On the Interactions between Amino Acids and Ionic Liquids in Aqueous Media

Luciana I. N. Tomé,[†] Montserrat Domínguez-Pérez,[‡] Ana Filipa M. Cláudio,[†] Mara G. Freire,[†] Isabel M. Marrucho,[†] Oscar Cabeza,[‡] and João A. P. Coutinho*[,†]

CICECO, Departamento de Química, Universidade de Aveiro, 3810-193 Aveiro, Portugal, and Departamento de Física, Facultade de Ciencias, Universidade da Coruña, 15071 A Coruña, Spain

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The understanding of the molecular-level interactions between biomolecules and ionic liquids (ILs) in aqueous media is crucial for the optimization of a number of relevant biotechnological processes. In this work, the influence of a series of amino acids on the liquid−liquid equilibria between 1-butyl-3-methylimidazolium tricyanomethane and water was studied to evaluate the preferential interactions between these three compounds. The solubility effects observed are dependent on the polarity, size, and charge distribution of the amino acid side chains and are explained in terms of a refined version of the model proposed earlier (Freire et al. *J. Phys. Chem. B* **2009**, *113*, 202; Tomé et al. *J. Phys. Chem. B* **2009**, *113*, 2815) for ion specific effects on aqueous solutions of imidazolium-based ILs. Although acting through different mechanisms, salting-in and salting-out phenomena possess a common basis which is the competition between water−amino acid side chain, IL−amino acid side chain, and water−IL interactions. The delicate balance between these interactions is dependent on the relative affinities of the biomolecules to water molecules or to IL cation and anion and determines the trend and magnitude of the solubility effect observed.

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Introduction

Due to their biochemical and industrial relevance, processes such as the separation and recovery of bioproducts from natural sources and fermentation media, enzyme activity and stability, kinetic resolution of racemates and biosynthesis are topics of utmost importance in the domain of biotechnology. The search for efficient and safe approaches to these procedures and the recognition of remarkable and advantageous properties in ionic liquids (ILs)[3] has led, in the past few years, to a growing interest in the use of these compounds as *green* alternatives to conventional solvents. In liquid−liquid extraction processes, for instance, the replacement of volatile and toxic organic solvents by ILs as extractors enabled us to overcome environmental, operational, and efficiency problems associated with the conventional application of such a technique.[4,5] An increasing number of works describing the use of ILs with low solubility in water as biphasic extraction media have been published in the past few years,[4,6−11] and encouraging results for the recovery of acetone, ethanol, and butanol from fermentation broths,[4,7] the extraction of antibiotics,[4,9] and the removal of organic contaminants from aqueous waste streams[8] have been reported. Besides separation and purification, the use of ILs as reaction media in processes involving biologically relevant compounds has been promising as well, as shown by their successful application in kinetic resolution,[12−15] biocatalysis,[16−18] and biosynthesis.[19] In these fields, much discussion has been generated around the role of ILs in the stabilization and activity of enzymes, crucial for the improvement of those processes.[14,15,20−23]

In spite of the widespread application and popularity, there are, however, critical unsolved issues concerning the use of ILs in the biotechnological processes mentioned above. In fact, to optimize and control the extraction of biomolecules by ILs from aqueous media or to improve the yields of biocatalyzed reactions by the enhancement of the stability and activity of enzymes in those solvents, an effective manipulation of the parameters that influence the solvation of the biocompounds in ILs and aqueous phases is required. These are, in turn, dependent on the interactions established in solution. Although some recent studies have provided correlations between the extraction of biomolecules and a few different factors[4,24] and some insight on the effects of IL nature and properties on the activity and stability of enzymes,[14,15,20−23] there still lacks the basic knowledge indispensable to improve these processes through the selection of the optimal conditions and the complete exploration of the potentialities of ILs as *designer solvents*. Furthermore, the molecular-level mechanisms which dictate the behavior of these systems, which are ultimately those responsible for the viability of these processes, are far from being established. There is thus the need to develop a systematic work aiming at understanding the interactions between ionic solvents and biomolecules to develop a well-supported molecular picture of the phenomena.

To get a solid and deep knowledge of the molecular mechanisms governing extractions from aqueous media or bioreaction processes using ILs as solvents, a detailed understanding of the phase behavior of biomolecule/water/IL mixtures is necessary. Liquid−liquid equilibrium (LLE) data and complementary microscopic results for aqueous solutions of ILs are thus precious sources of information, especially as far as systems containing salts, sugars, fermentation metabolites, amino acids, and enzymes (or proteins in general) are concerned. These biomolecules are usually present in the aqueous phases of biological media and can have a deep influence on the water−IL mutual solubilities. In previous works, we studied the mutual solubilities of water and ionic liquids[25,26] and explored the behavior of ternary mixtures composed by water, imidazolium-based ILs, and organic and inorganic salts.[1,2] In the face of the thermodynamic and spectroscopic data gathered, we were able to refute the long-held "structure maker/breaker" classical ideas

* Corresponding author. Tel.: +351-234-370200. Fax: +351-234-370084. E-mail address: jcoutinho@ua.pt.
[†] Universidade de Aveiro.
[‡] Universidade da Coruña.

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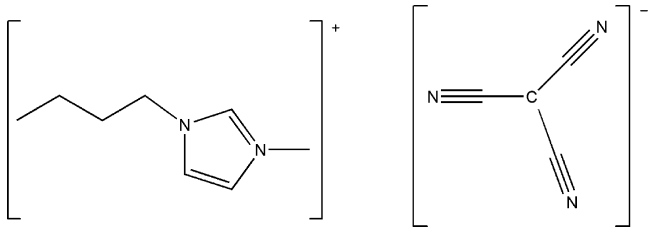

Figure 1. Chemical structure of the ions constituting the IL used in this work.

for the Hofmeister series regarding the effects of salts on macromolecule solubilities[27−30] and bring forward a well-supported model for the molecular-level phenomena dictating the action of salting-in and salting-out inducing ions in aqueous solutions of ILs. In the current work, we investigate the molecular mechanisms underlying the behavior of aqueous solutions of a partially miscible IL, 1-butyl-3-methylimidazolium tricyanomethane, $[C_4mim][C(CN)_3]$, in the presence of amino acids and discuss the influence of the chemical structure and properties of the biomolecules on the interactions established between all species in solution. For that purpose, the effect of 12 amino acids—alanine (Ala), arginine (Arg), aspartic acid (Asp), glutamic acid (Glu), glycine (Gly), isoleucine (Ile), leucine (Leu), lysine (Lys), phenylalanine (Phe), proline (Pro), threonine (Thr), and valine (Val)—on the phase behavior of aqueous mixtures of $[C_4mim][C(CN)_3]$ was studied. The magnitude of the observed salting-in/salting-out effects observed was evaluated and related to the nature, polarity, and chemical properties of the biomolecules.

By choosing amino acids, we intend to give a contribution toward the understanding of the solvation mechanisms which control the separation and purification processes by ILs, not only of these simple molecules but also of more complex biomolecules such as proteins and their influence on the recovery, activity, and stability of enzymes in these solvents. In fact, besides their commercial and biochemical relevance, amino acids are ideal compounds to be studied as model biomolecules, not only because they are the basis of proteins but also because their charged nature reproduces the behavior of many charged biomolecules. Moreover, it is possible to easily change their properties by increasing/decreasing their polarity, hydrophobicity, aromaticity, or charge, simulating a wide range of behaviors. The imidazolium ion is often used for extractions or as reaction media since, when combined with adequate anions, the hydrophobicity of the resulting IL constitutes an advantageous property when dealing with the recovery of products from aqueous environments[25] and a factor of stabilization and activation of enzymes.[22] Furthermore, when comprising a short alkyl chain on the cation, imidazolium-based ILs have low toxicities and thus a reduced water pollution risk.[31−33]

Experimental Section

Materials. The IL $[C_4mim][C(CN)_3]$ was supplied by Merck with mass fraction purity >99%. To reduce the water and volatile compounds content to negligible values, the IL was dried under constant agitation at vacuum (0.1 Pa) and moderate temperature (353 K) for a minimum of 48 h. After this procedure, its purity was checked by ^1H and ^{13}C NMR spectra. The chemical structure of the ionic liquid is depicted in Figure 1. The water used for the preparation of the amino acid solutions was double-distilled, passed by a reverse osmosis system, and further treated with a Milli-Q plus 185 water purification apparatus. It has a resistivity of 18.2 MΩ·cm and a TOC smaller than 5 $\mu g \cdot dm^{-3}$, and it is free of particles greater than 0.22 μm. The following amino acids were used as purchased without further purification: L-glycine (Riedel-de-Haen, >99.1 w/w %), L-alanine (BDH Chemicals, >99 w/w %), L-arginine (Merck, >99 w/w %), L-lysine (Fluka, >98 w/w %), L-leucine (Merck, >99 w/w %), L-isoleucine (Merck, >99 w/w %), (L-(+)-glutamic acid (Riedel-de-Haen, >99 w/w %), DL-aspartic acid (Fluka, >99 w/w %), L-valine (Merck, >99 w/w %), L-threonine (Riedel-de-Haen, >99 w/w %), L-proline (Sigma, > 99 w/w %), and L-phenylalanine (Merck, >99 w/w %).

Experimental Procedure. The effect of both the amino acid nature and concentration on the critical point of the binary system (IL + water) was evaluated. For that purpose, solutions of each amino acid in pure water were gravimetrically prepared within an uncertainty of $\pm 10^{-4}$ g and concentrations in the range [0−11.0] $mol \cdot kg^{-1}$, depending on the water solubility saturation of the amino acid under study. Sealed Pyrex-glass capillaries with a magnetic stirrer were filled with approximately 0.19 g of aqueous amino acid solution and 0.32 g of IL, so that the composition of the resulting aqueous solutions of IL was nearly the critical point ($wc_{IL} = 0.38$ for $T_c = 326$ K[2]). The onset of the liquid−liquid immiscibility (cloud point temperature) of ($[C_4mim][C(CN)_3]$ + water + amino acid) mixtures was measured by turbidimetry, according to the procedure described elsewhere.[2]

Results

Figure 2 shows the results obtained for the cloud-point temperatures of ($[C_4mim][C(CN)_3]$ + water + amino acid) mixtures as a function of the molality of the amino acid, at a nearly IL/water critical composition. Since decrease (salting-out), increase (salting-in), and negligible effects on the mutual solubilities of the IL/water are observed, in magnitudes strongly dependent on the nature and concentration of the amino acid, the experimental data were divided into three distinct figures (Figure 2(a), (b), and (c), respectively), and the critical temperature for the system (IL + water)[2] (amino acid concentration = 0 $mol \cdot kg^{-1}$) is also represented to better visualize those effects. The critical temperature is upper critical solution temperature, and therefore a decrease in the latest implies salting-in behavior, while an increase corresponds to decreasing the IL aqueous solubility.

The strongest salting-out inducing amino acid is Gly and, in general terms, the importance of this effect decreases in the order Gly > Ala ≈ Thr > Arg > Lys. The temperature dependence for the liquid−liquid miscibility of $[C_4mim][C(CN)_3]$ in the aqueous phases containing the first three species shows an almost linear dependence on the amino acid concentration, and the temperature shifts are actually very pronounced and proportional to the amino acid molality increase. In contrast, Arg and Lys behave quite differently, inducing a slight decrease of the IL/water mutual solubilities which, for Lys, is only detectable at high amino acid molalities. From the results presented, it might be suggested that the effect promoted by Arg is stronger and more concentration dependent than that of Lys, but the experimental data gathered are not enough to fully support this statement since the molality range studied for Arg was limited by its saturation solubility in water.

With a negligible influence on the mutual solubilities of IL and water, Asp and Glu are positioned in the middle of the rank of the amino acid series. Also in these cases, the low solubility of the amino acids limited the experiments to a narrow molality range.

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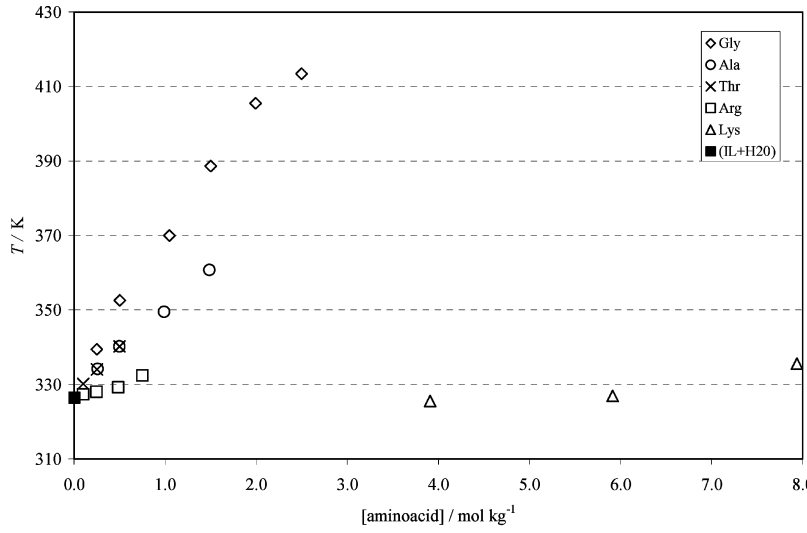
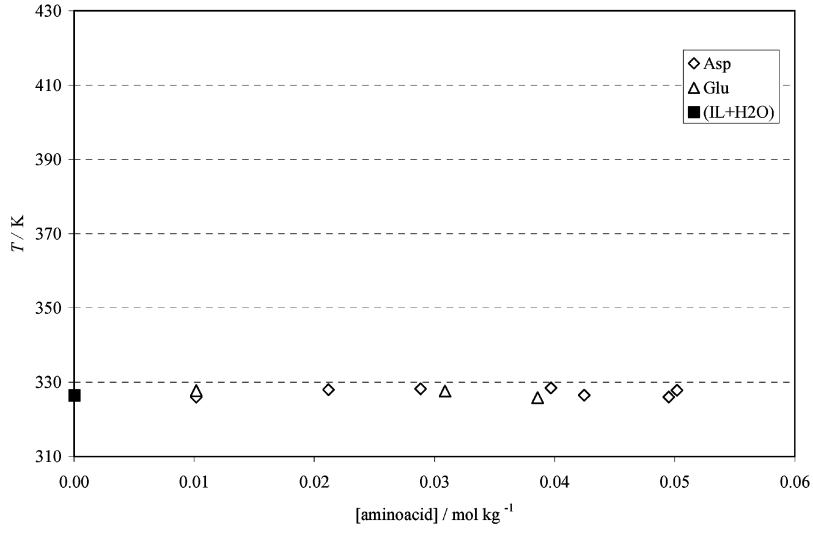
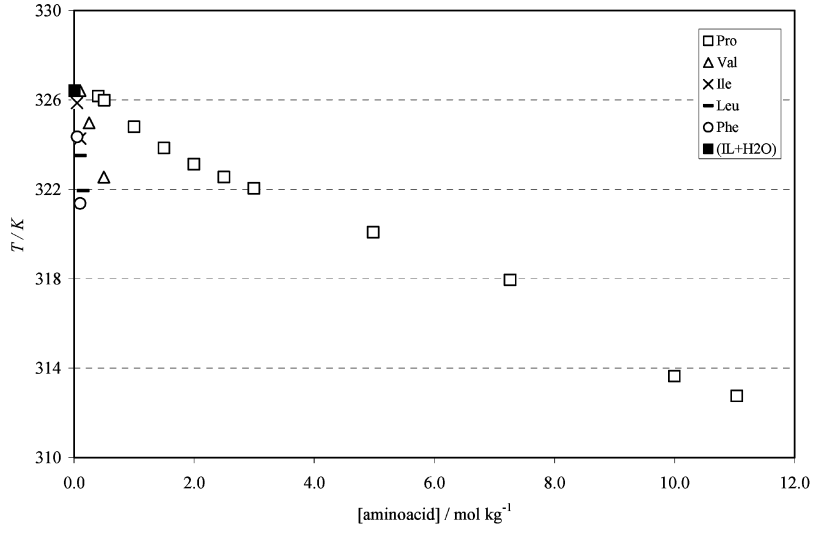


(a)



(b)



(c)

Figure 2. Effect of the amino acids on the near critical temperatures of the phase diagrams of ([C_4mim][$C(CN)_3$] + water) systems ($wc_{IL} = 0.38$) for (a) salting-out inducing amino acids; (b) amino acids with negligible solubility effects; and (c) salting-in inducing amino acids.

As far as salting-in effects are concerned (Figure 2(c)), the trend observed is Pro < Val < Ile < Leu < Phe, the latest being the amino acid which induces the most significant negative shift in the cloud point temperature. Except for Pro, the other salting-

in inducing amino acids promote a very strong increase of IL/water mutual solubilities, even at low concentrations.

It is worth noticing that the molality ranges studied were limited by the solubility of the amino acids in water. Nevertheless, the data are clear enough to enable the establishment of general trends. Another important point to note is related to the pH influence. In a previous work,[2] the effect of the pH on the $[C_4mim][C(CN)_3]$ solubility in water was evaluated. From the comparison of those results and the data obtained in the current work, it can be seen that this is negligible when compared to the effect of the added amino acids. Consequently, the observed results for the solubility behavior are not promoted by changes in the pH of the solutions, and thus the pH influence will be neglected in the discussion developed below.

Discussion

It is well-known that, depending on the pH of the solution, amino acids can be present in different equilibrium forms due to the ionization/protonation of their characteristic functional groups, $-COOH$ and $-NH_2$. Taking into account the pK_a of these groups, at the pH considered in this work ($\approx$7), the amino acids under study are all expected to be in their zwitterionic form. The majority of the biomolecules possesses, additionally, neutral side chains and, therefore, have no net charge. The exceptions are Arg, Lys, Asp, and Glu. Although being in their zwitterionic forms, these amino acids comprise acidic/basic groups in their side chains which, at pH = 7, are ionized/protonated. As a consequence, they acquire positive/negative net charges, depending on their acidic/basic character. The structure of the amino acids investigated and the pK_a of their functional groups are displayed in Table 1. From the analysis of this data and the results depicted in Figure 2, it is possible to establish the following trends. Salting-in inducing behaviors are only observed for amino acids with apolar and neutral alkylic side chains, and the longer this chain, the more pronounced is the increase in the IL/water mutual solubilities promoted. In extreme cases, the apolar chain is clearly predominant, and the amino acids are globally hydrophobic acting as cosolvents for the IL/water binary system. Salting-out is induced by amino acids with polar character, the stronger effect being observed for the simplest amino acid, Gly, where the side chain is absent. Significant decreases of the IL solubility in water are also induced by amino acids with a short side chain, such as Ala and Thr. The magnitude of salting-out decreases with the increase of the size of the neutral (polar) side chain. The least pronounced solubility effects are observed for amino acids whose side chains are not neutral: positively charged side chain amino acids (Arg and Lys) induce just a slight salting-out effect, while amino acids comprising side chains with negative formal charge have negligible solubility effects. It is, however, rather difficult to compare and distinguish these less noticeable effects because the concentration ranges studied are different since they were limited by the biomolecules solubilities.

From the exposed above, it can be perceived that the influence of the amino acids on the $[C_4mim][C(CN)_3]$/water liquid−liquid equilibria is dependent on the nature and properties of their side chain, namely, its size, polarity, and charge, the last determined by the acid/basic characteristics of the constituent groups. In an indirect form, this dependence is necessarily reflected and related to the hydrophobic/hydrophilic character of the amino acids. It is worth keeping in mind that since the amino acid molecules have the same charged end groups, the contribution of the electrostatic interactions of these groups should be approximately the same; consequently, there are the side chains that are responsible for the variation trends.

In recent investigations carried out in our working group,[1,2] it was observed that the effect of salts on IL solubilities in water follows the Hofmeister series and is analogous to that promoted on protein solubility in water. In face of the results obtained, we proposed a model to describe the specific ion effects on the aqueous solubility of ILs and were able to refute the long-held classical dogma of "structure makers/breakers" effects of salts on macromolecule solubilities in water. With ILs being dissociated into ions in aqueous solutions and amino acid charged particles, it is reasonable to apply the molecular interpretation given before[1,2] to the systems under study. As shown below, it provides a good basis to explain the results reported in this work.

According to the model referred,[1,2] salting-in and salting-out inducing ions operate essentially by different mechanisms. Salting-out effects on IL saline solutions were explained in terms of an entropically driven effect resulting from the formation of hydration complexes away from the solute hydrophobic moieties due to the preferential hydration of high charged density ions and the increase of surface tension of cavity formation. On the other hand, the salting-in phenomena promoted by low charged density ions were interpreted in terms of the direct binding of the charged species to the hydrophobic moieties of the IL cation, as a consequence of their unfavorable interactions with water. Following the approach adopted before,[1,2] the perturbation on the solubility of a solute caused by the addition of salts, at constant solute composition, can be described by the following equation

$$T = T_0 + kc + [B_{mx}K_Ac/(1 + K_Ac)] \quad (1)$$

where T_0 is the cloud-point temperature of the (IL + water) system in the absence of salt; c is the concentration of salt in molality; and K_A, B_{max}, and k are adjustable parameters describing the phenomena that affect the solute solubility. Applying eq 1, we obtained the fitted parameters K_A, B_{max}, and k for the systems under study (Table 2). It is worth noting that the last term was only used for salting-in inducing amino acids as Zhang et al. suggested.[34] The constants K_A, B_{max}, and k, which are a measure of the solubility effects observed, were related to the molar entropy of hydration, $\Delta_{hyd}S$, of the biomolecules. These were calculated from literature data and are presented in Table 3. The plot of k as a function of $\Delta_{hyd}S$ of the amino acids (Figure 3) shows a correlation between these two quantities for salting-out inducing amino acids that supports an entropically driven mechanism of salting-out. Unfortunately, surface tension data of the amino acid aqueous solutions are not available to further support the molecular interpretation of salting-out/salting-in inducing behavior. Nevertheless, and as discussed below, the LLE data gathered in this work, together with some quantitative treatment performed on the results, not only strongly support the mechanism proposed for the action of ions in aqueous solutions of ILs but also enable us to point out other aspects of the mechanism that were not so evident in the case of salts.

The LLE evidence here obtained for the behavior of salting-in inducing amino acids suggests, in fact, the same type of interpretation. Actually, since only amino acids with neutral apolar side chains provoke an increase of the IL + water mutual solubilities and these become more pronounced with the enhancement of the hydrophobicity of the alkylic side chain, it is fair to conjecture that salting-in phenomena are the result of direct interactions between the amino acid alkyl side chains and the hydrophobic moieties of the IL cation. Because water−amino acid interactions are weaker than those established between water molecules, the direct binding stabilizes the solute in water,

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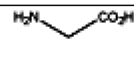
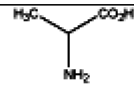
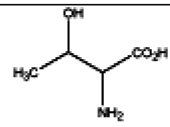
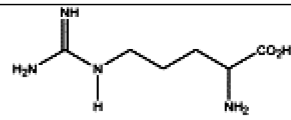
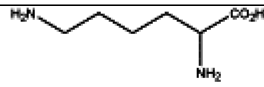
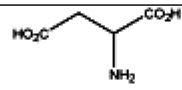
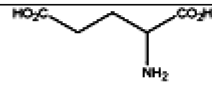
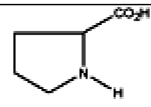
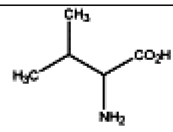
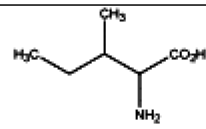
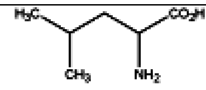
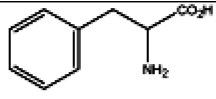
TABLE 1: Structure of the Amino Acids Studied and Their Dissociation Constants[24,40]

Amino acid	Structure	pK_a(COOH)	pK_a(NH_3)	pK_a(-R)
Gly		2.35	9.78	-
Ala		2.34	9.69	-
Thr		2.09	9.10	-
Arg		2.01	9.04	12.48
Lys		2.18	8.95	10.53
Asp		2.10	9.82	3.86
Glu		2.19	9.67	4.07
Pro		2.00	10.60	-
Val		2.32	9.62	-
Ile		2.32	9.76	-
Leu		2.36	9.60	-
Phe		2.13	8.62	-

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enhancing, therefore, its solubility, acting as a cosolvent. The more apolar the side chain, the stronger the interaction with the IL cation and, consequently, the magnitude of the salting-in. As the size (or hydrophobicity) of the alkylic chain of the amino acid decreases, not only its interaction with the hydrophobic moiety of the IL cation becomes weaker but it also starts to have some affinity with water. With a significant apolar part, Phe is the strongest salting-in inducing amino acid, while Pro, comprising a small aliphatic ring, is the weakest.

The LLE data reported for salting-out inducing amino acids are suggestive as well. Gly, the simplest amino acid with no side chain and a highly polar zwitterion portion, has clearly a preferential hydration, inducing the strongest salting-out influence. As a general trend, when the (polar) side chain becomes

TABLE 2: Fitted Values for k, K_A, and B_{max} for the Amino Acids at 298.15 K

amino acid	k (K·mol of amino acid^{-1})	$10^2 \cdot K_A$ (kg·mol of amino acid^{-1})	B_{max} (K)
Gly	38.48	-	-
Ala	23.93	-	-
Thr	28.00	-	-
Arg	7.681	-	-
Lys	0.755	-	-
Asp	≈0		
Glu	≈0		
Pro	8.988×10^{-8}	0.43	−290.41
Val	4.724×10^{-8}	1.83	−309.02
Ile	1.617×10^{-7}	1.06	−1894.8
Leu	1.651×10^{-7}	0.48	−4167.9
Phe	7.316×10^{-8}	7.16	−668.29

TABLE 3: Literature Values for Amino Acid Solubilities in Water (s)[40] and Molar Gibbs Energy of Hydration ($\Delta_{hyd}G_m$),[37] Molar Enthalpy of Hydration ($\Delta_{hyd}H_m$),[41] and Calculated Values for Molar Entropy of Hydration ($\Delta_{hyd}S_m$) of the Amino Acid Side Chains at 298.15 K[a]

amino acid	s g (100 g^{-1})[40]	$\Delta_{hyd}G_m$ kJ·mol^{-1}[37]	$\Delta_{hyd}H_m$ kJ·mol^{-1}[41]	$\Delta_{hyd}S_m$ J·K^{-1}·mol^{-1}[b]
Gly	24.99	10.0	-	-
Ala	16.65	8.1	−8.3	−55.0
Thr	very soluble	−20.4	−45.0	−82.5
Arg	15.00	−83.3	−99.4	−54.0
Lys	very soluble	−39.8	−48.8	−30.2
Asp	0.778	−45.8	−47.3	−5.0
Glu	0.864	−42.7	−50.7	−26.8
Pro	162.3	-	−10.2	-
Val	8.850	8.3	−13.7	−73.8
Ile	4.117	9.0	−17.1	−87.5
Leu	2.426	9.5	−17.1	−89.2
Phe	2.965	−3.2	−25.3	−74.1

[a] Values considered at pH = 7. [b] Calculated from $\Delta_{hyd}G_m$ and $\Delta_{hyd}H_m$.

longer (more alkyl groups in its constitution and, thus, more hydrophobic), the salting-out phenomena become less pronounced, as a consequence of the simultaneous decrease of its hydrophilic character and increase of its affinity with the hydrophobic moieties of the IL cation. This hypothesis can be verified with the cases of Ala and Thr. In fact, in spite of their different structure, the effects of Ala and Thr are practically undistinguishable. The reason for this behavior might be attributed to the balance between the interactions established by their side chains with water and with the hydrophobic moiety of the cation of the IL. Actually, when compared to Thr, Ala is less hydrophilic but, at the same time, has also less affinity to the IL cation because of its smaller side chain. The side chain of Thr, for its turn, is more likely to establish favorable interactions with water but also stronger binding to the IL cation since its side chain comprises more alkylic groups. There is therefore a compensation effect between the strength of those interactions (side chain−water and side chain−IL cation), and consequently, the magnitude of the salting-out promoted is similar for both the amino acids. Further evidence for the existence of such a balance can be obtained from the analysis of the cases of the amino acids with the least pronounced solubility effects. Actually, Ala and Lys contain polar and hydrophilic side chains and were therefore expected to promote pronounced salting-out effects because of their obvious preferential hydration. That is not the case, however. In fact, although polar and hydrophilic, Ala and Lys side chains contain a significant number of alkylic groups which also confer them affinity to the IL cation hydrophobic moieties. The balance between the competitive interactions (side chain−water) and (side chain−IL cation) will result in the decrease of the magnitude of the salting-out effect. This behavior is even better explained if we consider, additionally, that the interactions of the amino acids are established not only with the cation of the IL but also with the anion, an aspect of the mechanism which had already been claimed for the interpretation of specific ion effects in aqueous IL solutions.[2] Contrary to the amino acids considered so far, the side chains of Ala and Lys are not neutral due to the protonation of the basic functional groups present at pH = 7. As a consequence, important attractive electrostatic interactions can be additionally established with the anion of the IL, contributing to the less noticeable decreases of IL + water mutual solubilities. The same type of argument is valid for the acids, Asp and Glu, but in these cases, the additional factors to be considered to explain the decrease of the magnitude of salting-out are electrostatic interactions established by their negatively charged side chains with the cation of the IL, in addition to the dispersive interactions with the hydrophobic moieties of the latest. The binding of the amino acid side chains to the IL cation are thus reinforced, and the interactions amino acid−water and amino acid−IL are balanced, resulting in a negligible solubility effect. Amino acids with neutral polar side chains are not able to establish these additional electrostatic interactions with the IL cation; therefore, the binding only occurs at the level of the hydrophobic moieties of the IL cation through dispersive forces, and their hydration is still preferential. It is worth noting that recent studies[24] on the partition coefficients of amino acids between water and different ILs stressed the importance of electrostatic interactions between the cationic form of amino acids and the anions of the ILs.

The data obtained in this work seem therefore to indicate that the mechanisms underlying salting-in and salting-out effects of amino acids are essentially different but are dictated by a common basis which is the competition between water−side chain and IL−side chain interactions. Since the resulting balance is dependent on the relative affinity of the amino acids to water molecules or to IL ions, it is appropriate to relate some macroscopic constants which reflect the hydrophobic/hydrophilic nature of the amino acids to the k parameter to support the model proposed in a more quantitative perspective. The literature values taken for the solubility (s), molar Gibbs energy of hydration ($\Delta_{hyd}G$), and molar enthalpy of hydration ($\Delta_{hyd}H$) of the amino acids studied and used in the discussion below are presented in Table 3. The plots of k as a function of these quantities are displayed in Figures 4−6. For a better vizualisation, some of the points are labeled in the graphs.

The solubility of a species is a macroscopic property which reflects the strength of the interactions established with water molecules. Therefore, if the salting-out effect was caused solely by the different extent of hydration of the different amino acids, the trend observed for the decrease in the IL/water solubilities promoted would follow the order of the values of their solubility in water. Nevertheless, as can be seen from Figure 4, that is not what is verified. Actually, the strongest salting-out inducing amino acid is not the more water-soluble, and amino acids with less pronounced salting-out effects have considerable solubilities in water. These facts support the idea that there are other competitive interactions, namely, at the level of the IL cation/anion, that sometimes overcome or balance this hydration preference. For instance, although Thr and Lys are the most soluble amino acids, which is understandable by the presence of −OH and −NH_2 groups in their side chains, they are not the strongest salting-out inducing biomolecules. As discussed above, this behavior results from their equally high affinity to the IL cation due to the considerable apolar moieties of their side chains and, in the case of Lys, its additional charge. More supporting examples can be found if we consider the salting-in inducing amino acids. These have, actually, lower solubilities than the ones which promote salting-out effects, pointing to a more unfavorable hydration. Moreover, it is verified that the magnitude of the salting-in decreases with the increase of the values of the water solubility of the amino acid, indicating that its interaction with water becomes more important and starts to compete with its direct binding to the IL. The exceptional and particular case is Pro, more soluble than any of the salting-out inducing amino acids. Its short and compact alkylic side chain should have a rather favorable affinity to the IL cation, balancing this pronounced hydrophilic character.

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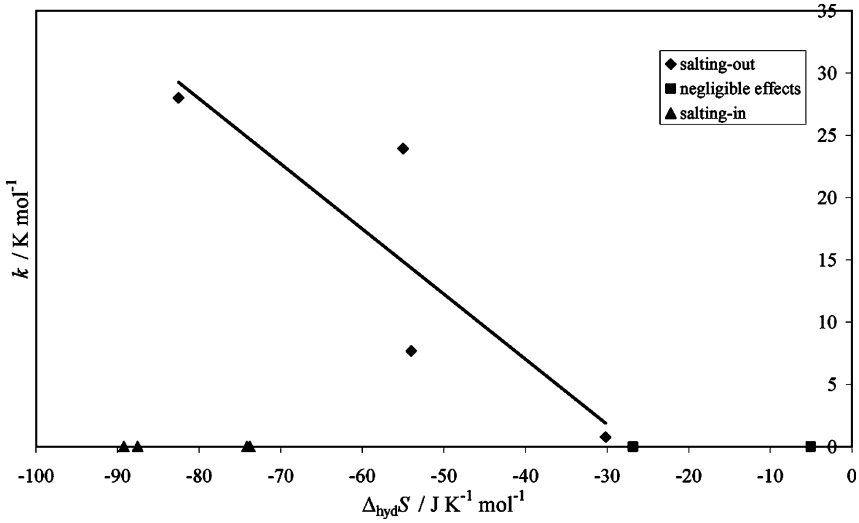

Figure 3. k parameter as a function of the molar entropy of hydration ($\Delta_{hyd}S$) of the amino acids. The line is drawn as a correlation for the salting-out amino acids.

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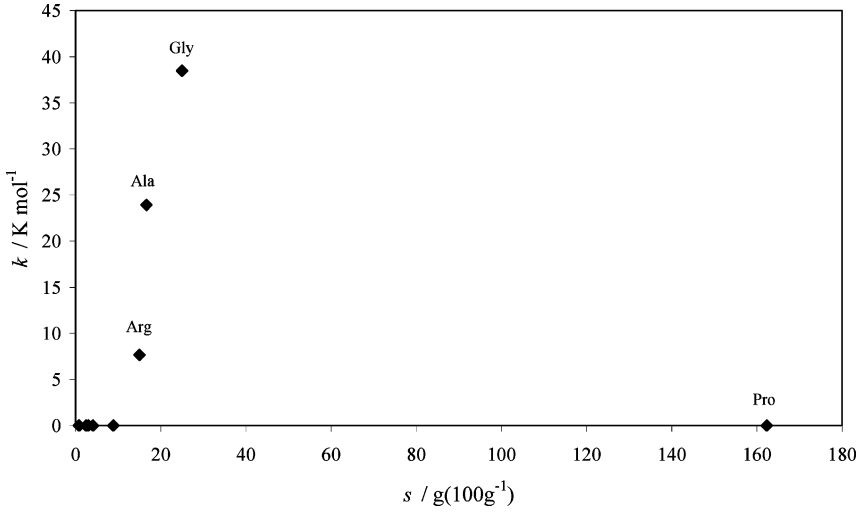

Figure 4. k parameter as a function of the solubility (s) of the amino acids in water. Thr and Lys are not represented because they are very water-soluble amino acids.

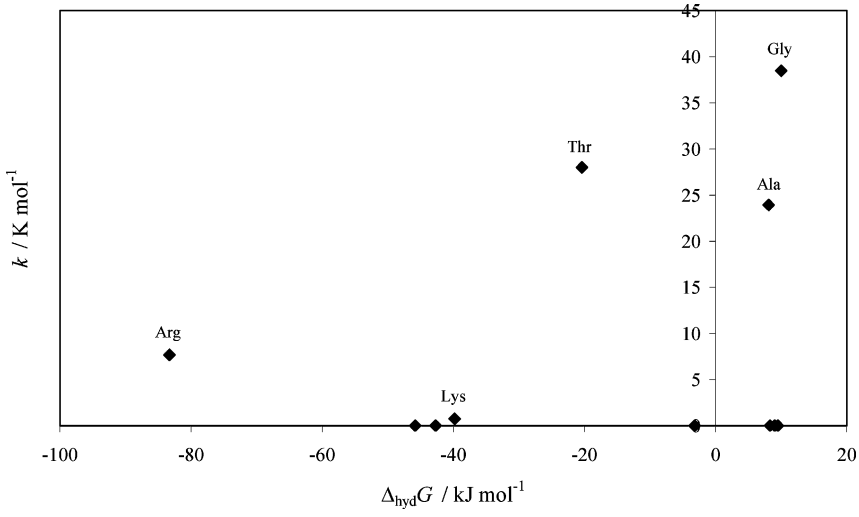

Figure 5. k parameter as a function of the molar Gibbs energy of hydration ($\Delta_{hyd}G$) of the amino acids.

The hydrophobicity of an amino acid, particularly of its side chain, has been often quantified by the measurement of $\Delta_{solv}H$/ $\Delta_{solv}G$ of the amino acids or by the enthalpy of transfer of amino acids from water to different aqueous media, $\Delta_{trs}H$. These thermodynamic properties have actually been commonly used as hydrophobicity scales and provided important information regarding the solute−solvent interactions.[35−37] As far as the current discussion is concerned, these might be helpful as well

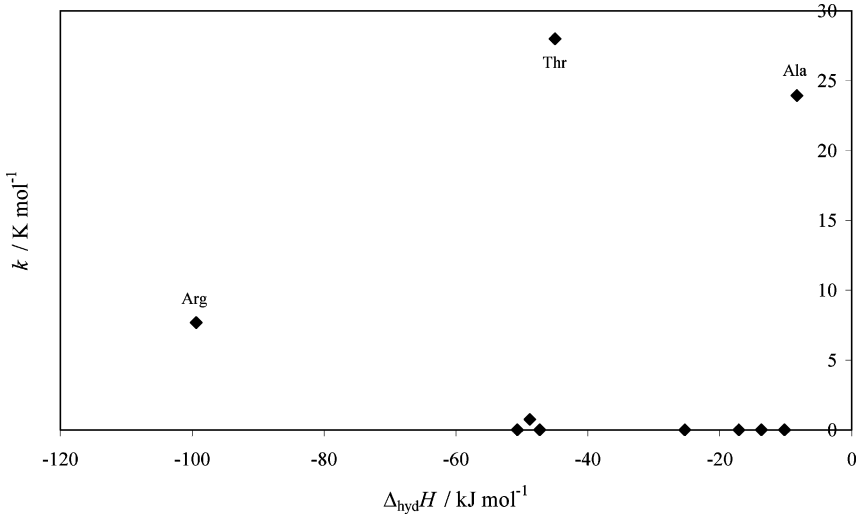

Figure 6. k parameter as a function of the molar enthalpy of hydration ($\Delta_{hyd}H$) of the amino acids.

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to elucidate some aspects of the mechanism proposed for the interaction of these biomolecules with water and ILs.

As can be seen in Figure 5, the trend observed for $\Delta_{hyd}G$ of the amino acids does not follow the order established in this work for the magnitude of salting-out effects. Actually, the more favorable hydration is that of Arg and Asp and the least of Ala and Gly, the strongest salting-out inducing amino acids. Curiously, the hydration of salting-in inducing amino acids is even more favorable than that of Gly. The trends observed in Figure 6 for $\Delta_{hyd}H$ do not reflect as well the order of solubility effects induced by the amino acids. Evidence of this type had already been found in the case of the water solubilities of the amino acids. The apparent contradiction is overcome if we assume that other interactions, besides the hydration tendency, are determining the solubility effects observed. The reasonability of such an argument can actually be supported by some literature studies on the enthalpy of transfer of amino acids from different aqueous media to water,[36,38] where the authors stress the roles of the different types of interactions established between the solute and the solvents in the transfer processes. Interestingly, results for the $\Delta_{trs}H$ of some amino acids from water to aqueous cationic surfactant solutions have inclusively been discussed in terms of a delicate balance of hydrophilic and hydrophobic interactions in the aqueous surfactant solutions and differences in the molecular structures of amino acids.[39] The surfactant concentration dependence has also been considered and explained in terms of the dominant type of interaction. Interactions between the hydrophobic groups of Ala and headgroup of the surfactant molecules, for instance, are considered determinant in the behavior of the system; the interactions of the polar groups of Thr and the ion headgroup of the surfactant molecules are also focused and pointed out as responsible for the decrease of the enthalpies of transfer with the increase of surfactant concentration. Following this reasoning, the linear and marked amino acid concentration dependence observed for the strongest salting-out inducing amino acids (Gly, Ala, and Thr) would lie on a delicate balance between the different types of interactions established: as the amino acid molality increases, both amino acid−water and amino acid−IL cation interaction become stronger, and the overall effect is a linear concentration dependence. As far as salting-in effects are concerned, the increase of amino acid molalities implies an enhanced availability of its side chains to the IL hydrophobic moieties and consequently a more pronounced decrease of IL solubilities in water.

To conclude, the results gathered in this work for amino acids inducing behaviors in water + IL mixtures constitute further evidence for the mechanism proposed for the interpretation of specific ion effects on aqueous IL solubilities but also point further to the existence of a competition between the interactions amino acid side chain−water and amino acid side chain−IL cation/anion, which, for its turn, is determined by the size, polarity, and charge distribution of the side chains of the amino acids. This feature of the mechanism, which was additionally supported by thermodynamic literature data available, was not observed in the case of ion effects since these are smaller and simpler than amino acids and do not offer the possibility of varying the size of alkyl chains.

Conclusion

The influence of the zwitterionic forms of amino acids on the mutual solubilities of $[C_4mim][C(CN)_3]$ and water was evaluated and discussed on the basis of a refined model of the molecular mechanism earlier proposed for ion specific effects on aqueous solutions of imidazolium-based ILs. The salting-in and salting-out effects observed can be understood in terms of a delicate balance between direct or indirect (water-mediated) interactions established by the amino acids and the solute, determined by the relative affinities of the biomolecule side chains to water and/or to IL ions, dependent, for their turn, on the polarity, size, and charge of the former. When hydration is preferential, as it happens with hydrophilic amino acids with absent or short alkylic chains, the salting-out phenomenon takes place. As the number of nonpolar groups present increases, the interaction with the IL cation hydrophobic moieties becomes more important and balances the interactions established with water, decreasing the magnitude of the solubility effect observed. If the side chain of the biomolecules is hydrophobic, the induced effect is salting-in. Less hydrophobic amino acids are able to form hydration complexes to a certain extent, the result being a less pronounced decrease of IL + water mutual solubilities. When the interactions water−side chain and water−IL are balanced, the amino acid has a pratically negligible solubility effect. This is observed for amino acids with non-neutral side chains, capable of establishing additional electrostatic interactions with the IL cation or anion, due to their acidic or basic character.

Since amino acids can be taken as models to reproduce the behavior of many other biochemical molecules, the mechanism

proposed might be extrapolated to the interpretation of the behavior of other (biological) systems.

In practical terms, the information on the factors and molecular-level phenomena that govern the interactions between biomolecules and ILs gathered in this work will help to establish optimal conditions for biomolecule extractions in environmentally friendly standards and will contribute to the improvement of other crucial processes in biotechnology, such as the stabilization of enzymes and their activities in ILs, constituting thus profitable knowledge susceptible of being appropriated in biochemistry, biology, and biotechnology, with possible economical impact.

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