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Calorimetric studies of hydrophobic interactions of alkanols in concentrated aqueous solutions of glucose Implications for the mechanism of protein stabilization by sugars

Giuseppina Castronuovo*, Vittorio Elia, Marcella Niccoli, Filomena Velleca

Department of Chemistry, University Federico II of Naples, Complesso Universitario Monte S. Angelo, via Cintia, 80126 Naples, Italy

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Abstract

Calorimetric measurements were carried out at 298 K on concentrated aqueous solutions of glucose, ranging from 0 to 5 mol kg^{-1}, containing alkan-1-ols, alkane-1,2-diols and alkane-α,ω-diols from C_2 to C_7. The purpose of this study is to obtain more information about the influence of glucose on hydrophobic hydration and interactions. The pair-wise interaction coefficients of the virial expansion of the excess enthalpies were evaluated, and the results rationalized according to the preferential configuration model. At increasing glucose concentration, alkanols and 1,2-diols maintain almost unaltered the coefficients they have in water, while α,ω-diols show a smooth decrease only at 3 mol kg^{-1} glucose. Urea and glycine have been studied in concentrated glucose, too. Their coefficients indicate that glucose is able to reduce hydrophilic interactions. Then, the invariance of the coefficients for alkanols means that both hydrophilic and hydrophobic interactions are attenuated in the presence of high concentrations of the cosolvent. The data show that the nature of the hydration cosphere of the alkyl chain does not change, so that the same interaction mechanism operates in water and in concentrated glucose solutions. The results obtained are compared with those found for diols in concentrated solutions of urea and ethanol: some comments are made on the possible mode of action of these substances on the stability of proteins in solution.

Keywords: Excess enthalpies; Aqueous mixed solvents; Hydrophobic interactions; Preferential configuration model; Protein stabilization

1. Introduction

The folding, structural stability, and dynamics of globular proteins are thought to be extensively controlled by the interactions of the macromolecule with water. Various added substances affect these interactions and consequently alter the structural stability of proteins. Many investigations have shown that polyhydric alcohols and sugars increase the thermal stability of proteins or reduce the extent of denaturation by other reagents [1–5]. Some authors correlate the stabilizing effect of sugars with the number and positions of hydroxyl groups. In other cases, the preferential hydration of proteins, a consequence of the increase in the surface free energy of water, is indicated as the origin of the stabilizing effect of some sugars and polyols [6–9].

The thermodynamic properties associated with the stabilization process in the presence of a large amount of sugars are difficult to interpret, because of the large

* Corresponding author. Tel.: +39-81674239; fax: +39-81674090.
E-mail address: castronuovo@chemistry.unina.it (G. Castronuovo).

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number of interactions that can occur and which contribute to the overall thermodynamic properties of the protein in each state. Studies on simple compounds that model some specific aspect of a protein can provide estimates of the contributions from particular functional groups on the protein. We are interested in gaining information about the mechanism underlying the denaturation process of proteins in aqueous solutions, especially about modifications of hydrophobic interactions induced by nonelectrolytes. These last substances have been phenomenologically classified as: (a) hydrophobic structure makers ($g_{xx} < 0, h_{xx} > 0$ e $Ts_{xx} < 0$); (b) hydrophilic structure breakers ($g_{xx} < 0, h_{xx} < 0$ e $Ts_{xx} > 0$); (c) hydrophilic structure makers ($g_{xx} > 0, h_{xx} > 0$ e $Ts_{xx} > 0$), where g_{xx}, h_{xx}, and s_{xx} are the pair-wise interaction coefficients of the excess Gibbs free energy, enthalpy and entropy, respectively [10,11]. Hydrophobic structure makers promote ice-like structure of water through a mechanism which excludes direct solute–solvent hydrogen bonds, while hydrophilic structure makers promote structuring of the solvent for the increased solute–solvent hydrogen bonds. On the contrary, hydrophilic structure breakers reduce the total content of hydrogen bonds for the enhancement of solute–solvent interactions and the simultaneous attenuation of the solvent–solvent ones. Hydrophobic structure makers and hydrophilic structure breakers act as denaturing agents of proteins, reducing their thermostability. However, 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.

In recent papers, we presented the results of studies about the effect of concentrated urea or concentrated ethanol on the behavior of aqueous solutions of α-amino-acids [12] and hydroxylated substances [13,14]. The conclusion was that urea reduces both hydrophobic and hydrophilic interactions. The number of water molecules in the hydration cosphere of the alkyl chain is reduced, but the nature of the cosphere does not change, so that water still plays the major role in the pair interaction. Instead, the presence of the cosolvent ethanol, which lowers the relative permittivity of the solvent medium, enhances the forcing action of hydrophilic interactions, making the hydrophobic interactions more cooperative than in pure water.

In the present paper, we report a study of the enthalpic behavior of aqueous solutions of alkan-1-ols, alkane-1,2-diols, alkane-α,ω-diols, glycine and urea in concentrated aqueous solutions of glucose. The behavior of glycine and urea allows to analyze the effect of glucose on hydrophilic interactions and then to account for their importance on the overall interaction between two hydrated molecules. Alkylated substances are model molecules that mimic the alkyl side chains of amino acid residues in proteins: understanding the effect of a sugar on their side chains can give a further insight into the problems related to the stability of proteins as a function of the nature of the medium. Since denaturation or thermal stabilization of a protein requires high concentration of the cosolute, in such conditions the solvent must be considered as a mixed solvent with properties quite different from those of pure water. Through the present work, we want to get information on how such a mixed solvent will modify like solute-like solute interactions also on the basis of the results obtained for mixed (like solute-unlike solute) interactions in water between hydroxylated compounds and glucose [15].

2. Experimental

Alkanols and diols were Sigma and Aldrich products of the highest commercially available purity and were used without further purification. Water used to prepare solutions was glass-distilled and filtered on millipore, and aqueous solutions of glucose were mass-prepared. Measurements of heats of dilution were carried out using a Thermal Activity Monitor from Thermometric. Solutions of different concentrations were prepared automatically starting from a concentrated original solution using a GP 10 gradient programmer, a 500-μl mixing chamber, a PSV 50 electrovalve and a P3 peristaltic pump from Pharmacia, for automatic preparation and pumping of solutions into the cells of the calorimeter. 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 pair-wise 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 agreement with the literature values ($h_{xx} = -350 \pm 13$ J kg mol^{-2} for urea [16] and

$h_{xx} = 2955 \pm 55$ J kg mol^{-2} for hexane-1,2-diol [17]). Dilution enthalpies, $\Delta_{dil}H$, were obtained from:

$$\Delta_{dil}H = \frac{(dQ/dt)}{P_s}$$

where (dQ/dt), the heat evolved or absorbed per unit time, is normalized to the total mass flow-rate of mixed solvent per unit time, P_s. $\Delta_{dil}H$ is expressed 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. According to the treatment of solution properties originally proposed by McMillan and Mayer [18] and specifically applied to those of aqueous solutions of nonelectrolytes by other authors [19–22] an excess thermodynamic property, J^E, of a solution containing n solutes, can be expressed as a virial expansion of molalities of pair and higher order interaction coefficients, j, as follows:

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

In the case of the excess enthalpy for binary and ternary solutions, the virial coefficients can be easily obtained from the dilution enthalpy, $\Delta_{dil}H$, which is related to the corresponding excess enthalpy, H^E, as follows:

$$\Delta_{dil}H = H^E(m_x^f, m_y^f, \ldots) - \left(\frac{m_x^f}{m_y^f}\right) H^E(m_x^i, m_y^i, \ldots) \quad (2)$$

where x, y, ... are the solutes, and m_x^f and m_x^i are the molalities of the solute x after and before the dilution process, respectively. After the substitution of Eq. (2) in Eq. (1), the following relation is obtained:

$$\Delta_{dil}H = \sum_x \sum_y h_{xy} m_x^f m_y^f = \left(\frac{m_x^f}{m_x^i}\right)\left(\sum_x \sum_y h_{xy} m_x^f m_y^f\right) + \cdots \quad (3)$$

According to the McMillan and Mayer approach [18], 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 also for all variations of solvent–solvent and solute–solvent interactions. Since glucose is a cosolvent, each glucose solution can be regarded as a binary one, therefore Eq. (3) reduces to:

$$\Delta_{dil}H = h_{xx} m_x^f (m_x^f - m_x^i) + \text{higher terms} \quad (4)$$

The values of the coefficients of the dilution enthalpies were obtained by fitting $\Delta_{dil}H$ by a least-squares method. The fitting was tried with polynomials of increasing degree, choosing finally the one of the highest degree for which all the coefficients are significant with respect to their own 95% confidence limits. For all systems examined the concentration ranges studied were limited, so that only the second enthalpic interaction coefficients are necessary to best-fit the experimental data.

The pair-wise enthalpic interaction coefficients for alkan-1-ols, alkane-1,2-diols and alkane-α,ω-diols in water and at increasing concentrations of glucose at 298 K are given in Tables 1–3, respectively, together with their own 95% confidence limits. Whatever the concentration of glucose (up to 5 mol kg^{-1}), for all series of substances, dilution is an exothermic process: therefore, all the coefficients are positive. For alkan-1-ols, the terms with the shorter alkyl chains up to butan-1-ol show a coefficient that increases at increasing glucose concentration. Instead, pentan-1-ol and hexan-1-ol exhibit a constant value of the coefficients up to 1 mol kg^{-1} glucose: afterwards, they show a decrease. Enthalpic pair coefficients for alkane-1,2-diols are almost invariant up to 3 mol kg^{-1} glucose. Only at 5 mol kg^{-1} glucose 1,2-hexandiol shows a significant decrease of the enthalpic coefficients. For the longer terms of alkane-α,ω-diols, the decrease in the coefficients is more evident starting from 3 mol kg^{-1} glucose. An overall picture of the behavior of the investigated substances at increasing glucose concentration is shown in Fig. 1.

In Tables 4 and 5, the pair-wise and triplet enthalpic interaction coefficients are reported for glycine and urea in glucose solutions. The coefficients are negative and tend toward less negative values at increasing concentration of glucose. This trend resembles that

Table 1
Pair-wise enthalpic interaction coefficients, h_{xx}[a], of alkan-1-ols in water and in aqueous solutions of glucose at various concentrations ($m_{glucose}$), at 298 K

	$m_{glucose}$[b]			
	0	1.0	3.0	5.0
Ethanol	243 ± 10[c]	261 ± 5	322 ± 6	–
1-Propanol	559 ± 19[c]	619 ± 7	710 ± 11	–
1-Butanol	1003 ± 15[c]	1325 ± 22	1356 ± 24	–
1-Pentanol	2159 ± 56	2128 ± 54	1965 ± 116	–
1-Hexanol	2401 ± 70[d]	2354 ± 20	2133 ± 192	1423 ± 119

[a] Units: J kg mol^{-2}. Errors reported represent the 95% confidence limits.
[b] mol kg^{-1}.
[c] From [21].
[d] From [13].

Table 2
Pair-wise enthalpic interaction coefficients, h_{xx}[a], of alkane-1,2-diols in water and in aqueous solutions of glucose at various concentrations ($m_{glucose}$), at 298 K

	$m_{glucose}$[b]			
	0	1.0	3.0	5.0
Ethanediol	415 ± 30[c]	350 ± 14	311 ± 4	–
1,2-Propanediol	589 ± 1[d]	570 ± 36	627 ± 24	–
1,2-Butanediol	923 ± 5[d]	1008 ± 72	1026 ± 38	–
1,2-Pentanediol	1777 ± 30[e]	1864 ± 92	1732 ± 36	–
1,2-Hexanediol	2955 ± 55[e]	3175 ± 108	3035 ± 82	2518 ± 28

[a] Units: J kg mol^{-2}. Errors reported represent the 95% confidence limits.
[b] mol kg^{-1}.
[c] From [22].
[d] From [23].
[e] From [17].

Table 3
Pair-wise enthalpic interaction coefficients, h_{xx}[a], of alkane-α,ω-diols in water and in aqueous solutions of glucose at various concentrations ($m_{glucose}$), at 298 K

	$m_{glucose}$[b]			
	0	1.0	3.0	5.0
1,3-Propanediol	523 ± 9[c]	488 ± 10	424 ± 6	–
1,4-Butanediol	787 ± 2[c]	704 ± 18	786 ± 58	–
1,5-Pentanediol	1335 ± 25[c]	1226 ± 32	1047 ± 36	–
1,6-Hexanediol	2222 ± 20	2262 ± 42	1896 ± 54	1380 ± 11
1,7-Heptanediol	4017 ± 84[d]	3952 ± 68	3266 ± 54	–

[a] Units: J kg mol^{-2}. Errors reported represent the 95% confidence limits.
[b] mol kg^{-1}.
[c] From [23].
[d] From [24].

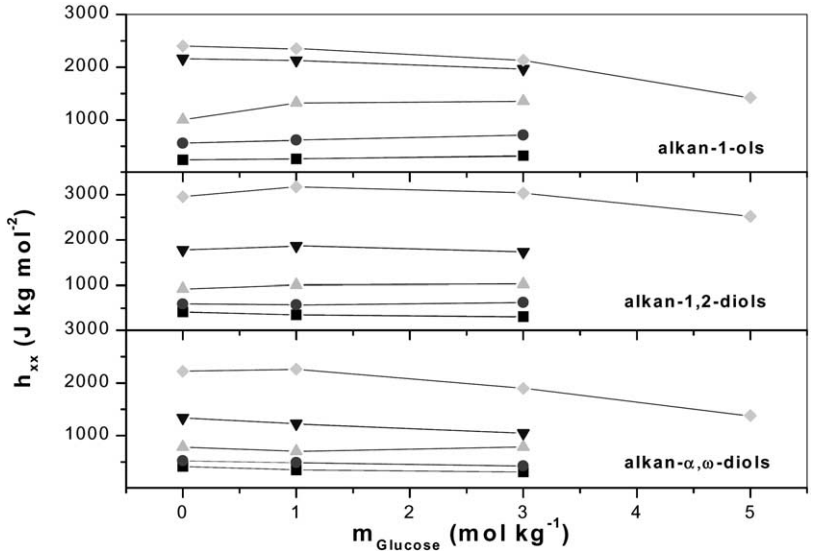

Fig. 1. Enthalpic interaction coefficients, h_{xx}, as a function of glucose concentration, $m_{glucose}$ (mol kg^{-1}), for alkan-1-ols, alkane-1,2-diols, and alkane-α,ω-diols at 298 K. The symbols refer to the length of the alkyl chain of the alkanols: C_6 (●); C_5 (▼); C_4 (▲); C_3 (●); C_2 (■).

Table 4
Pair-wise and triplet enthalpic interaction coefficients, h_{xx}[a] and h_{xxx}[b], of glycine in water and in aqueous solutions of glucose at various concentrations ($m_{glucose}$) at 298 K

	$m_{glucose}$[c]							
	0	0.20	0.40	0.60	1.0	3.0	5.0	7.0
h_{xx}	−404 ± 9[d]	−413 ± 14	−408	−362 ± 20	−339 ± 10	−206 ± 14	−46 ± 1	−35 ± 2
h_{xxx}	–	205 ± 50	165 ± 18	128 ± 26	–	80 ± 24	–	–

[a] Units: J kg mol^{-2}. Errors reported represent the 95% confidence limits.
[b] J kg^2 mol^{-3}.
[c] mol kg^{-1}.
[d] From [12].

Table 5
Pair-wise and triplet enthalpic interaction coefficients, h_{xx}[a] and h_{xxx}[b] of urea in water and in aqueous solutions of glucose at various concentrations ($m_{glucose}$), at 298 K

	$m_{glucose}$[c]						
	0	0.20	0.40	0.60	1.0	3.0	5.0
h_{xx}	−350 ± 13[d]	−399 ± 28	−379 ± 12	−364 ± 16	−344 ± 14	−181 ± 4	−85 ± 4
h_{xxx}	–	94 ± 50	64 ± 18	68 ± 23	185 ± 34	–	–

[a] Units: J kg mol^{-2}. Errors reported represent the 95% confidence limits.
[b] J kg^2 mol^{-3}.
[c] mol kg^{-1}.
[d] [16].

obtained in a preceding study for the same solutes in the presence of urea as a cosolvent [12].

4. Discussion

The results presented in this work aim to give a contribution to the understanding of the phenomena occurring upon the stabilization of proteins by high concentrations of glucose. Comparison between the interactive properties of solutes, such as alkanols, in water and in glucose–water mixtures is useful for obtaining a better understanding of the factors promoting the stabilization of biopolymers in such solutions. However, Gibbs energy data would be necessary for a more satisfactory interpretation of systems containing concentrated glucose.

According to a classification previously given, saccharides are hydrophilic structure makers ($h_{xx} > g_{xx} > 0$), where the positive sign of the virial coefficient of the excess Gibbs free energy, g_{xx}, suggests that the contributions to the excess free energy of sugar–sugar interactions are unfavorable. That supports the hypothesis that solute–solute interactions are screened by the more favorable sugar–water interactions, leading to a very stable hydration cosphere [10,11]. The signs and values of the second coefficients for saccharides must be attributed mainly to a rearrangement of water in the hydration cospheres, a process involving little changes in the energy and degree of freedom for water released from the more structured hydration cospheres of the saccharides to the bulk. Analysis of the enthalpic cross-interaction coefficients for the interaction of monosaccharides interacting with hydrophilic, destructuring solutes as urea and biuret led to the proposal of a more complex model for the hydration of saccharides [25]: besides, the cage hydration stabilized by the hydroxyl groups, a distorted domain exists in their hydration cosphere, due to the geometry of the hemiacetalic oxygen, which orients the water molecules in a manner not compatible with the tetrahedral arrangement. Hence, the interaction of pyranoses with water is a complex interaction in which the ordered arrangement of water molecules promoted by the hydroxyl groups is disturbed by the hydration of the other polar group: part of the cosphere fluctuates between an ordered and a distorted arrangement. The release of water from the distorted domain of the sugar cosphere should be the main factor determining the signs and values of the enthalpic cross coefficients in the presence of hydrophilic, destructuring solutes.

The data shown in Tables 4 and 5, relative to the self-interaction of glycine and urea in concentrated glucose, indicate that the interaction between the hydrophilic domains is modified by the presence of the cosolvent glucose: it becomes thermochemically less favorable. This is a rather unexpected result, since hydrophilic interactions should be strengthened in a medium of lower permittivity as glucose solutions: the enthalpic coefficients of hydrophilic solutes as glycine and urea should become more negative at increasing concentration of glucose as it occurs in the presence of ethanol as a cosolvent [26]. Actually, the coefficients tend towards positive values, indicating that the effect of glucose is to reduce the hydrophilic, destructured cosphere of glycine and urea. The hypothesis is that an interaction occurs between the destructured domain of glucose and the hydrophilic substance, or that glucose subtracts water when increasing its concentration. The consequence would be the same in either case, namely the presence of high concentrations of glucose provokes attenuation of hydrophilic interactions. The same effect is shown by glycine in concentrated urea: the interaction becomes thermochemically less favorable, probably for the increased hindrance of the functional group prevailingly solvated by urea [12].

The overall, most important feature of the self-interaction of alkan-1-ols, alkane-1,2-diols and alkane-α,ω-diols in concentrated glucose is that the three series of substances maintain almost unaltered the coefficients they have in water. This invariance is an indication that the attenuation of hydrophilic interactions is accompanied by the simultaneous attenuation of the hydrophobic ones. However, some minor but significant modulations in the values of the coefficient were detected, which depend on the particular series considered. In concentrated glucose, the shorter terms of alkan-1-ols are characterized by coefficients increasing as respect to those in water, while pentan-1-ol and hexan-1-ol vary slightly with the concentration of cosolvent. In fact, only in 5 mol kg^{-1} glucose hexan-1-ol shows a significant decrease in the coefficient. It can be envisaged that for the shorter terms, at increasing glucose concentration, the variation of the hydrophilic contribution to the coefficient prevails with the consequent enhancement of the coefficient. For the longer

terms, the coefficients are the same as those in water, probably because the gain of the positive contribution coming from the attenuated hydrophilic interactions is compensated by the attenuation of hydrophobic interactions between the alkyl residues. For alkane-1,2-diols and for alkane-α,ω-diols, coefficients are the same in water and in 1 mol kg^{-1} glucose, and then significantly decrease for the α,ω-isomers at 3 mol kg^{-1} glucose. Probably be presence of the two hydroxyl groups at both ends of the molecule causes attenuation of hydrophobic interactions, thus providing a smaller positive contribution to the coefficient.

For hydroxylated substances in water, the analysis of the enthalpic self-interaction coefficients indicated that two interacting molecules prefer to be oriented in a ''side-on'' configuration, stabilized by the maximum number of preferred interactions between groups having the same action on water structure (hydrophobic–hydrophobic, hydrophilic–hydrophilic) [11]. Mixed interactions (hydrophilic–hydrophobic) are unfavored, in agreement with free energy parameters which indicated that cross-interaction is unfavored compared with homogeneous ones [27]. Then, other configurations, in which hydrophilic–hydrophobic interactions could occur, cannot be excluded but are less probable. In the side-on configuration the CH_2 groups near the functional groups, αCH_2, are remote, meaning that their contributions to the hydrophobic interaction are small. Taking into account the number of carbons in positions α to the carbon atoms bearing functional groups or hydroxy groups a unifying linear dependence was obtained for alkan-*m*-ols or alkan-*m*,*n*-diols, from plotting the h_{xx} values versus N:

$$h_{xx} = a + bN \tag{5}$$

where N is given by:

$$N = n_{CH_2}(n_{CH_2} - n_{\alpha CH_2})(n_{CH_2} - n_{CH_2OH}) \tag{6}$$

Here n_{CH_2} is the total number of equivalent CH_2 groups ($CH_3 \equiv 1.5CH_2$, $CH \equiv 0.5CH_2$), $n_{\alpha CH_2}$ is the number of equivalent CH_2 groups at positions α to a carbon atom bearing a functional OH group, and n_{CH_2OH} is the number of equivalent CH_2 groups bearing hydroxy functional groups. Notwithstanding its roughness, Eq. (6) works as a powerful predictive method through the parameter N, that accounts for the role of hydrated hydrophobic groups which can actually juxtapose and are available for hydrophobic interactions with another molecule. When the enthalpic interaction coefficients are reported versus the abscissa N, the following linear trends are obtained for alkan-1-ols, alkane-1,2-diols and alkane-α,ω-diols in water (Eq. (7a)), in 1 mol kg^{-1} (Eq. (7b)) and in 3 mol kg^{-1} aqueous solutions of glucose (Eq. (7c)):

$$h_{xx} = (305 \pm 80) + (20 \pm 1)N \tag{7a}$$

$$h_{xx} = (314 \pm 62) + (20.1 \pm 0.8)N \tag{7b}$$

$$h_{xx} = (386 \pm 67) + (16.9 \pm 0.8)N \tag{7c}$$

Then, the parameters obtained in the presence of 1 mol kg^{-1} glucose are exactly equal to those characterizing the same substances in pure water. It is to be noted that the above fits are obtained with the exclusion of hexan-1-ol, whose coefficient is very near to that of pentan-1-ol, whatever the solvent employed. The rationalization for this behavior has been already given elsewhere [28]. Since the slope is bound to the contribution to the coefficients of every added CH_2 group, we can conclude that, at this concentration of cosolvent, hydrophobic interactions for the three series of substances are the same as those acting in water.

In 3 mol kg^{-1} glucose, the slope of the linear trend is smaller. However, a closer look at the data shows that this decrease is mainly due to alkan-α,ω-diols. In fact, reporting h_{xx} versus N for that series only, a linear trend is obtained (Eq. (8)) whose slope is very near to that of Eq. (7c):

$$h_{xx} = (384 \pm 68) + (16.3 \pm 0.7)N \tag{8}$$

Thus, for these substances hydrophobic interactions are attenuated in 3 mol kg^{-1} glucose, an indication that the presence of the two hydroxyl groups at the ends of the alkyl chain makes less effective the juxtaposition of interacting molecules as respect to alkane-1,2-diols having the two hydroxyl groups confined at one end of the molecule.

The linear trend exhibited in water and in glucose clearly means that the model proposed to rationalize the enthalpic behavior in water is appropriate also for that in the presence of the cosolvent. Namely, the nature of the hydration cosphere of the alkyl chain does not change: alkyl chains are preferentially hydrated so that water still plays the major role when two molecules interact. Glucose affects only to a limited extent the hydration cosphere of the alkyl chain. The interaction mechanism is the same as that

in water: the preferred side-on configuration plays the major role, determining the extent of water relaxation from the hydrophobic cospheres to the bulk.

It is interesting to remember that, for alkan-*m*,*n*-diols, the presence of 7 mol kg^{-1} urea determines an analogous behavior described by the following linear fit [13]:

$$h_{xx} = (338 \pm 86) + (13.3 \pm 1.2)N \quad (9)$$

Then urea, as glucose, is able to attenuate both hydrophilic and hydrophobic interactions, a consequence of a different balance of the involved interactions in the presence of the two cosolvents. Evidences for this similarity were found from the study of ternary systems in water: the interaction between D-glucose and ethanol, propanol and butanol is explained in terms of a mechanism not different from the one proposed for the urea–alkanol interaction in water. There is a prevailing release of water from the more labile hydrophobic cosphere of the alkanol, which is partially destroyed in this interaction [15].

For alkanols in the presence of concentrated ethanol, instead, the deviation from the linear trend was found to start from a concentration of 1 mol kg^{-1} of cosolvent [14]. The enthalpic interaction coefficients are larger than those in water, reaching a maximum at 2–3 mol kg^{-1} ethanol, and then decreasing with the increasing structuring effect of the cosolvent on water. We concluded that the mechanism through which interaction occurs is different from that in pure water: at low concentration of ethanol, the alkyl chains of the diols are preferentially hydrated, while at higher concentration the solvation cospheres are ''ethanolated'', so that the alkyl chains tend to interact with ethanol and interactions between molecules of the same species are attenuated.

Knowledge of the enthalpic interaction coefficients of model molecules of biological interest in pure water [11] and in concentrated urea [12,13], ethanol [14] and glucose [15], together with the results reported by other authors [29–31], allows us to hypothesize a possible mode of action of these substances on globular proteins. It is important to underline that our approach is tentative and oversimplified, since only enthalpic information are available. Many proofs in the literature indicate a strong interaction between urea or ethanol and globular proteins. When interacting with a substance containing a polar and a hydrophobic group, urea solvates preferentially the polar groups, as the contribution to the Gibbs free energy of the mixed urea–alkyl chain interaction is positive [27]. Then, it competes with water in solvating the hydrophilic groups on the surface of the globular proteins so that the hydrophilic interactions between these groups are depressed. Intermolecular, favorable interactions with peptide groups disrupt the intramolecular peptide–peptide hydrogen-bonding interactions, while 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 also in agreement with the findings coming from molecular dynamics simulation studies [32]. On the other hand, denaturation of proteins is provoked also by high concentration of alcohols [33]: the mechanism of denaturation must be necessarily different from that acting in the presence of urea. The unstabilizing effect of monohydric alcohols on the native structure of proteins has been generally interpreted in terms of the interaction between protein and alcohol [34]. It can be envisaged that, in concentrated solutions of alcohols, the hydrophobic core of a protein, hidden in water, could adapt well to the solvent medium: the alkanol, in fact, can give hydrophobic interactions with the exposed alkyl residues of the protein. The ethanolation of the solvation cospheres for model systems in concentrated ethanol supports this hypothesis [14].

Sugars and polyhydric alcohols are widely known to have a stabilizing effect on the native structure of proteins [1–9]. However, there are no evidences of a direct binding of these substances with the protein. For instance, it is reported that the binding constant for glucose to lysozyme is very small [35] or that glucose and maltose do not bind measurably to lysozyme [36]. The small value of the constant for the complex formation is evidence that other interactions must exist which stabilize the native conformation of a protein in the system protein–sugar–water. The results of isopiestic measurements on model systems indicate that sucrose solutions are more unfavorable solvent than water for aminoacid with aliphatic and aromatic side chains [37,38]. Consequently, it would require much more work for nonpolar groups in the interior of the protein to be exposed in the sucrose solution than in water; they would thus be caused by the sucrose

solution to enter into the interior of the protein. Our present study clearly shows that alkyl chains are preferentially hydrated, maintaining almost unaltered the properties they have in pure water. That is in agreement with studies by other authors who found that proteins are preferentially hydrated in lactose, glucose and sucrose systems [6–9]: that is a common feature of aqueous sugar systems within the sugar concentration range ordinarily employed, regardless of the kind of protein and the solvent conditions used. In a three-component system, preferential hydration of a protein is a good indication that the third component is a stabilizer of the structure of the macromolecule.

As a conclusion, denaturation in urea could be ruled prevailingly by hydrophilic interactions between the polar regions of the protein and the denaturant. In ethanol, exposure to the solvent of the alkyl groups of the hydrophobic core should be favored, and denaturation could be determined mainly by hydrophobic interactions of these residues with the cosolvent. In glucose, instead, resistance to thermal denaturation could be due to the inertness of glucose in reacting with protein: that allows the protein to maintain its hydration cosphere. The preferential exclusion of sugars from the domain of the proteins reflects their very reduced tendency to react with other substances, even with themselves (their pair-wise coefficient to the excess Gibbs free energy is positive). It could be safely hypothesized that, at increasing concentration of a sugar, bulk water keeps saving its properties, the prevailing process being probably a rearrangement of water in the saccharide hydration cosphere whose extension changes at varying concentration of the sugar.

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