Specific electrolyte effects on Hemoglobin in denaturing medium investigated through Electron Spray Ionization Mass Spectrometry.

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Highlights

° Protein in denaturing medium shows highly charged ions in ESI/MS spectra

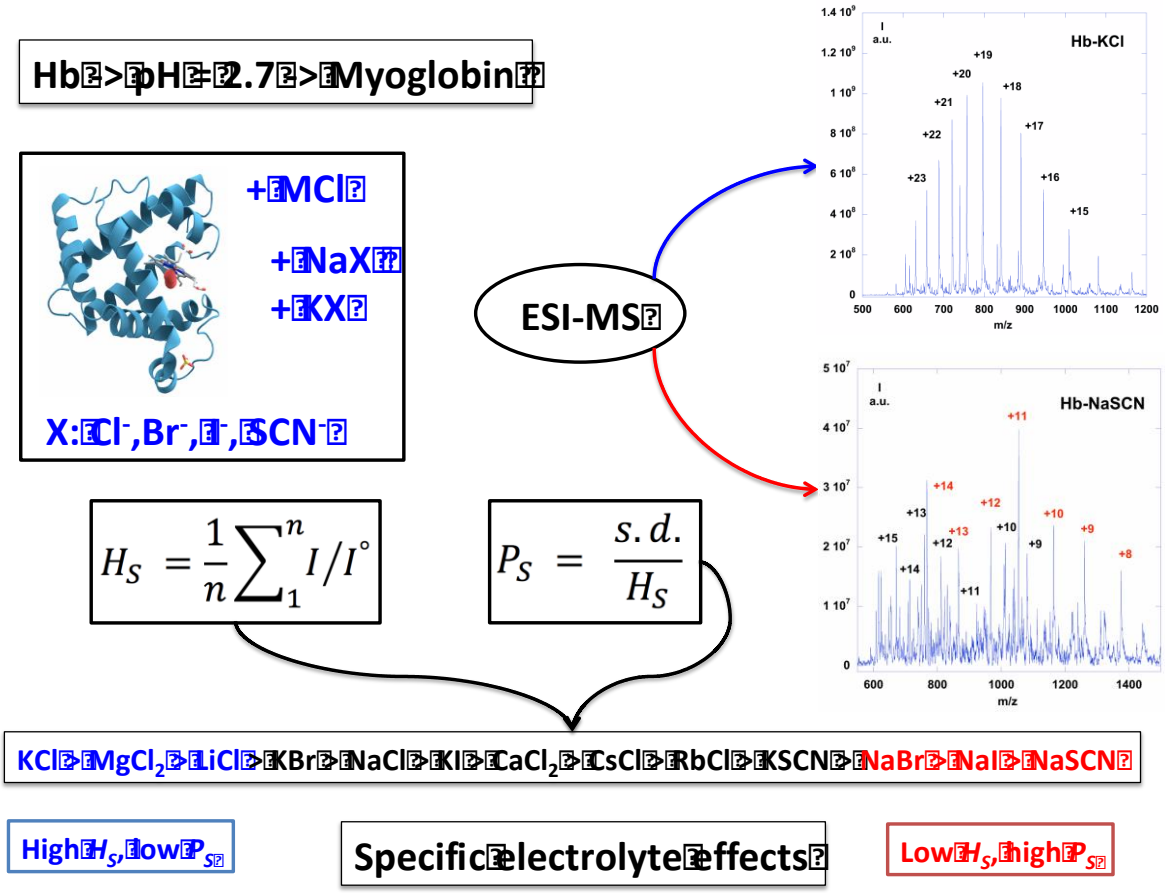
° Myoglobin α-chain is the main detected profile in ESI/MS spectra

° KCl, $MgCl_2$ and LiCl stabilize Myoglobin ions against fragmentation/hydolysis at acid pH

°Electrolyte specific effects may improve clinical molecular biology analysis

° Hofmeister effects are quantified by new specific electrolyte perturbation parameters

Graphical Abstract



Synopsis

Hemoglobin at pH = 2.7 produces a Myoglobin unfolded ion that is stabilized by KCl, and highly fragmented by NaSCN

Abstract

We examine Hofmeister specific ion effects of electrolytes added to protein solution under conditions minimizing electrostatic attraction between cations and positively charged protein. Hemoglobin (Hb) in aqueous solution at the denaturing pH = 2.7 is investigated in the presence of several metal chlorides, along with sodium and potassium bromides, iodides and thiocyanates, using electrospray ionization mass spectrometry (ESI-MS). Salt concentration was varied to maximize peak intensity and bell-shaped profile in the ESI-MS spectrum. The α-chain of myoglobin is identified as the main pattern of the ESI-MS spectra in all Hb-salt systems. Both peak intensity and quality of the bell-shaped profile of the protein spectrum decrease in the cation order: $K^+ >> Mg^{2+} > Li^+ >> Na^+ > Ca^{2+} \approx Cs^+ > Rb^+$ for Hb-Metal Chloride systems, and decrease in the anion order: $Cl^- > Br^- > I^- > SCN^-$ for systems of both Hb-NaX and Hb-KX salts. To quantify salt addition effects two Hofmeister specific electrolyte parameters H_S, and P_S are proposed. H_S is the mean (Hb-salt)/Hb peak intensity ratio, measured for the nine peaks used for ESI-MS spectra deconvolution, taken at the same m/z values of the Hb profile. P_S is the ratio between H_S standard deviation and H_S, and provides a specific perturbation parameter measuring the loss of protein structure. These two Hofmeister parameters give clear evidence of the effects induced either by KCl, $MgCl_2$ and LiCl that enhance protein peak intensity, or by NaBr, NaI, NaSCN and KSCN that induce the protein fragmentation, due to electrolyte-mediated dissociation.

Keywords: Hemoglobin; Myoglobin; ESI-MS; Specific electrolyte effects

1. Introduction

In 1888 Franz Hofmeister studied the effect of adding salts on the aggregation of egg white proteins.[1–3] He sorted the salts, having the same cation but different anion, according to their ability to promote precipitation or increase solubility of a protein in aqueous solution. A conventional Hofmeister series is: $HPO_4^{2-} > SO_4^{2-} > F^- > Cl^- > Br^- > I^- > NO_3^- > ClO_4^- > SCN^-$.

Anions promoting precipitation (left side of the series) are strongly hydrated (kosmotropic) while those facilitating solubility (right side of the series) are weakly hydrated (chaotropic). A similar series was also observed for cations: $Cs^+ > NH_4^+ > Rb^+ > K^+ > Na^+ > Li^+ > Mg^{2+} > Ca^{2+}$.

The conventional cation series shows a substantial difference compared to the anion one. In fact, the cations that promote precipitation (e.g. Cs^+) are weakly hydrated while those that facilitate solubility (e.g. Mg^{2+}) are strongly hydrated. Basically, anions and cations give the opposite effect, suggesting that ionic hydration is not the only factor responsible for the observed effects. A huge number of investigations followed since then. Ion specificity has been observed in many kinds of fluids spanning from simple solutions to complex colloidal and macromolecular systems, even in nonaqueous solutions.[4–6] Indeed, nature and concentration of ions affect significantly several physico-chemical properties of solutions, namely viscosity, surface tension, solubility, etc. [7–11]

Due to the importance of proteins and their activity in biological systems, ion specificity has been demonstrated, through many specific techniques and computational studies, either at a macroscopic or molecular level, to be extremely relevant.[12–19]

Several approaches have been used to explain these particular Hofmeister effects, including the empirical rule proposed by Collins and known as "the law of matching water affinity" (LMWA).[20] This law is based on the classification of ions according to the degree of hydration (kosmotropic and chaotropic). Water affinity is quantified in terms of ion hydration enthalpy.

An ionic pair can only be formed if the two ions have similar values of hydration enthalpy (kosmotropic-kosmotropic or chaotropic-chaotropic). A theoretical approach due to Ninham explains the specific ion-ion, ion-water, ion-surface interactions as a result of electrostatic and non-electrostatic (dispersion) interactions.[3] Ion dispersion forces are the result of a delicate interplay between hydration, ion size, and polarizability.[21]

Hemoglobin (Hb) has been one of the first model proteins for the investigation of the Hofmeister series.[22] For this protein some previous papers, based on peculiar techniques and modelling, highlighted Hofmeister effects and the role of weak ion-protein specific interactions. Surprisingly, it was pointed out that in the range of pH 4.5-9.5 the Hofmeister series for cations followed a non-monotonic trend, with Li^+ and Cs^+ giving similar aggregation curves.[23] These results were rationalized in a following theoretical work by quantifying non-electrostatic interactions in terms of competing chemisorption and physisorption, where particularly potassium cation was found to promote Hb aggregation via strong attractive interactions.[24]

In the last two decades, electro-spray ionization mass spectrometry (ESI-MS) techniques have received great attention for the potential in investigating proteins and their features in terms of stability and interactions. ESI-MS technique was found to be particularly informative in specific and sophisticated molecular biology analysis of clinical interest. [25,26] In this work, we present the results of an ESI-MS investigation performed on hemoglobin (Hb). Here, we will focus not only on the specific ion effects due to added electrolytes, but also on the role of a denaturing environment choosing an acidic pH = 2.7, placing our protein well below the isoelectric point (for Hb pI ≈ 7), where Hb is positively charged. These conditions address two important aims of the investigation: i) to ensure the typical bell-shaped profile in the ESI-MS spectra, characterized by a large number of charged peaks at different *m/z* values, without

using the supercharging additives, that were employed in other papers;[27–33] ii) to minimize the electrostatic attraction between the cations and the positively charged protein that can mask Hofmeister specific cation effects. Indeed anions (A) are expected to quickly evaporate, at least partially, during the ESI-MS experiment through a ion evaporation model (IEM, see below par. 1.2 and Eq. 1) as H^+A^-, due to either the small size of the ions or the decrease of surface tension (see below Results and discussion and Table S1 in SI).[34] A background on ESI-MS technique, needed to understand our experimental results, is shortly described in the following paragraph.

1.2 ESI-MS main features

Electrospray ionization (ESI), introduced by Fenn in 1989[35] is a soft ionization technique that produces multiple intact charged species, particularly on biologically important macromolecules such as proteins and nucleic acids, without significant fragmentation as a result of ionization.[35–37] It has been demonstrated that even weak noncovalent interactions can be preserved in the gas phase.[38] Several articles and reviews have accurately described the technique as well as the technical protocol used to set the instrumental parameters and to analyze the effects of ionization on different kind of molecules.[39–45] Many ESI-MS instruments with a variety of performance characteristics, in terms of ionization power, mass range and resolution along with ESI-MS instrument design, have been developed in the last decades. Konermann's group has produced very interesting and fundamental contributions to the development and to the applications of ESI-MS technique, particularly to investigate protein behavior.[28,33,46–49] The process is summarized in the schematic diagram of the ESI-MS experiment taken from ref.[46], reported in SI (Scheme 1).

The sample droplets coming out of the Taylor cone are highly charged and, before entering

the MS device, tend to shrink because of the solvent evaporation. A redistribution of charge density on the droplets occurs until surface tension is counterbalanced by electrostatic repulsion. The classical situation is defined as the Rayleigh limit, and the number of elementary *e* charges z_R is given by the equation proposed by Rayleigh [50]

$$z_R = (8\pi/e)(\varepsilon_0 \gamma R^3)^{0.5} \quad (1)$$

where *R* is the droplet radius, ε_0 the vacuum permittivity, and γ the surface tension of the aqueous solution. Assuming the value of surface tension γ = 72 mN/m (pure water), eq (1) can be approximated, according to De La Mora [51], to

$$z_R \approx 0.078\ M^{0.5} \quad (2)$$

where M is the molecular mass in Dalton. With reference to proteins in aqueous media, diverse ion release mechanisms have been proposed.[46] Here, we can briefly cite the main features: I) the charge residue model **CRM** where unfolded proteins undergo liquid-gas phase transition upon solvent evaporation, when the droplet charge approaches the value of z_R; II) the chain ejection model **CEM** where unfolded proteins come out of the droplet as a result of Coulombic rebalancing between the droplet and the polypeptide protruding chain; III) the collision induced dissociation **CID** which occurs when either high energies or sample composition favour dissociation; IV) ion evaporation model **IEM** that occurs for small ions that quickly come out of the charged droplet.

The diverse mechanisms may occur also as a result of suitable additives. It can be emphasized that in any case many parameters may affect the electrospray ionization process, particularly the addition of volatile compounds such as acetonitrile, the ionic strength and pH of the aqueous medium, as well as the presence of weak (e.g. buffers) and strong electrolytes that can modify the viscosity and surface tension of the aqueous environment (see eq. 1). Depending on the design of the ion source and type of mass spectrometer (MS), the amount

of added electrolyte must be appropriately calibrated since several reactions or specific interactions may occur between the charged proteins and the dissociated electrolytes. A high ESI-MS response due to the formation of highly charged protein ions has been related to the addition of substances able to induce denaturation or to raise surface tension. Salts are commonly added to enhance the response of ESI-MS protein spectra, and in some papers Konermann et al[33,47,48] investigated the role of different cations on the release of charged protein ions. However, a systematic study of the effect of electrolytes, particularly anions, in the framework of the Hofmeister series, has not previously been reported.

In this work the effect of several metal-chlorides, along with sodium and potassium bromides, iodides and thiocyanate, added to Hb 19 μM solutions at pH = 2.7, is investigated. ESI-MS spectra were acquired in the positive ion mode, keeping constant all experimental conditions apart from the salt concentrations used to maximize the quality of the ESI-MS spectrum. Since the salt/protein molar ratios vary, it is useful to evaluate the charge state distribution (CSD) through the average charge, defined by the relation [52]:

$$Z_{av} = (\Sigma z_i \times I_i)/ (\Sigma I_i) \qquad (3)$$

where z_i is the charge at each m/z value and I_i the associated intensity. This relation can provide information related to the ion-induced disorder of the protein, since the CSD is strongly affected by the compactness of the whole protein during the liquid-gas transition. [52–55] In addition the CSD has been related to both molecular mass (M) and solvent accessible surface area (A_s), through power laws with various coefficients.[37,41,51–53,56,57] Different relationships have been found depending on the folded-unfolded structure of the protein in the aqueous solution.[52,53,56–59] The A_s values can be calculated through the following eq. (4) [58]:

$$A_s = 4.84\, M^{0.76} \qquad (4)$$

where *M* is the molecular mass (in Dalton) of the protein. It is worth citing that Eq. (4) has been validated by several authors and for a large number of proteins in a wide range of molecular mass.[52,53,56–59] Interestingly, the Grandori group developed significant relationships between Z_{av} and mass *M*, as well as between Z_{av} and A_s, able to evaluate the state of the protein, in terms of folded or unfolded structure.

2. Materials and Methods

2.1 Chemicals

Human hemoglobin (Hb) was purchased from Sigma-Aldrich (H7379) and used without further purification. Formic acid (85%), NaCl (98%), KCl (99.5%), KBr (99%), KI (99.5%), and CsCl (99.5%) were from Carlo Erba (Italy); LiCl (99%) was from Janssen; RbCl (99%) and NaI (99%) were from Aldrich; NaBr (99%) was from Acros; $MgCl_2.6H_2O$ (98%) was Alfa-Aesar; Acetonitrile (99.9%), NaSCN (98%), KSCN (99%) and $CaCl_2$ (99.99%) were from Sigma Aldrich (Milan, Italy). MilliQ water was used to prepare all samples.

2.2 Mass spectrometry

Mass spectra were recorded using a triple quadrupole QqQ Varian 310-MS mass spectrometer using the atmospheric-pressure ESI technique.[60,61] The sample solutions were infused directly into the ESI source using a programmable syringe pump at a flow rate of 1.25 mL h^{-1}. A dwell time of 14 s was used, and the spectra were accumulated for at least 30 min in order to increase the signal-to-noise ratio. Mass spectra were recorded in the *m*/*z* 50 - 2000 range. The experimental conditions were needle voltage 5500 V, shield 600 V, source temperature 60 °C, drying gas 150 °C, drying gas pressure 20 psi, nebulizing gas pressure 20 psi, detector voltage 1800 V. The mass spectrometer was calibrated with ESI Tuning Mix (Agilent

Technologies) before the measurements. All ions listed in this manuscript correspond to the monoisotopic masses.

The samples were prepared by weighing an appropriate amount of protein (Hb), equal to about 76 mg (uncertainty ±0.1 mg) and adding small concentrations (mM) of chloride solutions with different cation (Na^+, K^+, Li^+, Cs^+, Rb^+, Mg^{+2}, Ca^{+2}) or different sodium and potassium salts (Br^-, I^-, SCN^-). Subsequently, each sample was brought up to a volume of 5 mL with H_2O and HCOOH 0.4 %, thus creating an acid environment (pH = 2.7). An aliquot of 850 µL of this solution was diluted with 150 µL of CH_3CN, obtaining a final volume of 1000 µL to be analyzed by ESI-MS.

2.3 Samples for ESI-MS experiments

All samples were prepared using a solution of Hb 1.9 x 10^{-5} M in milliQ water containing HCOOH and CH_3CN 0.4 % and 15.0 % w/w respectively (pH = 2.7). This protein concentration was selected to provide the highest quality of the ESI-MS spectra in terms of peak intensity and bell-like shape. The amount of each salt was carefully chosen to generate an ESI-MS profile as close as possible, in terms of resolution and peaks intensity, to that obtained for Hb in the absence of salts. The main criterium for selection of salt concentration was the preservation of the bell shape of the distribution of peaks. The deconvolution of the mass spectra pattern was performed through the ESIprot online program:[62] this procedure enables a mass and *z* charge to be assigned to each peak.

3. Results and discussion

3.1 ESI-MS data

ESI-MS experiments where conducted at pH = 2.7, where the Hb protein is positively charged in a denaturing environment. As described in the experimental section, different concentrations were used for the various salts in order to maximize the intensity of the protein profile and to preserve the profile shape. See par.2 and 3 in SI where ESI/MS spectra of some Hb-salt systems, collected at different salt concentrations, can clarify the criteria used in the choice. We found that the addition of salts induced important modifications in the intensity profile of the peaks of ESI-MS spectra. But in no case we observed new peaks due to stable adducts of protein-cation/anion, likely because of the low salt/protein molar ratio. In some cases there were significant shifts in the *m/z* values of the highest peak detected. All ESI-MS spectra of Hb in the absence and in the presence of the various salts are reported in the Supporting Information (SI).

Figure 1 shows the ESI-MS spectrum of Hb in the 500-1200 *m/z* range, along with the attribution of *z* charges obtained through the deconvolution based on the 9 peaks around the highest one at *m/z* = 797.1, from which a molecular mass M = 15127.3 ± 0.6 Da was calculated. The ESI-MS spectrum of Hemoglobin shows the typical bell-shaped profile of a highly charged protein which, according to literature[26,28,29,46,63,64], can be identified as the myoglobin unit at acidic pH.

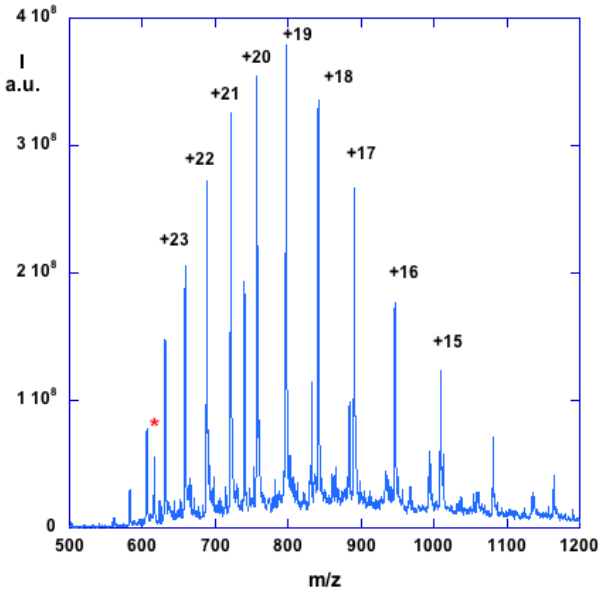


Figure 1. ESI-MS spectrum showing Intensity vs. *m/z* in the range *m/z* = 500-1200 with the attribution of *z* charges obtained through deconvolution based on the 9 peaks around the highest one. Hb 1.9 x 10^{-5} M in H_2O containing HCOOH (0.4% w/w) and CH_3CN (15.0% w/w), pH = 2.7. The red * indicates heme group at *m/z* = 616.1.

The calculated molecular mass value is close to the mass *M* = 15257.405 Da, reported for α–globin otherwise referred as Hemoglobin alpha-chain HBA1 which is constituted of 142 residues (https://go.drugbank.com/polypeptides/P69905), though less close than the value *M* = 17183.725 Da, reported for myoglobin (MB) with 154 residues (average a.a. residue MW ≈ 112 Da) (https://go.drugbank.com/polypeptides/P02144). Notably, our spectra are very similar to those reported for myoglobin at pH = 2 or pH = 3.4 [28,29], where the α-chain signals are always much more intense than those assigned to β-chain.

Table 1 summarizes the relevant features of the ESI-MS spectra (reported in SI) of Hb alone and in the presence of different chlorides, and of Na^+ and K^+ salts with the anions Br^-, I^-, SCN^-.

Table 1. Hb systems: Concentrations and Salt/Hb molar ratio. Highest Peak data: *m/z* values, Intensity I, *z* charges; Mean Mass (s.d. ≈ 0.6%) from deconvolution of the 9 peaks around the highest one, in the range *m/z* = 600-1100; Quality of ESI-MS spectra.

Hb – Salt Sample	**Conc. (mM)**	**Salt/Hb Molar ratio**	***m/z***	**I x 10^8 a.u.**	***Charge z***	**Mean Mass Da**	***Quality ESI-MS**
Hb no salt	0.019	-	797.2	3.5	19	15126	high
LiCl	0.013	0.69	797.2	6.7	19	15128	high
NaCl	0.083	4.39	721.3	3.3	21	15128	high
KCl	0.015	0.79	797.2	10.5	19	15126	high
RbCl 1°	0.024	1.25	1009.1	0.74	15	15120	very low
RbCl 2°			1054.4	0.53	12	12679	
CsCl	0.023	1.2	890.6	1.8	17	15122	low
$MgCl_2$	0.835	43.49	797.1	7.7	19	15124	high
$CaCl_2$	0.846	44.06	841.2	2.0	18	15122	acceptable
NaBr 1°	0.041	2.16	760.0	0.47	19	14257	very low
NaBr 2°			767.5	0.50	10	7587	
NaI 1°	0.072	3.79	759.9	0.27	15	11106	very low
NaI 2°			767.5	0.43	10	7568	
NaSCN 1°	0.045	2.37	1054.6	0.55	11	11306	very low
NaSCN 2°			759.8	0.22	13	9887	
KBr	0.014	0.74	841.3	3.2	18	15122	good
KI	0.029	1.53	797.0	2.2	19	15122	good
KSCN 1°	0.014	0.74	1009.4	0.49	15	15126	very low
KSCN 2°			1054.5	0.4	13	13817	

*The notation 'very low' is used when both low intensity of the peaks and loss of bell-shaped profile are observed. See ESI/MS spectra in SI.

It should be remarked that the optimal salt concentration was found to be very low for LiCl (0.013 mM)[65] and higher for $MgCl_2$ (0.835 mM) and $CaCl_2$ (0.846 mM), as reported in the first column of Table 1. The salt/protein molar ratio is lower than one only in the case of LiCl, KCl, KBr and KSCN added to Hb (second column in Table 1). The choice of salt/Hb optimal ratios

was carried out based on several preliminary experiments. It is not actually clear why each salt has its own optimal concentration. Indeed the main goal of adjusting the concentration was to maximise the quality of the ESI-MS spectra, as demonstrated by ESI-MS spectra reported in SI, rather than to examine conventional Hofmeister series at identical ionic strengths. Mg^{2+} and Ca^{2+} chlorides were included in this investigation because of their relevance in biology. However, the need for such a wide variation in the salt/protein molar ratio required to obtain the ESI-MS spectra can be attributed to multifactorial parameters specifically determined by every cation-anion pair. This is not easily reduced to a single mechanism, but we will furthermore deepen this important issue below in par. 3.4.

Considering either the quality of ESI-MS spectra (see SI) or the data reported in Table 1, the dramatic effect of several salts on protein pattern is clearly demonstrated by the figures related to the highest peak as well as to the mean mass values. In the case of RbCl, NaBr, NaI, NaSCN and KSCN two profiles that were associated to a different mean mass M were detected. Interestingly, although the fluctuations observed for m/z and z charge values of the highest peak, the calculated mean mass in most systems is $M \approx 15.12$ kDa. Introducing the mean mass M in eq. 2 (salt addition does not modify water surface tension significantly due the low concentration) we can calculate z_R values significantly diverse from our z charges. These findings rule out the possibility of a protein ionization according to the CRM mechanism, as expected in consideration of the highly denaturing environment.[46] We observe anomalous masses driven by NaBr, NaI, NaSCN and also a different fragment profile induced by RbCl and KSCN, which we interpret as salt-assisted fragmentation of the myglobin.

3.2 Salt effects

In order to highlight the effect of salts on the intensity profiles as a function of z charges, the *m/z* value and the intensity *I* of the highest peak is reported in Figure 2A. Figures 2B, 2C and 2D for Hb in the presence of Cl^- salts, Na^+ salts and K^+ salts, respectively, summarize the details of the intensity profile due to the 9 peaks around the highest one observed in the ESI-MS spectra. Before commenting on the details of the profiles presented in Figure 2, it is useful to recall that globular folded proteins generally produce peaks in the ESI-MS spectra having relatively low intensity whereas even a partial unfolding of the protein allows for a significant increase of the peaks' intensity.[33,46] For instance, due to the denaturing environment (pH = 2.7) in Hb system, the ESI-MS spectrum reveals substantially the MB unity since the quaternary structure is lost and likely unfolding occurs before the liquid-gas phase transition. Added electrolytes may induce peculiar macroscopic effects such as increase or decrease of peaks' intensity at each *m/z* value. If the profile of the peaks is maintained in terms of *m/z* values, we can ascribe the intensity to higher or lower unfolding as a result of weak interactions between ions and charged protein amino acids, and following charge rebalancing. If the protein profile is lost or significantly altered, collision induced dissociation, promoted by specific ion interactions, even with uncharged and polar protein residues, may be called into play. It is worth noticing that LiCl, KCl and $MgCl_2$ produce a significant increase of the intensity of the peaks with respect to Hb without salts in the order $K^+ >> Mg^{2+} > Li^+$. All the other salts produced instead a decrease of the peaks' intensity, however NaCl, KBr, KI and also $CaCl_2$ still allow for a relatively high-quality bell-shaped profile. Moreover, the addition of Li^+, K^+ and Mg^{2+} chlorides does not modify the *m/z* and *z* values of the highest peak, which remains at *z* = +19. NaCl, on the other hand, moves the highest peak to a higher *z* ion charge (z = + 21) while RbCl, CsCl and $MgCl_2$ shift the highest peak to the lower charges, +15, +17 and +18, respectively.

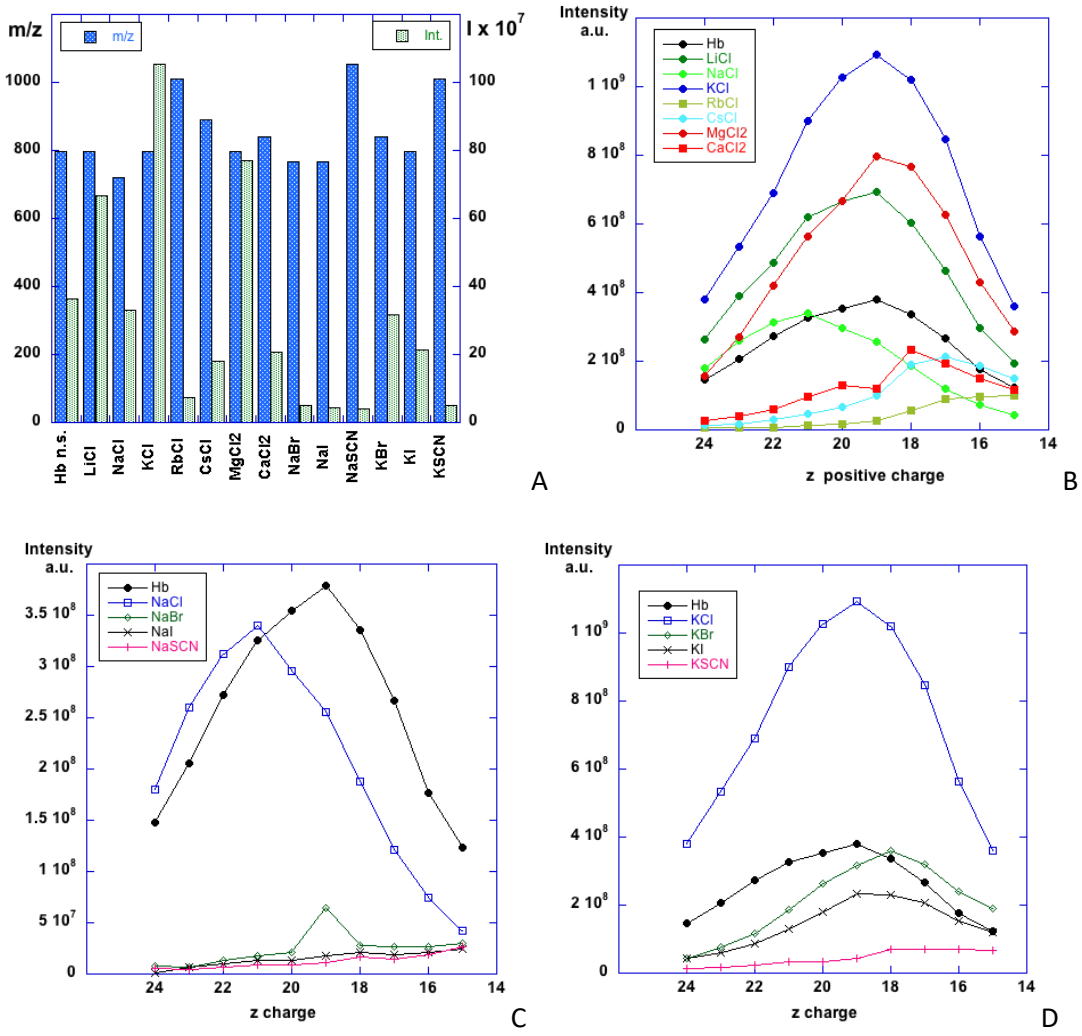


Figure 2: Hb Systems: **A)** *m/z* values and Intensity I of the highest peak. The profile of peak intensity **I vs *z***, in the range *m/z* = 600-1100 for **B**) Hb-Metal Chlorides; **C**) Hb-Na salts (Cl, Br, I, SCN); **D**) Hb-K salts (Cl, Br, I, SCN). Hb profile: black symbols.

Replacing chloride for Na^+ and K^+ salts with Br^-, I^-, or SCN^- anions generally decreases both intensity and quality of ESI-MS spectra as shown in the ESI-MS spectra profiles (Figs. 2C and 2D, see also original spectra in SI). Only KBr and KI retain a bell-shaped profile of the peaks.

As to the effect of cations in Hb-Metal chloride systems, it can be observed that peak intensities and goodness of the bell-shaped profile decrease in the order $K^+ >> Mg^{2+} > Li^+ >>$

$Na^+ > Ca^{2+} \approx Cs^+ > Rb^+$. As to the effect of anions in the Hb-NaX salts and Hb-KX salts systems, both peak intensities and quality of the spectra decrease in the order $Cl^- > Br^- > I^- > SCN^-$. Remarkably, in the case of K^+ salts only SCN^- anion produces significant modifications of the spectrum and fragmentation as shown in Table 1 (highest peak of the main pattern *z* = + 15 and *z* = + 13 for the fragment). In the case of Na^+ salts all anions except chloride cause deep modifications, being the highest peaks observed at the positive *z* charges of +10 for Br^- and I^-, and +11 for SCN^-.

The macroscopic effect of anions can easily be rationalized in terms of increased chaotropicity in the Hofmeister series, although we should remark the joined contribution of the cation. Indeed KBr and KI do not promote fragmentation of myoglobin unit as NaBr or NaI. In addition, the most chaotropic SCN^- anion, that is expected to strongly interact with the protein positively charged sites, causes high fragmentation of myoglobin only in the case of Na^+ counterion, whereas allows for a whole charged chain ejection (CEM mechanism) of the protein when associated to K^+ counterion. In order to justify the effects of cations a combination of macroscopic and microscopic effects induced by the different polarizability, hydration enthalpy of the ions, as well as by the nature of amino acids that constitute the polar surface domain of myoglobin should be considered.[12,66,67] According to literature data[68] the most abundant residues of myoglobin are lysine (22, pI = 9.74) glutamic acid (19, pI = 3.22, anionic), leucine (18, pI = 6, apolar), glycine (13, pI = 5.97, polar), aspartic acid (10, pI = 2.98, anionic). The glutamic and aspartic acids, which, along with other polar amino acids, are located at the protein-water interface in the liquid samples, may be significantly involved in strong or weak interactions with cations, thus producing modifications of the charge distribution during the liquid-gas phase transitions. On the other hand, it is known that

chaotropic ions such as Cs^+ and Rb^+ cations or Br^-, I^-, and SCN^- anions can strongly interact with uncharged polar sites also.[23,24,66]

3.3 Effects of added electrolytes on the CSD Z_{av} and solvent accessible surface area A_s.

To obtain more information on the structural modifications of the myoglobin α-chain in the presence of our salts, we can consider the Z_{av} calculated through eq. 3, and the A_s values calculated through eq. 4 using the mean mass M reported in Table 1. The radius R of the proteins was also calculated from A_s values, assuming a spherical shape.

Table 2 reports the values of Z_{av} (eq. 3), A_s (eq.4) and the related radius R. Considering data in Table 2, we can notice that the A_s and R values are very similar for Hb – Metal chloride systems because of the very similar average masses. Particularly, A_s values are relatively close to the value A_s = 8071 $Å^2$ obtained for equine myoglobin (M ≈ 17 kDa) by Kaltashov.[55] A radius around 2.4 nm is reliable for an approximately globular shape of myoglobin without the heme group, which occurs as a single peak located at m/z = 616.1 (see Figure 1).

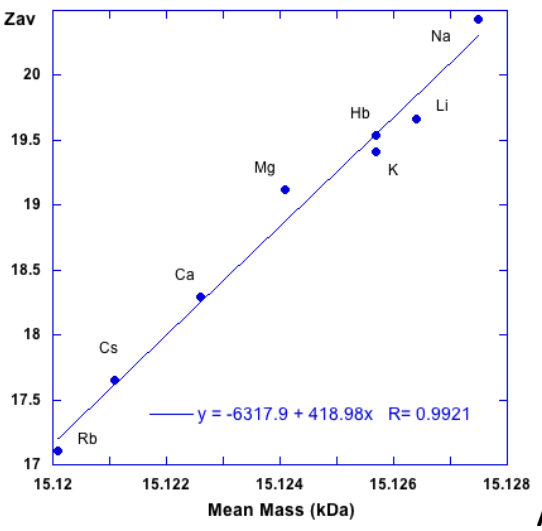
Interestingly, considering the Hb-Metal chloride systems only, almost linear relations between Z_{av} and average mass values (see Fig. 3A) and between log Z_{av} and log A_s (see Fig. 3B) are observed. Na^+ and K^+ salts did not exhibit a clear correlation.

As reported in the literature,[53,57] a power law relationship is expected between Z_{av} and M, cf. Eq.2, i.e. linear on a log scale. The observation of linearity demonstrates that salt introduces only small perturbations away from pure Hb mass, likely due to anion adsorption or possibly a small ion-induced change in protein hydration.

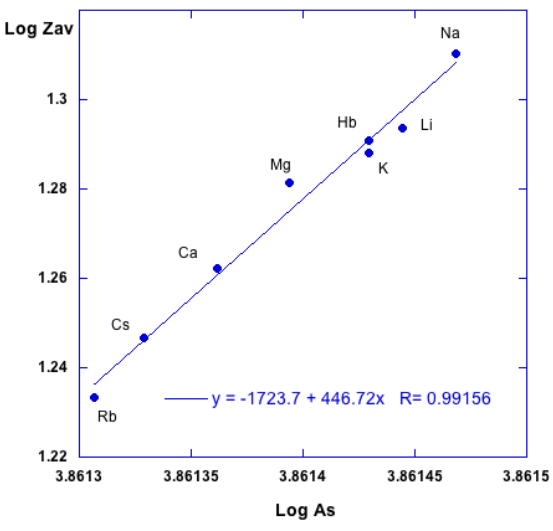
In terms of mechanism it can be suggested that all investigated chlorides promote the liquid-gas transition of the unfolded Myoglobin unity of Hb as a whole according to the CEM model, with the exception of RbCl that also induces the formation of a fragment, likely due to a collision induced dissociation (CID mechanism) of myoglobin unit.

Table 2. Hb systems: Charge State Distribution Z_{av} of the peaks in the range m/z = 600-1100, examining the 9 peaks with Z positive charges in the range 24-15; solvent accessible surface area A_s, radius R.

Hb - salt	Zav	As (Å^2)	R (Å)
Hb-no salt	19.54	7268.24	24.0
LiCl	19.66	7268.49	24.1
NaCl	20.43	7268.90	24.1
KCl	19.41	7268.24	24.0
RbCl	17.11	7266.19	24.0
CsCl	17.65	7266.56	24.0
$MgCl_2$	19.12	7267.65	24.0
$CaCl_2$	18.29	7267.11	24.0
NaBr	18.55	9223.99	27.1
NaI	18.20	3999.33	17.8
NaSCN	18.05	6740.32	23.2
KBr	18.58	7266.81	24.0
KI	18.69	7266.81	24.0
KSCN	18.14	10742.71	29.2



A



B

Figure 3. Hb-Chlorides Systems. **A)** Z_{av} vs. Mean Mass. **B)** Log-log plot Z_{av} vs. As.

In terms of mechanism it can be suggested that all investigated chlorides promote the liquid-gas transition of the unfolded Myoglobin unity of Hb as a whole according to the CEM model, with the exception of RbCl that also induces the formation of a fragment, likely due to a collision induced dissociation (CID mechanism) of myoglobin unit.

Considering both the quality of the ESI-MS spectra and data reported in Tables 1 and 2, the systems with KBr and KI salts should also undergo the transition according to the CEM model, but including the data of these two systems into the regression of Figure 3, the linear trend is lost (r = 0.95), as a result of mass values too low or Z_{av} values too high. As for the other salts, whenever two different peak profiles are observed, but at least one profile brings to calculate a mean mass of about 15 Da, it can be hypothesized that the addition of salts, in very acidic medium, promotes partial dissociation. Hence the charged ejection mechanism (CEM model) coexists with a collision induced dissociation (CID) mechanism; these mechanisms occur at a varying extent depending on the specific ion pair interaction. The addition of NaBr, NaI and NaSCN favors the CID mechanism only.

3.4 Quantification of Hofmeister Electrolyte Specific Effects

Clearly, the investigated salts cause very complex multifactorial effects. In regards to the varying concentrations used to optimize ESI-MS spectra, it may be suggested that the much higher concentration needed particularly in the case of Mg^{2+} and Ca^{2+} chlorides (salt/Hb ≈ 43-44), besides possible electrostatic interactions with the protein chain, can be related to the higher hydration enthalpy with respect to monovalent ones (See Table S1 in SI).[66] Moreover, the increase of surface tension due to Mg^{2+} and Ca^{2+} chloride addition might also play a role (See Table S1 in SI).[34] Clearly, this is an additional Hofmeister effect itself that can also be

related to the high kosmotropicity of the two divalent cations. On the other hand considering the data reported in Table S1 (SI), we notice that the two divalent cations show not only very high hydration enthalpies but also very high molar surface tension increments that may impede the approach of these ions to the water/air interface, thus decreasing their participation in the cation/anion mediated charge equilibration of the unfolded protein chain. This last process is responsible for the formation of the highly charged ion that will undergo the liquid-gas phase transition. During the formation of the unfolded protein ion the role of anions should also be considered. According to Collins' law of matching water affinity [20] we can expect that the chaotropic positively charged amino groups as well as hydrophobic sites located along the protein chain may bind the anions in the chaotropic decreasing order SCN^- > I^- > Br^- > Cl^-. Anion binding, however, is in competition with the ion evaporation model of the pairs H^+A^- that implies an increase in their concentration at the water/air interface. This occurs in the same decreasing order as the binding to the protein chain, namely HSCN > HI > HBr > HCl. A greater reduction in surface tension corresponds to a higher concentration of the ion pair at the air/water interface (see salt and HA surface tension increments in Table S1 in SI).

To quantify the interplay between cations and anions, in terms of chaotropic-kosmotropic balance, we define two specific electrolyte parameters. The first is H_S, defined as Hofmeister Specific Electrolyte parameter, which is given by the average value of the (Hb-salt)/Hb ratios of peak intensities (i.e. hemoglobin peaks measured with salt, relative to the hemoglobin peaks measured without added salt) evaluated for the nine peaks used for ESI-MS deconvolution in the our systems. H_S may represent a detailed quantification of salt effects (see Tables S2 and Graphs-Figure S1 in SI) since it measures the average specific electrolyte shift in the peak intensity profile of the protein, considered as a whole intact charged ion. H_S is defined as follows:

$$H_S = \frac{1}{n}\sum_1^n I/I° \quad (5)$$

where *I/I°* is the (Hb-salt)/Hb intensity ratio of each peak and *n* is the number of considered peaks (we apply *n* = 9). The second parameter P_S can be defined as the Specific Perturbation parameter, defined as the relative variance (standard deviation) of the *I/I°* ratios:

$$P_S = \frac{s.d.}{H_S} \quad (6)$$

here s.d. is the standard deviation calculated for H_S. P_S provides a measure of the significance of H_S and represents a measure of the loss of structure in the charged protein ESI-MS profile, caused by specific electrolyte perturbations. A lower P_S parameter can be expected to correlate with a higher value of Hofmeister electrolyte specificity H_S (>1), indicating that protein structure has been fortified by salt, resulting in a higher ESI-MS signal. A low value of H_S (<1) with large variance (hence, high P_S) corresponds to a loss of protein structure, indicating fragmentation. The calculated H_S and P_S parameters are reported in Table 3 along with the corresponding values divided by salt/Hb molar ratio, to provide a normalized series with respect to the different salt concentrations.

Table 3. Hofmeister specific electrolyte parameters: H_S, its associated standard deviation H_S s.d. , P_S, and the corresponding values normalized with respect to salt/Hb molar ratio.

Salt	H_S	H_S s.d.	P_S	H_S/mol.ratio	P_S/mol.ratio
LiCl	1.788	0.103	0.058	2.591	0.083
NaCl	0.796	0.352	0.442	0.181	0.101
KCl	2.875	0.238	0.083	3.639	0.105
RbCl	0.213	0.271	1.272	0.170	1.018
CsCl	0.454	0.431	0.949	0.378	0.791
MgCl2	1.904	0.478	0.251	0.044	0.006
CaCl2	0.479	0.294	0.614	0.011	0.014
NaBr	0.099	0.068	0.687	0.046	0.318
NaI	0.066	0.057	0.864	0.017	0.228

NaSCN	0.059	0.061	1.034	0.025	0.436
KBr	0.842	0.435	0.517	1.138	0.698
KI	0.576	0.251	0.436	0.376	0.285
KSCN	0.198	0.158	0.798	0.268	1.078

To provide the sequence of the Hofmeister electrolyte effect, the two parameters H_S and P_S, associated with the various salt systems, are reported in Figure 4 in the decreasing order with respect to H_S and H_S /(molar ratio).

Figure 4A suggests that a division between structure enhancing and protein structure breaking can be identified at $P_S \approx 1/2$. Notably, Figure 4A gives clear evidence, in terms of the specific electrolyte parameter H_S, of the high stability of myoglobin charged ion in the presence of KCl, $MgCl_2$ and LiCl ($H_S > 1$, and low P_S), and the protein fragmentation (cfr. Table 1) induced by RbCl, NaBr, NaI, NaSCN and KSCN (low H_S, and $P_S > 0.5$). Figure 4A also shows that anions decrease the quality of ESI-MS spectra in the conventional Hofmeister order $Cl^- > Br^- > I^- > SCN^-$, for both Na and K salts, with Cl^- salts generating higher quality spectra and SCN^- the most degraded.

Figure 4B, where *y* axes are in log scales, shows some differences in the sequence of salts. Particularly, due to the very high concentrations used for $MgCl_2$ and $CaCl_2$, the two trends differ significantly. Also the position of NaCl changes significantly, being its salt/Hb molar ratio the highest among monovalent cations. In fact, in Figure 4B we can notice that the stabilizing effect of KCl and LiCl is still highlighted by the H_S*/(molar ratio)* values greater than unity, whereas the stabilizing effect of $MgCl_2$ can be suggested considering that for this salt the lowest value of the perturbation parameter P_S*/(molar ratio)* is calculated. In addition, the sequence of electrolytes observed in Figure 4B highlights the stabilizing role of potassium cation even in the case of the most chaotropic thiocyanate anion.

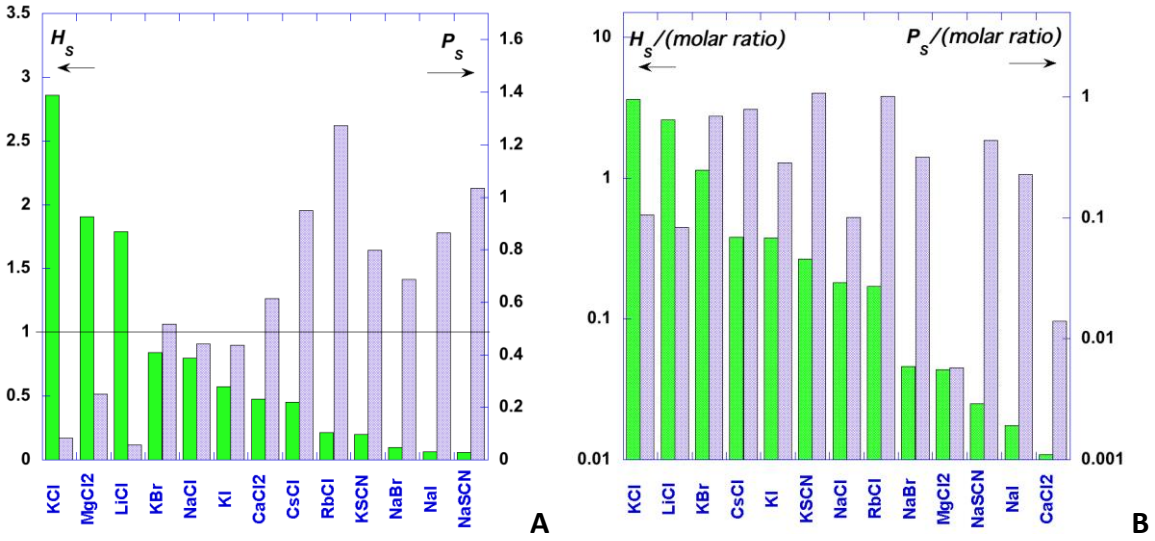


Figure 4. Hofmeister specific electrolytes parameters: **A)** ***H*S** *(green)* and ***P*S** *(violet)* parameters; **B)** The same ***H*S** *(green)* and ***P*S** *(violet)* parameters normalized with respect to salt/Hb molar ratio (y axes in log scale)

The observation of the conventional anion series is not unexpected since electrolyte conditions maintain a positive protein charge (pH < IEP), with anions interacting as regular counterions, modulated by anion-specific nonelectrostatic dispersion interactions. The cation specific effect is unconventional, suggesting a specific cation interaction, perhaps specific chemisorption,[15,16] in spite of the positive electrostatic environment.

4. Concluding remarks

This work focused on the effect of several added salts on the protein hemoglobin in denaturing conditions (pH = 2.7), giving evidence of several peculiar features. First of all the very acidic medium favored the loss of the quaternary structure of Hb, thus the highly charged myoglobin α-chain, having molecular mass M ≈ 15.12 kDa, was identified as the main species in ESI-MS spectra. It is not actually clear why each salt has its own optimal concentration. Tentatively, the hydration enthalpy and surface tension increment related to the each salt can be called into play, particularly in the case of the divalent cations. It has been suggested that a reduction

in surface tension favors an increase of concentration of the ion pair at the air/water interface and vice versa: this means, for instance, that particularly anions are in competition between binding to protein sites and coming out of the charged droplet as H^+A^- moieties (IEM model). The addition of salts modified the typical bell-shaped profile of the protein significantly, nevertheless no significant modifications of *M* values were observed at least in the presence of metal chloride salts. Indeed, for Hb-metal chloride systems a linear correlation between the mean *M* values and the average charge state Z_{av} of the main peaks was found. The solvent accessible surface area A_s, assuming an approximately spherical shape of the highly charged protein, indicated a radius $R \approx 2.4$ nm for the α-chain of Myoglobin. On the basis of experimental data and of the highly denaturing medium, the CEM model, where unfolded proteins come out of the droplet as a result of charge rebalancing between the droplet and the slightly unfolded protein, dominates the liquid-gas phase transition in several Hb-salts systems. In the case of NaBr, NaI, NaSCN and KSCN addition, myoglobin undergoes collision induced dissociation that causes the formation of fragments having lower molecular masses. Interestingly, these effects are not observed in the presence of KBr and KI, likely due to the specificity of K^+ cation.

Finally we can emphasize the role of the various ions. The anion effects reflect the Hofmeister series, with intensity of peaks and quality of ESI-MS spectra decreasing in the order $Cl^- > Br^- > I^- > SCN^-$ for both Na^+ and K^+ salts systems. As to the effect of the cations, the conventional Hofmeister series, as observed in many other systems, is not respected since in our systems we could not use the same concentration of added salts. In Hb-Metal chlorides systems, the intensity of the peaks and the quality of the bell-shaped profiles decrease in the order $K^+ >> Mg^{2+} > Li^+ >> Na^+ > Ca^{2+} \approx Cs^+ > Rb^+$. In addition, it should be remarked that the addition of K^+, Mg^{2+} and Li^+ chlorides increases the intensity of the peaks significantly. Hence these salts

contribute to stabilize the unfolded myoglobin charged ion during the transition to the gas phase. Indeed for these salts the highest and the lowest values of H_S (values > 1) and P_S, respectively, were determined. Different behaviour was reported for Hb in the presence of K^+, Rb^+, Cs^+, Ca^{2+} and Mg^{2+} chlorides in a paper by Konermann, where monovalent cations had no significant effects while divalent cations stabilized protein complexes.[47] However, in that case pH was 6.8, and much higher salt concentrations (in terms of salt/protein molar ratio) than in this work were used.

Remarkably, the interplay of very complex interactions induced by the considered ion pairs can be quantified by H_S and P_S proposed parameters that provide a new vision of the Hofmeister series approach, where the concept of kosmotropicity-chaotropicity associated to anions and cations is replaced by specific electrolyte effects. This new approach may be of great interest also for specific clinical analysis.

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