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# Interactions of aminoacids in concentrated aqueous solutions of urea or ethanol. Implications for the mechanism of protein denaturation

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**Abstract**

Calorimetric measurements were carried out at 298.15 K on binary aqueous solutions of glycine, and on binary and ternary solutions containing the L and D forms of the α-aminoacid leucine at different concentrations of urea or ethanol as cosolvents. The derived pairwise interaction coefficients of the excess enthalpies were rationalized according to the preferential configuration model. The behaviour shown by glycine, when the concentration and the nature of the cosolvent changes, allows important observations on the influence of the medium on hydrophilic interactions. For leucine, differences were found between the values of the homochiral and heterochiral pairwise enthalpic interaction coefficients. This chiral recognition depends on the nature and concentration of the cosolvent, which influences differently hydrophilic and hydrophobic interactions. The results obtained are compared with those found for other model compounds in concentrated aqueous solutions of urea or ethanol: some comments are made on the possible mode of action of ethanol and urea as protein denaturants. 

*Keywords:* α-aminoacids; Molecular interactions; Excess functions; Mixed solvents; Calorimetry

## 1. Introduction

It is well known that, in the solid state, physical properties of racemic mixtures differ markedly from those of the pure D or L forms. This is essentially due to the different distances and topological correlations between the groups of atoms in the molecules [1]. As a consequence, all thermodynamic properties that depend on the solid state energetics, as enthalpies of solution [2], fusion [3], or solubility, must be influenced. In the presence of a solvent without chiral properties, differences between excess properties, which depend on solute–solute interactions, are likely to occur. Instead, limiting properties, which depend on solute-solvent interactions, must be identical. Differences in the energetics of interactions between L–L (or D–D) or D–L stereoisomers are usually referred to as chiral recognition [4–8], chiral selectivity [9] or chiral interaction [10]. In apolar solvents [11–13] or in DMF [10,14], chiral recognition has been clearly detected for protected α-aminoacids bearing alkyl side chains. In that case, it was attributed to the possibility of formation of dimeric peptides via H–bonds between CO and the NH group. On the contrary, many authors have been claiming that in aqueous solutions of free α-aminoacids, where dipolar ions predominate, coulombic forces screen the subtle differences in the interactions between molecules of the same or different

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chirality [9,15,16]. For these reasons attention was mainly focused on non-ionic α-aminoacids derivatives, obtaining encouraging results.

Studies from this laboratory on α-aminoacids [4,5,17,18] in aqueous solutions, have shown that the presence of dipolar ions is the main factor that determines chiral recogniton. In fact, this effect was not detected when α-aminoacids, bearing unsubstituted alkyl chains, were investigated in acidic aqueous solutions, pH 0.13, where dipolar ions disappear [19]. Moreover, when the side chains of an α-aminoacid bears a hydrophilic functional group, the synergetic action of hydrophilic and hydrophobic interactions determines a larger chiral recognition. In such a case, the effects occurring upon the failure of this interaction are more pronounced [8,17]. Then, the failure of earlier researchers must be attributed to non-systematic studies of homologous series of aminoacids bearing unsubstituted alkyl chains. On these bases, an interaction model has been proposed, different from a simple statistical approach [20]. Namely, two hydrated, interacting molecules bearing hydrophilic and hydrophobic domains, interact through a preferential configuration, stabilized by the best juxtaposition of hydrophilic and hydrophobic domains [20]. The most probable configuration maximizes favorable interactions between similar domains, while minimizing unfavorable interactions between unlike domains. For α-aminoacids the preferential configuration model has allowed to explain the chiral recognition detected in their aqueous solutions [4,5,17,18].

Urea and ethanol act as denaturing agents of proteins and reduce their thermostability. However, being the former a hydrophilic structure breaker solute and the latter a hydrophobic structure maker solute, the mechanisms through which they act must be different, and the delicate balance of the contrasting forces that determine the native conformation must be differently influenced. Many working, often contrasting hypotheses have been formulated concerning the effect of urea or ethanol on the hydrophobic interaction, which is generally assumed to be one of the main driving forces stabilizing the native conformation of proteins. In order to gain a better understanding of these problems, we are carrying out a research programme on the physical chemical properties of concentrated aqueous solutions of urea or ethanol containing model molecules, such as α-aminoacids. Here, we want to go further in the studies concerning chiral recognition by examining the enthalpic behaviour of aqueous solutions of the L and D forms of leucine in concentrated aqueous solutions of urea or ethanol, with the aim of giving a contribution to the problem of the stability of proteins as a function of the nature of the medium. Since denaturation requires high concentrations of the cosolute, in such conditions the solvent must be considered as a mixed solvent, with properties quite different from those of pure water. Glycine has also been investigated in the same experimental conditions, to get information about the behaviour of a hydrophilic solute representative of the functional group of α-aminoacids, i.e., the zwitterion.

The second coefficient of the virial expansion of the excess enthalpy as a function of molalities is an useful quantity to get information about the interaction mechanism. Through the analysis of homo- and heterochiral enthalpic interaction coefficients we aim to ascertain whether the hypothesis of preferential configurations holds for these systems, what are the various contributions acting in the interaction, and how the mixed solvent influences chiral recognition detected for leucine in water.

## 2. Experimental

Solutes employed were Sigma and Aldrich products. The purity of L- and D-leucine was assessed by the company to be greater than 98–99%. They were used without further purification. Measurements of the heats of dilution were carried out using an LKB flow microcalorimeter and a thermal activity monitor from Thermometric. Calorimeters were equipped with a GP 10 gradient programmer, a 500 μl mixing chamber, a PSV 50 electrovalve and a P3 peristaltic pump (all from Pharmacia) for the authomatic preparation and the pumping of solutions into the cells of the calorimeters. The method has been tested through known systems. Enthalpies of dilution in water of urea and hexane-1,2-diol have been determined, and the evaluated pairwise enthalpic interaction coefficients ($h_{xx} = -331 \pm 3$ J kg mol$^{-2}$ for urea and $h_{xx} = 2999 \pm 46$ J kg mol$^{-2}$ for hexane-1,2-diol) were in a very good agreement with the literature values ($h_{xx} = -350 \pm 13$ J kg mol$^{-2}$ for urea [21] and $h_{xx} = 2955 \pm 46$ J kg mol$^{-2}$ for hexane-1,2-diol [20]. The

values of the dilution enthalpies, $\Delta H_{dil}$, were obtained from:

$$\Delta H_{dil}(m_x^i \rightarrow m_x^f) = \frac{(dQ/dt)}{P_S}$$

where $(dQ/dt)$, the heat evolved or adsorbed per unit time, is normalized to the total mass flow-rate of mixed solvent per unit time, $P_S$ and $m_x^i$ and $m_x^f$ are the initial and final molalities, respectively. $\Delta H_{dil}$ is given in J $kg^{-1}$ of solvent in the final solution.

## 3. Results

Molecular interactions can be studied through the analysis of the excess thermodynamic properties, which are defined as the difference between the values of that function referred to a real and an ideal solution. Then, the excess enthalpy is obtained by:

$$H^E = H(m) - H^{id} = H(m) - (H_s^\circ - {}_x\bar{H}_s^\circ) \qquad (1)$$

where $H(m)$ and $H^E$, the absolute and the excess enthalpy respectively, both refer to 1 kg of solvent and $m_x$ moles of each solute; $H^{id}$ is the enthalpy of the ideal solution; $H_s^\circ$ is the enthalpy of 1 kg of solvent and $\bar{H}_x^\circ$ is the limiting partial molal enthalpy of each solute. According to the treatment of solution properties originally proposed by McMillan–Mayer [22] and specifically applied to those of aqueous solutions of nonelectrolytes by Kauzmann [23] and other authors [24–26], an excess thermodynamic property, of a solution containing $n$ solutes, $J^E$, can be expressed as a virial expansion of molalities of pair and higher order interaction coefficients, $j$, as follows:

$$J^E = \sum_{i=1}^{n} j_{ik} m_i m_k + \text{higher terms} \qquad (2)$$

For ternary solutions containing the x and y solutes, virial coefficients of the power series of the excess enthalpies, $h_{xy}$, as a function of molalities can be derived from the enthalpies of dilution, $\Delta H_{dil}$, as follows:

$$\begin{aligned}\Delta H_{dil}(m_x^i, m_y^i \rightarrow m_x^f, m_y^f) &= H^E(m_x^f, m_y^f) \\ &- (m_x^f/m_x^i) H^E(m_x^i, m_y^i) = h_{xx} m_x^f (m_x^f - m_x^i) \\ &+ h_{yy} m_y^f (m_y^f - m_y^i) + 2h_{xy} m_x^f (m_y^f - m_y^i) \\ &+ \text{higher terms} \end{aligned} \qquad (3)$$

where $m_x^i$, $m_y^i$, $m_x^f$, $m_y^f$ are the molalities of the x and y solutes before and after the dilution process, respectively. According to the Mc Millan–Mayer approach [22], the $h$ coefficients appearing in Eq. (3) represent the enthalpic contributions to the Gibbs free energy coefficients characterizing the interaction between pairs, triplets or higher order interactions. They implicitly account for all variations of solvent–solvent and solute–solvent interactions. Considering as solvent the mixture of ethanol-water or urea-water, a solution of the solute x can be regarded as a binary one and then Eq. (3) reduces to:

$$\Delta H_{dil}(m_x^i \rightarrow m_x^f) = h_{xx} m_x^f (m_x^f - m_x^i) + \text{higher terms} \qquad (4)$$

where $\Delta H_{dil}$ is expressed in J $kg^{-1}$ of mixed solvent, and the molalities are calculated as moles per kg of mixed solvent.

Two-solute solutions, as the D and L forms of an aminoacid, are, instead, ternary solutions which are characterized by cross-coefficients evaluated by means of an auxiliary function $\Delta H^{**}$:

$$\begin{aligned}\Delta H^{**} &= \Delta H_{dil}(m_D^i, m_L^i \rightarrow m_D^f, m_L^f) \\ &\quad - \Delta H_{dil}(m_D^i \rightarrow m_D^f) - \Delta H_{dil}(m_L^i \rightarrow m_L^f) \\ &= 2h_{DL} m_D^f (m_L^f - m_L^i) + \text{higher terms} \\ &= 2h_{DL} m_L^f (m_D^f - m_D^f) + \text{higher terms}\end{aligned}$$

where $h_{DD} \equiv h_{LL}$ and $h_{DL}$ are the homochiral and heterochiral pairwise enthalpic interaction coefficients, respectively, in the mixed solvent used. The values of the coefficients of the excess enthalpies were obtained by fitting $\Delta H_{dil}$ or $\Delta H^{**}$ by a least-squares method. The fitting was tried with polynomials of increasing degree, choosing finally one with the highest degree for which all the coefficients are significant with respect to their own 95% confidence limits. For most of the examined systems the concentration ranges studied are limited, so that only the second homochiral or heterochiral coefficients, $h_{LL}$ and $h_{DL}$, are to be determined.

Enthalpic pair interaction coefficients of glycine in concentrated aqueous solutions of urea are reported in Table 1 [27]. The coefficients increase at increasing concentrations of the cosolvent, passing from negative to positive. Homo- and heterochiral enthalpic interaction coefficients for aqueous solutions containing L

Table 1
Enthalpic pair interaction coefficients for glycine[a] in concentrated aqueous solutions of of urea, at 25°C

| $(m_U)^b$ | $(h_{xx})^b$ | $(m_U)^b$ | $(h_{xx})^c$ |
|---|---|---|---|
| 0 | $-404 \pm 9$ | 5.00 | $-15 \pm 5$ |
| 0.100 | $-334 \pm 5$ | 7.00 | $53 \pm 8$ |
| 1.00 | $-298 \pm 6$ | 9.00 | $109 \pm 3$ |
| 3.00 | $-140 \pm 5$ | | |

[a] The data reported are taken from [18].
[b] Concentration of urea, mol $kg^{-1}$.
[c] Uncertainties reported are 95% confidence limits, J kg $mol^{-2}$.

and/or D forms of leucine and various concentration of urea as cosolvent are reported in Table 2. The homochiral coefficient decreases at increasing concentration of urea, while the heterochiral coefficient shows a pronounced maximum at 3 m urea. At 7 m urea differences between the two coefficients disappear.

In Table 3, enthalpic pair interaction coefficients for aqueous solutions of glycine in concentrated aqueous solutions of ethanol are reported. At increasing concentration of ethanol, the coefficient for glycine shows an opposite behaviour with respect to that exhibited in the presence of urea; namely, it goes rapidly toward values much more negative than in water. In Table 4, the homo- and heterochiral coefficients for leucine at different concentrations of ethanol are given. The former shows a maximum, while the latter diminishes rapidly at increasing concentration of ethanol. Both coefficients change sign at 9 m ethanol. Chiral recognition detected in ethanol is opposite in sign with respect to that exhibited in urea. In Fig. 1, the enthalpic

Table 3
Enthalpic pair interaction coefficients for glycine in concentrated aqueous solutions of ethanol, at 25°C

| $(m_{EtOH})^a$ | $(h_{xx})^b$ | $(h_{xxx})^c$ | $n^d$ | c.r.[e] |
|---|---|---|---|---|
| 0 | $-404 \pm 9$[f] | – | | |
| 1.00 | $-339 \pm 10$ | – | 27 | 0.28–0.16 |
| 2.00 | $-746 \pm 18$ | – | 24 | 0.13–0.07 |
| 3.00 | $-1055 \pm 50$ | $378 \pm 78$ | 16 | 0.22–0.15 |
| 5.00 | $-1148 \pm 42$ | $737 \pm 70$ | 40 | 0.20–0.07 |
| 7.00 | $-1299 \pm 20$ | – | 24 | 0.11–0.07 |
| 9.00 | $-1693 \pm 20$ | – | 45 | 0.24–0.15 |
| 11.0 | $-2324 \pm 52$ | $1254 \pm 138$ | 27 | 0.14–0.08 |

[a] Concentration of ethanol, mol $kg^{-1}$.
[b] Uncertainties reported are 95% confidence limits, J kg $mol^{-2}$.
[c] J $kg^{-2}$ $mol^{-3}$.
[d] No. of experimental data.
[e] Concentration range.
[f] [18].

coefficients for leucine and glycine are reported as a function of the concentrations of urea (Fig. 1a) and ethanol (Fig. 1b).

## 4. Discussion

The results presented in this work aim to give an indirect contribution to the understanding of the phenomena occurring upon the denaturation of proteins by high concentrations of urea or ethanol. Comparison between the properties of solutes in water and in water-cosolvent mixtures is useful for obtaining a deeper insight into the factors promoting the unfolding of biopolymers in such solutions. It must be under-

Table 2
Homo- and heterochiral enthalpic pair interaction coefficients for leucine in concentrated aqueous solutions of urea at 25°C

| $(m_{Urea})^a$ | $(h_{LL})^b$ | $n^c$ | c.r.[d] | $(h_{DL})^b$ | $n^c$ | c.r.[d] |
|---|---|---|---|---|---|---|
| 0 | $1269 \pm 9$[e] | | | $1059 \pm 36$ | | |
| 1.00 | $1122 \pm 14$ | 20 | 0.054–0.034 | $1305 \pm 26$ | 20 | 0.046–0.034 |
| 3.00 | $957 \pm 10$ | 20 | 0.054–0.035 | $1669 \pm 36$ | 20 | 0.05–0.032 |
| 5.00 | $1153 \pm 20$ | 13 | 0.054–0.035 | $1328 \pm 36$ | 20 | 0.052–0.034 |
| 7.00 | $1116 \pm 12$ | 32 | 0.057–0.037 | $1149 \pm 22$ | 20 | 0.046–0.026 |
| 9.00 | $1177 \pm 28$ | 12 | 0.040–0.021 | $1105 \pm 50$ | 20 | 0.040–0.022 |

[a] Concentration of urea, mol $kg^{-1}$.
[b] Uncertainties reported are 95% confidence limits, J kg $mol^{-2}$.
[c] No. of experimental data.
[d] Concentration range.
[e] [4].

Table 4
Homo- and heterochiral enthalpic pair interaction coefficients for leucine in concentrated aqueous solutions of ethanol at 25°C

| $(m_{EtOH})^a$ | $(h_{LL})^b$ | $n^c$ | c.r.[d] | $(h_{DL})^b$ | $n^c$ | c.r.[d] |
|---|---|---|---|---|---|---|
| 0 | $1269 \pm 9$[e] | | | $1059 \pm 36$ | | |
| 2.00 | $1555 \pm 16$ | 16 | 0.10–0.061 | $680 \pm 70$ | 16 | 0.060–0.036 |
| 5.00 | $1332 \pm 34$ | 13 | 0.054–0.035 | $874 \pm 40$ | 20 | 0.052–0.034 |
| 7.00 | $753 \pm 13$ | 20 | 0.057–0.037 | $415 \pm 30$ | 20 | 0.048–0.032 |
| 9.00 | $-628 \pm 8$ | 16 | 0.044–0.028 | $-999 \pm 70$ | 16 | 0.026–0.016 |

[a] Concentration of ethanol, mol $kg^{-1}$.
[b] Uncertainties reported are 95% confidence limits, J kg $mol^{-2}$.
[c] Number of experimental data.
[d] Concentration range.
[e] [4].

lined that the conclusions which will be drawn about the model systems presently investigated are based only on enthalpic data: Gibbs energy data, however, would be necessary for a more satisfactory interpretation.

Non-bonding interactions in aqueous solutions of non-electrolytes have reached a good qualitative understanding from the analysis of the excess thermodynamic properties using a statistical group additivity approach [21]. The analysis of the Gibbs free energy coefficients through this approach has led to the conclusion that the interaction between similar domains, having the same effect on water structure, is thermodynamically favourable [28]. From that we hypothesized the existence of a preferential configuration in solution between two hydrated interacting molecules: this configuration allows the best juxtaposition of the hydrated functional groups and, contemporaneously, of the hydrophobically hydrated alkyl chains [20]. This working model, referred to as the 'preferential configuration model', accounts qualitatively for the sign and magnitude of the parameters used to characterize non-bonding interactions in aqueous solutions of nonelectrolytes, namely the pair coefficients of the excess thermodynamic properties [20]. The physical meaning of a pair interaction coefficient is related to the changes in the thermodynamic property when two hydrated molecules are brought from an infinite distance, where only solute-solvent interactions are operating, to a finite distance where hydrated solute-hydrated solute interactions occur. This working model was successfully employed to explain chiral recognition occurring in aqueous solutions of α-aminoacids bearing unsubstituted alkyl side chains [4,5]. Among the various favorable configurations of two interacting molecules having the same or different chirality, the one juxtaposing linearly the charged groups of zwitterions is supposed to prevail. This side-on configuration, that well juxtaposes also hydrophobic domains, should mainly contribute to building up the pairwise homo or heterochiral coefficients. Significant differences between homochiral and heterochiral interactions must not be expected. On the contrary, the configuration responsible of chiral recognition should be that juxtaposing the four electrical charges simultaneously [4,5,8,17,18]. When two hydrated, interacting molecules have the same chirality, the side chains lye in the same half-space determined by the imaginary plane containing the charged groups. The opposite occurs when molecules of different chirality are involved. The two configurations determine different interactions between the side chain residues. This is obviously an extreme representation and many intermediate cases must necessarily exist. When chiral recognition is not detected in water, using a cosolvent able to influence differently the intensity of hydrophilic and hydrophobic interactions should allow to detect that effect.

The present study aims at verifying the effect of urea or ethanol on the chiral recognition exhibited by leucine in water. The enthalpic interaction coefficients for the aminoacid in water are positive, thus, indicating a typical behaviour of prevailingly hydrophobic solutes [4]. In concentrated aqueous solutions of urea, the homochiral coefficient undergoes a flat decrease: then, in the homochiral interaction, the attenuation of hydrophobic interactions throughout the entire range

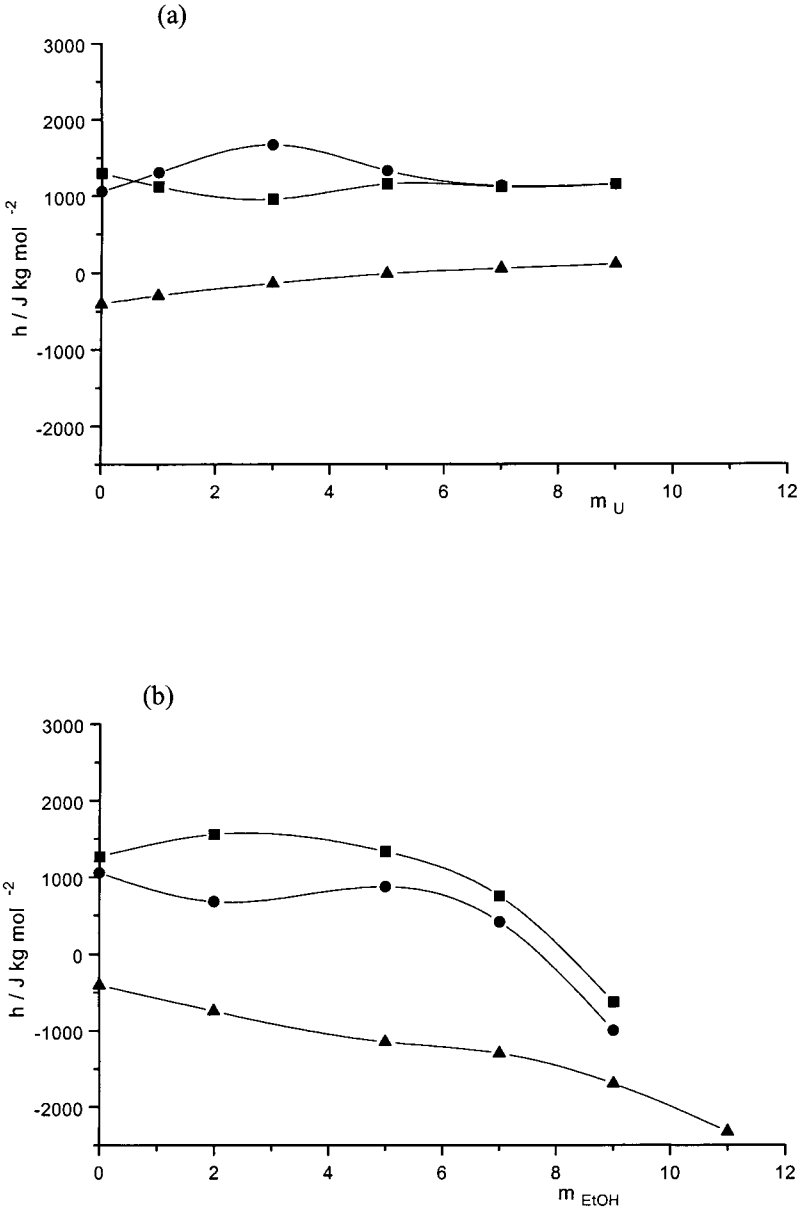


Fig. 1. a Homochiral (■) and heterochiral (●) enthalpic interaction coefficients for leucine and for glycine (Δ) as a function of urea molality, $m_U$ , and *b*) as a function of ethanol molality, $m_{EtOH}$.

of concentration of urea is the prevailing effect. The same occurs for alkan-1-ols [29], alkan-m,n-diols [29], N-acetylamides of α-aminoacids in 7 mol $l^{-1}$ [30] or 8 mol $l^{-1}$ urea [31], and amides in 6 mol $l^{-1}$ guanidinium chloride [32]. In contrast, the heterochiral coefficient becomes at first more positive, then decreases (Table 2, Fig. 1a), and at 9 mol $kg^{-1}$ of urea, it reaches the same value as in water. This marked variability indicates that the action of urea on the hydrophilic domains is larger for the heterochiral pair, which is less stabilized by hydrophobic interactions, compared to the homochiral pair. At low concentration

of urea, the attenuation of hydrophilic interactions is the prevailing effect: at higher concentration of cosolvent, the attenuation of hydrophobic interactions makes the coefficient to diminish, reaching almost the same value as in water. The different trends of the coefficients determine a chiral recognition, which disappears at high concentrations of urea (about 7 mol $kg^{-1}$). In these conditions, the interaction between zwitterions becomes almost athermal, as unravelled in a preceding study on α-aminoacids in concentrated aqueous urea [27]. There, evidence was given that the contribution of the functional group varies from a negative to an almost null value, underlining a transition towards a thermochemically unfavorable behavior at increasing urea concentration. The same features are shown by glycine in urea, whose literature coefficients are reported in Table 1 [27] and Fig. 1a. That behaviour could be due partially to the increased dielectric constant of the medium determined by urea, an effect reducing the electrostatic interactions between the functional groups, and then their contribution in forcing interactions between hydrophobic domains. The consequent loss of enthalpic favourable contributions determines a reduced ability of imposing a configuration leading to chiral recognition, hence, the disappearance of the last effect. The large values of the enthalpic coefficients, comparable to those obtained in pure water, indicate that water released to the bulk undergoes a large enthalpic and entropic change, being the final state (bulk) less structured than pure water for the presence of urea. To conclude, while zwitterions become 'inactive' towards chiral recognition, hydrophobic interactions are still effective, even in concentrated urea (7–9 mol $kg^{-1}$), thus, determining identical and positive values for the homo and heterochiral coefficients. The same phenomenon occurs in 1 mol $l^{-1}$ HCl, where this aminoacid does not present chiral recognition because of the absence of the zwitterion [19].

The behaviour shown by glycine in concentrated aqueous solutions of ethanol evidentiates that the nature of the medium greatly influences the strength of non-bonding interactions. As reported in Table 3 and Fig. 1b, at increasing ethanol concentration, the coefficients become much more negative than in pure water. Ethanol, less polar than water, lowers the dielectric constant of the medium potentiating hydrophilic and, in particular, electrostatic interactions, which give a negative contribution to the enthalpy. For leucine, the change in the structure of the solvent and the decrease of the dielectric constant determine two opposite effects. Hydrophilic interactions improve, and the consequent enhanced cooperativity of hydrophobic interactions between the alkyl side chains determine a higher homochiral coefficient and a lower heterochiral one (Table 4, Fig. 1b). On the other hand, ethanol, a well known structure maker solute promotes the ice-like structure of liquid water. Then, its presence as cosolvent lowers the energetic level of the bulk, and solvent molecules released upon pairwise interaction, relaxing from the hydration cospheres to a more structured medium, undergo a smaller enthalpic and entropic change than in the presence of urea. At increasing concentrations of ethanol that leads both coefficients to be smaller than those obtained in water, and determines the inversion of their sign at $m_{EtOH} > 7$ mol $kg^{-1}$ of ethanol (see Table 4). These results agree with those for diols in highly concentrated aqueous solutions of ethanol (9 mol $kg^{-1}$) [33]. There, a new phenomenological behaviour occurs: diols, typical hydrophobic structure maker solutes ($h_{xx} > 0$ in water), are described by negative enthalpic pairwise coefficients, which usually characterize typical hydrophilic structure breaker solutes ($h_{xx} < 0$ in water). Since the hydrophobic interation is commonly considered as entropically driven because $Ts_{xx} > h_{xx} > 0$ in water, then, at increasing concentration of cosolvent, the driving force must change from entropic to enthalpic [33]. In some way, when the increase in the structure of the medium becomes relevant, diols behave as hydrophobic structure breaker solutes. For leucine, the very different trends of homo and heterochiral coefficients determine a chiral recognition constantly more relevant than in pure water, especially at low concentrations of ethanol. Since the heterochiral coefficient behaves qualitatively as that for glycine in the same conditions, then it is representative of a system in which hydrophilic interactions play a major role. In our opinion, this is a strong evidence of the presence of the configuration stabilized by the interaction between zwitterions and with the alkyl chains laying in two different half-spaces. On the contrary, the homochiral coefficient presents the same trend as for diols, namely solutes whose hydrophobic interactions are forced by the enhanced hydrophilic interactions. As a conclu-

sion, the study in mixed solvents confirms the validity of the preferential configuration model used to explain the results obtained in water.

The picture provided here has strong implications with the mechanism of chemical denaturation of globular proteins induced by concentrated aqueous solutions of urea or ethanol. The delicate balance of intra- and inter-molecular interactions responsible for the only biologically active conformation in water is modified at increasing concentration of a denaturant. The high concentrations required for urea or ethanol to be effective indicate that they are non-specific agents, which probably operate through different mechanisms on the various elements of macromolecular structures. For globular proteins, many working hypotheses have been formulated concerning the effect of urea on the hydrophobic interaction [34], which is generally assumed to be one of the main driving forces stabilizing the native conformation [35,36]. However, doubts have been raised about the major importance of that interaction: it has been shown that hydrophilic interactions are highly directional, they depend on the specific sequence of the aminoacids along the chain, and they are specific to the solvent. Then, they are as important as hydrophobic interactions in highly specific processes such as protein association, protein folding and molecular recognition [37–39]. The strong repulsion between apolar residues and peptide backbone (CONH-phobicity) should also be taken into account [40]. The knowledge of the enthalpic coefficients relative to model systems of biological interest, in pure water [20], in concentrated urea [27,29] and in ethanol [33], together with the results reported by other authors [31,32], allows us to make some considerations on the possible mode of action of these chemical denaturants on globular proteins. It must be clearly underlined that our approach is tentative and oversimplified, since only enthalpic information is available. When interacting with a substance containing both polar and hydrophobic groups, urea solvates preferentially the polar groups, being positive the contribution to the Gibbs free energy of the mixed urea-alkyl chain interaction [20,28]. Then, it competes with water in solvating the hydrophilic peptide groups on the surface of globular proteins. These favourable intermolecular interactions disrupt the intramolecular peptide-peptide hydrogen-bonds: at the same time, also hydrophobic interactions are attenuated by the presence of that amount of urea. The protein swells exposing the hydrophobic residues to water, and the penetration of water into the interior destabilizes the compact native conformation causing denaturation. This view is in agreement with the findings coming from molecular dynamics simulation studies [41]. On the other hand, the denaturation mechanism provoked by high concentrations of alcohols and glycols [42] must be necessarily different from that acting in the presence of urea. For diols, evidences have been given that, in highly concentrated aqueous ethanol, the solvation cospheres are ‘ethanolated’ [33]. Namely, the alkyl residues tend to interact with ethanol, and interaction between molecules of the same species are attenuated. Then, for a protein in the same experimental conditions, it can be envisaged that the hydrophobic core, hidden in water, could adapt well to the solvent medium, and the denaturation determined mainly by hydrophobic interactions of the alkyl residues with the cosolvent.

Work is in progress concerning the influence of other cosolvents on the interactions in the aqueous solutions of model molecules of biological interest. Among these cosolvents, saccharides are especially interesting, because they act as stabilizers of the native conformation of a protein against thermal denaturation.

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## References

[1] G. Barone, P. Cacace, G. Castronuovo, V. Elia, J. Solution Chem. 13 (1984) 625.
[2] M. Matsumoto, K. Amaya, Bull. Chem. Soc. Japan 56 (1983) 2521.
[3] S. Miyagishi, S. Matsumara, K. Murata, T. Asakawa, M. Nishida, Bull. Chem. Soc. Japan 58 (1985) 1019.
[4] G. Barone, G. Castronuovo, P. Del Vecchio, V. Elia, S. Puzziello, J. Solution Chem. 18 (1989) 1105.
[5] G. Castronuovo, V. Elia, M. Magliulo, Can. J. Chem. 69 (1991) 794.
[6] G. Barone, G. Castronuovo, V. Elia, C. Giancola, J. Thermal Anal. 30 (1985) 1367.

[7] G. Barone, G. Castronuovo, P. Del Vecchio, V. Elia, C. Giancola, Thermochim. Acta 122 (1987) 105.
[8] S. Andini, G. Castronuovo, V. Elia, F. Velleca, J. Solution Chem. 24 (1995) 485.
[9] G.M. Blackburn, T.H. Lilley, P.J. Milburn, J. Chem. Soc. Chem. Comm., 1985, 299.
[10] A.H. Sijpkes, G. Somsen, J. Chem. Soc., Faraday Trans. 86 (1990) 1461.
[11] M.T. Cung, M. Marraud, J. Néel, Biopolymers 15 (1976) 2081.
[12] M.T. Cung, M. Marraud, J. Néel, Biopolymers 16 (1977) 715.
[13] M.T. Cung, M. Marraud, J. Néel, A. Aubry, Biopolymers 17 (1978) 1149.
[14] A.H. Sijpkes, G. Somsen, J. Chem. Soc., Faraday Trans. I 85 (1989) 2563.
[15] M.K. Kumaran, G.R. Hedwig, I.D. Watson, J. Chem. Thermodyn. 14 (1985) 93.
[16] E.J. Cohn, T.J. Edsall, Proteins, Aminoacids and Peptides, Reinhold, New York, 1943.
[17] G. Castronuovo, V. Elia, F. Velleca, J. Solution Chem. 24 (1995) 1209.
[18] G. Castronuovo, V. Elia, F. Velleca, J. Solution Chem. 25 (1996) 51.
[19] G. Castronuovo, V. Elia, C. Giancola, S. Puzziello, J. Solution Chem. 19 (1990) 855.
[20] G. Castronuovo, V. Elia, F. Velleca, Curr. Top. Solution Chem. 2 (1997) 125.
[21] J.J. Savage, R.H. Wood, J. Solution Chem. 5 (1976) 733.
[22] W.G. Mc MillanJr., J.E. Mayer, J. Chem. Phys. 13 (1945) 276.
[23] J.J. Kozak, W.S. Knight, W. Kauzmann, J. Chem. Phys. 48 (1968) 675.
[24] H.L. Friedman, C.V. Krishnan, J. Solution Chem. 2 (1973) 119.
[25] F. Franks, M.D. Pedley, D.S. Reid, J. Chem. Soc., Faraday Trans. I 72 (1976) 359.
[26] T.H. Lilley, in: M.N. Jones (Ed.), Biochemical Thermodynamics, Elsevier, Amsterdam, 2nd edn., 1988, pp. 1–52.
[27] S. Andini, G. Castronuovo, V. Elia, A. Pignone, F. Velleca, J. Solution Chem. 25 (1996) 837.
[28] B.Y. Okamoto, R.H. Wood, P.T. Thompson, J. Chem. Soc., Faraday Trans. I 74 (1978) 1990.
[29] G. Castronuovo, G. d'Isanto, V. Elia, F. Velleca, J. Chem. Soc., Faraday Trans. 92 (1996) 3087.
[30] G. Barone, C. Giancola, Pure Appl. Chem. 62 (1990) 57.
[31] A.H. Sijpkes, G. Somsen, T.H. Lilley, J. Chem. Soc., Faraday Trans. 86 (1990) 2943.
[32] G.R. Hedwig, T.H. Lilley, H. Linsdell, J. Chem. Soc., Faraday Trans. 87 (1991) 2975.
[33] G. Castronuovo, V. Elia, V. Moniello, S. Perez Casas, F. Velleca, Phys. Chem. Chem. Phys. 1 (1999) 000.
[34] W. Kauzmann, Adv. Protein Chem. 14 (1959) 1.
[35] W. Kauzmann, Nature London 325 (1987) 763.
[36] R.L. Baldwin, Proc. Natl. Acad. Sci. USA 83 (1986) 8069.
[37] A. Ben-Naim, Biopolymers 29 (1990) 567.
[38] K.R. Shoemaker, P.S. Kim, E.J. York, J.M. Stewart, R.L. Baldwin, Nature London 326 (1987) 563.
[39] M. Vasquez, M.R. Pincus, H.A. Sheraga, Biopolymers 26 (1987) 351.
[40] M. Bloemendal, Y. Marcus, A.H. Sijpkes, G. Somsen, Int. J. Peptide Protein Res. 34 (1989) 405.
[41] A. Wallqvist, D.G. Covell, D. Thirumalai, J. Am. Chem. Soc. 120 (1998) 427.
[42] T.H. Herskovits, B. Gadegbeku, J. Jaillet, J. Biol. Chem. 245 (1970) 2588.