffer was titrated with mixtures of KCl : $CaCl_2$ (1:1) and NaCl : $CaCl_2$ (1:1) qualitatively different thermograms were acquired. Whereas the dilution process of $CaCl_2$: KCl mixture is endothermic the dilution of $CaCl_2$: NaCl mixture is exothermic. The explanation of this difference has been offered previously (Kaczynski et al. 2014). Assuming that mixtures of $CaCl_2$ and NaCl salts produce homogenous solvents their dilution thermograms can be approximated with the sum of dilution enthalpies of each salts individually. The situation is different when mixtures of $CaCl_2$ and KCl are diluted. The quantity of energy released in this case does not differ significantly from the energy released when the KCl solution is diluted alone, indicating that in this case the solution is not homogeneous. The effect was explained by the proposition that the solution produced by mixtures of $CaCl_2$ and KCl salts contain dilution resistant clusters of interacting ions. Panel C in Figure 2 and Figure 3 demonstrate the difference between the two mixtures of salts. It has been proposed previously that stable ionic Ca^{2+} : Cl^- : K^+ clusters are formed in sufficiently high potassium concentrations preventing the change of Ca^{2+} hydration upon dilution (Kaczynski et al. 2014).

In order to highlight differences between titration experiments plots of cumulative enthalpy changes are fitted with linear function and the slope is then calculated and presented in Figure 3.

Figure 3 quantitatively summarizes results obtained for each titrant, expressed as abovementioned slopes, derived using the regression analysis, for ions and their mixtures titrated into buffer, protein solution or lipid suspension. Data presented in Figure 3 shows that energetics of $CaCl_2$: KCl (1:1) dilution with the buffer, albumin solution or suspension of liposomes is qualitatively similar. This indicates that postulated ionic clusters remain intact in the aqueous phase at biologically relevant concentrations of water-soluble proteins and lipid membranes therefore supporting the preposition that ionic clusters containing Ca^{2+} and K^+ cations may reduce the energy cost of the intracellular signaling mediated by spatio-temporal fluctuations of Ca^{2+} ion concentration. No such effect is observed when mixtures of Ca^{2+} and Na^+ cations are used. Despite the lack of qualitative effects of proteins or membranes, on the overall character of the dilution thermodynamics of salts and their mixtures there are some quantitative differences. The presence of albumin and lipid membrane results with the elevated enthalpies of calcium dissolutions by 11% and 17%, respectively. This indicates that there are some interactions between calcium and/or chlorine ions with proteins and lipid surfaces. This result agrees with other studies, which have demonstrated that polyions bind with variety of compounds and structures (Ahyayauch et al. 2009; Arias-Moreno et al. 2010; Morton et al. 2010; Parsons et al. 2011). The effect is bigger when lipid bilayers are present in the solution. Analysis of NaCl and KCl dilutions demonstrate that the effect of protein on the two ions is small (8% and 5%, respectively) as compared to the effect of lipid surfaces. The effect of lipid surface on the sodium dilution (changed by 55%) is significantly stronger then on the potassium dilution (changed by 34%) showing that interaction between sodium and lipid surfaces is significant. The result agrees with data presented in literature (Vlachy et al. 2009; Yang et al. 2010; Klasczyk et al. 2010; Przybylo et al. 2014).

When mixtures of potassium and calcium are diluted in the presence of albumins and lipid membranes, the absolute value of dilution enthalpy is reduced in a meaner similar to that of potassium chloride alone showing that ionic environment make calcium unavailable for interaction with crowding agents. When the mixture of sodium and calcium is added to proteins and membranes the resulting dilution enthalpy is elevated by 46% and 66%, respectively. This result shows that the two cations interact with both proteins and membranes with little mutual interferences.

There are two factors, which need to be taken into account when analyzing phenomena occurring in crowded and/or concentrated solutions. First, ions are different with the respect to their charge, surface charge density and hydration what affects their solubility and, as demonstrated previously, propensity to form ionic complexes (Collins 1995, 1997; Collins et al. 2007). The ionic complexes may alter the Debye effect, especially at charged endomembrane surfaces (Langner and Kubica 1999; Vlachy et al. 2009; Yang et al. 2010). In this case the main assumptions of the Gouy-Champman theory may not be satisfied limiting dramatically applicability of the theory when intracellular processes are analyzed (Ben-Yaakov et al. 2011). Any local alteration of ionic composition may change hydrophobic/hydrophilic balance of surrounding macromolecules leading to their precipitation or aggregation as dramatically demonstrated by Hofmeister effect (Baldwin 1996; Kunz et al. 2004; Hey et al. 2005). Whereas the effect of monovalent ions on macromolecules solubility and/or propensity to aggregate is relatively weak, the effect of divalent ions can be dramatic. For example, calcium may induce fusion of negatively charged membranes present in high quantities in the cell cytoplasm (Michelangeli et al. 2005; Boettcher et al. 2011; Naranjo and Mellstrom 2012). The presence of ionic clusters containing calcium may contribute to the preservation of the electrostatic balance when the calcium concentration rises upon cell stimulation therefore ensuring the topological stability of endomembrane systems. The calcium release may induce intracellular molecular rearrangements, which may result from direct interaction of calcium ion with proteins and/or membranes triggered by the altered intracellular ionic composition (Tojyo et al. 2014). Data presented in the paper shows that the thermodynamics of ionic mixtures dilution is not qualitatively altered by the presence of interfaces presented by membranes and proteins showing that underlying molecular processes are likely to be preserved in a crowded intracellular environment.

Conclusions

The ionic composition of the cytoplasm determines its stability (hydrophobic/hydrophilic balance) and facilitates the transmission of signals through the cell volume. Whereas the major elements of the ionic homeostasis are qualitatively and quantitatively well characterized, the exact ionic composition of cell has not been convincingly justified. Recently, it has been proposed that the specific composition of the cellular space is determined by the thermodynamics of the collective hydration of ions. It has been demonstrated that the thermodynamics of dilution of ion mixtures dramatically depends on their ionic composition. Specifically, the dilution of calcium and potassium chlorides mixture is qualitatively different from all other mixtures. It has been speculated that the difference is a result of ionic

clusters formation, which may ensure stable level of calcium hydration regardless on their concentration. The significance of such ionic clusters in biological space filled with tightly packed soluble proteins and membranes depend on the cluster stability at the presence of various hydrated surfaces. Results of calorimetric experiments shown in the paper demonstrate that the presence of biologically relevant density of hydrophilic proteins and lipid bilayers do not influence the dilution thermodynamics of ionic mixtures containing calcium cation. This result shows that the ionic composition of the cytoplasm may play critical role in the stability of crowded biological space by ensuring the solubility of various components of cell volume.

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