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Analyzing the effects of protecting osmolytes on solute-water interactions by solvatochromic comparison method: I. Small organic compounds

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Solvent properties of water (dipolarity/polarizability, hydrogen bond donor (HBD) acidity, and hydrogen bond acceptor (HBA) basicity) in aqueous solutions of osmolytes (sorbitol, sucrose, trehalose, and trimethylamine N-oxide (TMAO)) were studied at different osmolytes concentrations using solvatochromic comparison method. The solvent properties of aqueous media in the coexisting phases of aqueous dextran-PEG-sodium/potassium phosphate buffer (0.01 M K/NaPB, pH7.4) two-phase system (ATPS) containing 0.5 M osmolyte additive (sorbitol, sucrose, trehalose, TMAO) and osmolyte-free ATPS were characterized. Partitioning of 30 low molecular weight polar organic compounds was examined in aqueous dextran-PEG-0.01M K/NaPB ATPS containing 0.5 M sorbitol. The solute-specific coefficients for the compounds examined were determined from the data obtained here and those reported previously. The results obtained demonstrate that the osmolytes examined affect the partition behavior of organic compounds in ATPS by influencing solvent properties of the media and not by direct association with the compounds.

Introduction

Small organic compounds known as protective or stabilizing osmolytes are used by many biological systems to protect their cellular components against denaturation under environmental stresses. Stabilizing osmolytes are commonly grouped into three major classes: polyols (e.g., sorbitol, glycerol, mannitol, sucrose, and trehalose), amino acids and their derivatives (e.g., L-proline, L-serine, α-alanine, β-alanine, glycine, sarcosine, and taurine), and methyl ammonium compounds (e.g., betaine and trimethylamine N-oxide (TMAO)).[1] One of the well- established effects of protective osmolytes is their ability to stabilize folded globular proteins under physiological conditions *in vitro* without substantial changes in protein structure and function.[1, 2] There are different views on the mechanisms of stabilizing effects of osmolytes on proteins in solution. The dominant hypothesis is based on the preferential solvation model, according to which osmolytes are excluded from protein surface and increase the Gibbs free energy change associated with protein unfolding.[3, 4] According to this model, the aqueous osmolyte solution is a media unfavorable for the unfolded forms of globular proteins. This view is based on the observations that the aqueous solubility of non-polar amino acids and peptides decreases in solutions of osmolytes relative to that in pure water.[5-9] Solubility and other physicochemical properties of various other compounds are known to be affected in solutions of different osmolytes in the osmolyte-specific manner. Therefore it is hypothesized that the water structure is altered in osmolyte solutions, and numerous studies of this issue were reported.[10-19] The data accumulated so far are sometimes contradictory, but the conclusion that the water properties in osmolyte solutions are changed relative to those of pure water seems unavoidable.[10-19] The recently reported observation that sorbitol may form aqueous two-phase system in mixture with Triton X-100,[20] in our view, serves as an unambiguous indication that sorbitol does change the structure and properties of water. We previously reported analysis of the effects of sucrose, trehalose, and TMAO on protein-water interactions using the solvent interaction analysis (SIA),[21, 22] which is based on analytical application of partitioning of solutes in aqueous two-phase systems (ATPSs).[23]

ATPSs are formed in aqueous mixtures of different polymers or a single polymer and a specific salt.[24, 25] When two certain polymers, for example, dextran and Ficoll, are mixed in water above certain concentrations, the mixture separates into two

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immiscible aqueous layers. There is a clear interfacial boundary separating two distinct aqueous-based phases, each preferentially rich in one of the polymers, with the aqueous solvent in both phases providing conditions suitable for biological products.[24, 25] ATPSs are unique in that each of the phases typically contains well over 80% water on a molal basis, and yet they are immiscible and differ in their solvent properties.[26-32] Differences in solute–solvent interactions in the two phases lead to uneven solute distribution which is readily quantified by the partition coefficient, designated as *K*, and may be exploited for sensitive detection of changes in the solute structure.[27, 29-32] The partition coefficient *K* of a solute is defined as the ratio of the solute concentrations in the two phases.

It has been established that the solvent properties of aqueous media in the coexisting phases of ATPS can be quantified using two approaches. The first of these approaches is based on the solvatochromic comparison method developed by Kamlet, Taft and others.[33-35] Here, a set of solvatochromic dyes is used to characterize the solvent dipolarity/polarizability, solvent hydrogen bond donor (HBD) acidity, and solvent hydrogen bond acceptor (HBA) basicity of the media in the two phases. The second approach is based on analysis of partitioning of a homologous series of charged compounds with varied length of aliphatic alkyl chain, such as sodium salts of dinitrophenylated amino acids. The data obtained are used to characterize the difference between the electrostatic and hydrophobic properties of the two phases (see in refs.[21, 22]).

The partition coefficient of a solute in an ATPS can be described as:[27, 29-32]

$$logK_i = S_s\Delta\pi^*_i + B_s\Delta\alpha_i + A_s\Delta\beta_i + C_sc_i \quad (1)$$

where *K* is the solute partition coefficient; $\Delta\pi^*$ is the difference between the solvent dipolarity/ polarizability of the two phases, $\Delta\alpha$ is the difference between the solvent HBD acidity of the two phases, $\Delta\beta$ is the difference between the solvent HBA basicity of the two phases; *c* is the difference between the electrostatic properties of the two phases; S_s, A_s, B_s, and C_s are constants (solute specific coefficients) quantifying the complementary interactions of the solute with the solvent media in the coexisting phases and representing the relative contributions of these interactions into partition coefficient of the solute; the subscript *s* designates the solute; the subscript *i* denotes the ATPS used; the difference for each solvent property is determined as the one between the upper and lower phases.

The solute specific coefficients may be determined for a given compound (including proteins) by the analysis of partition coefficients of this compound in multiple ATPSs with different polymer but same ionic composition with established solvent properties of the phases. Once $\Delta\pi^*$, $\Delta\alpha$, $\Delta\beta$, and *c* parameters in multiple ATPSs are determined, the solute specific coefficients are calculated by multiple linear regression analysis using Eq. 1. The partition coefficient of a compound with pre-determined solute specific coefficients in a "new" ATPS with established solvent properties of the phases can be predicted with 90-95% accuracy.[30]

It is important to emphasize that the partition coefficients of a solute in multiple ATPSs with different additives would fit Eq. 1 only if the solute-solvent interactions would vary due to different solvent properties of the phases and there would be no association of additives with the solute. It was established that while the minimal number of different ATPSs to be used for determination of solute-specific coefficients is five, using a set of 10 different ATPS provides much more reliable values of the solute-specific coefficients.[27, 28] The purpose of this study was to examine solvent properties of water in aqueous solutions of different osmolytes (sorbitol, sucrose, TMAO, and trehalose). We also examined solvent properties in the dextran-PEG ATPSs containing 0.5 M osmolyte (sucrose, TMAO, and trehalose, see refs.[21, 22] and sorbitol – this work). Then, we studied partitioning of 30 small organic compounds in the dextran-PEG-0.01M K/NaPB- 0.5M sorbitol ATPS, and attempted to estimate the solute-specific coefficients for all the compounds with the purpose to explore if all the osmolytes utilized affect the solute partition behavior solely by affecting the solvent properties of the aqueous media.

Materials and Methods

Materials

Polymers. Polyethylene glycol PEG-8000 (Lot 091M01372V) with an average molecular weight (M_n) of 8000 was purchased from Sigma-Aldrich (St. Louis, MO, USA) and Dextran-75 (Lot 119945) with an average molecular weight (M_w) 75,000 by light scattering were purchased from USB Corporation (Cleveland, OH, USA).

Amino acids. Dinitrophenylated (DNP) amino acids – DNP-glycine, DNP-alanine, DNP-norvaline, DNP-norleucine, and DNP-α-amino-*n*-octanoic acid, were purchased from Sigma-Aldrich. The sodium salts of the DNP-amino acids were prepared by titration.

Organic compounds. Adenine, adenosine, benzyl alcohol, caffeine, coumarin, 3-hydroxybenzaldehyde, methyl anthranilate, p-nitroanisole, p-nitrophenol, p-nitrophenyl-α-D-glucopyranoside, 2-phenyl ethanol, vanillin, tryptophan, glutamic acid, lysine, and sorbitol were from Sigma; phenol, phenylalanine, leucine, arginine, aspartic acid, asparagine, valine, threonine, glycine, alanine were from MB Biomedicals (Solon, OH, USA), and glutamine was provided by Bachem (King of Prussia, PA, USA) and used without further purification. Reichardt's carboxylated betaine dye was kindly provided by Professor C. Reichardt (Philipps University, Marburg, Germany).

Other chemicals. All salts and other chemicals used were of analytical-reagent grade and used without further purification.

Methods

Solvatochromic studies. All polymer solutions were prepared in 0.01 M sodium/potassium phosphate buffer, pH 7.4 (K/NaPB) by weight. The osmolytes solutions were prepared in water. The solvatochromic probes 4-nitroanisole, 4-nitrophenol and Reichardt's carboxylated betaine dye were

used to measure the dipolarity/polarizability π^*, HBA basicity β, and HBD acidity α of the media in the osmolytes solutions and separated phases of ATPS. Aqueous solutions (ca. 10 mM) of each solvatochromic dye were prepared and 5-15 μL of each was added separately to a total volume of 500 μL of osmolyte solution or a given phase of ATPS. An illustrative example of the absorption spectra of the Reichardt's betaine dye measured in the coexisting phases in a sample ATPS is shown in Supplementary Materials (see Figure S1). A strong base was added to the samples (~5 μL of 1 M NaOH to 500 μL of the osmolyte solution or a given phase) containing Reichardt's carboxylated betaine dye to ensure a basic pH. A strong acid (~10 μL of 1 M HCl to 500 μL of the solution) was added to the samples containing 4-nitrophenol in order to eliminate charge-transfer bands of the phenolate anion that were observed in some solutions. The respective blank solutions without dye were prepared separately. The samples were mixed thoroughly in a vortex mixer and the absorption spectra of each solution were acquired. To check the reproducibility, possible aggregation and specific interactions effects, the position of the band maximum in each sample was measured in five separate aliquots. A UV-VIS microplate reader spectrophotometer SpectraMax Plus384 (Molecular Devices, Sunnyvale, CA, USA) with a bandwidth of 2.0 nm, data interval of 1 nm, and high resolution scan (~0.5 nm/s) was used for acquisition of the UV–Vis molecular absorbance data. The absorption spectra of the probes were determined over the spectral range from 240 to 600 nm in each solution. Pure osmolyte solutions or phase of ATPS containing no dye (blank) were scanned first to establish a baseline. The wavelength of maximum absorbance in each solution was determined as described by Huddleston *et al.*[36] using PeakFit software package (Systat Software Inc., San Jose, CA, USA) and averaged. Standard deviation for the measured maximum absorption wavelength was ≤0.4 nm for all dyes in all solutions examined.

The behavior of the probes (4-nitrophenol, and Reichardt's carboxylated betaine dye) in several solvents (water, n-hexane, methanol) was tested in the presence and absence of HCl (for 4-nitrophenol) and NaOH (for the betaine dye) at different concentrations of the probes, acid or base, and the maximum shifts of the probes were compared to reference values found in the literature and were within the experimental errors in all cases (data not shown). The results of the solvatochromic studies were used to calculate π^*, α, and β as described by Marcus.[37]

The values of the solvent dipolarity/polarizability were determined from the wave number (ν_1) of the longest wavelength absorption band of the 4-nitroanisole dye using the relationship:

$$\pi^* = 0.427(34.12 - \nu_1) \quad (2)$$

The values of the solvent hydrogen-bond acceptor basicity β were determined from the wave number (ν_2) of the longest wavelength absorption band of the 4-nitrophenol dye using the relationship:

$$\beta = 0.346(35.045 - \nu_2) - 0.57\pi^* \quad (3)$$

The values of the solvent hydrogen-bond donor acidity α were determined from the longest wavelength absorption band of Reichardt's betaine dye using the relationship:

$$\alpha = 0.0649E_T(30) - 2.03 - 0.72\pi^* \quad (4)$$

The $E_T(30)$ values are based on the solvatochromic pyridinium N-phenolate betaine dye (Reichardt's dye) as probe, and are obtained directly from the wavelength (λ, nm) of the absorption band of the carboxylated form, as:

$$E_T(30) = (1/0.932)\times[(28591/\lambda) - 3.335] \quad (5)$$

Aqueous two-phase systems (ATPS). Stock solutions of PEG 8000 (50 wt.%), Dex-75 (~42 wt.%), and sorbitol (2.0 M) were prepared in deionized (DI) water. Stock sodium/potassium phosphate buffer (K/NaPB; 0.5 M, pH 7.4) was prepared by mixing appropriate amounts of KH_2PO_4 and Na_2HPO_4. A mixture of polymers was prepared as described elsewhere[38] by dispensing appropriate amounts of the aqueous stock polymer solutions into a 1.2 mL microtube using a Hamilton Company (Reno, NV, USA) ML-4000 four-probe liquid-handling workstation. Appropriate amounts of stock solution of sorbitol, stock buffer solutions, and water were added to give the ionic, polymer, and osmolyte composition required for the final system (after the sample addition – see below) with total weight of 0.5g (total volume 457±2 μL). All the aqueous two-phase systems used had the same polymer composition of 6.0 wt.% PEG-8000 and 12.0 %wt. Dex-75 and same ionic composition of 0.01 M K/NaPB, pH 7.4 with 0.5 M sorbitol.

Partitioning. An automated instrument for performing aqueous two-phase partitioning, the Automated Signature Workstation, ASW (Analiza, Inc., Cleveland, OH, USA), was used for the partitioning experiments. The ASW system is based on the ML-4000 liquid-handling workstation (Hamilton Company, Reno, NV, USA) integrated with a FL600 fluorescence microplate reader (Bio-Tek Instruments, Winooski, VT, USA) and a UV-VIS microplate spectrophotometer (SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA). Solutions of all compounds were prepared in water at concentrations of 0.5–5 mg/mL depending on the compound solubility. Varied amounts (e.g. 0, 15, 30, 45, 60 and 75 μL) of compound solution and the corresponding amounts (e.g. 75, 60, 45, 30, 15 and 0 μL) of water were added to a set of the same polymers/buffer mixtures with and without sorbitol. The systems were then vortexed in a Multipulse vortexer and centrifuged (Jouan, BR4i, Thermo Fisher Scientific, Waltham, MA, USA) for 60 min at 3500×g at 23°C to accelerate phase settling. The top phase in each system was removed, the interface discarded, and aliquots from the top and bottom phases were withdrawn in duplicate for analysis. An illustrative example of the output of the partition experiments is shown in Figure S2.

For the analysis of free amino acids partitioning, aliquots of 30 μL from both phases were transferred and diluted with water up to 70 μL into microplate wells. Then, the microplate was sealed, shortly centrifuged (2 min at 1500 rpm) and following moderate shaking for 45 min in an incubator at 37ºC, 250 μL of o-phthaldialdehyde reagent was combined. After moderate shaking for 4 min at room temperature, fluorescence was

determined using a fluorescence plate reader with a 360 nm excitation filter and a 460 nm emission filter, with a sensitivity setting of 100-125.

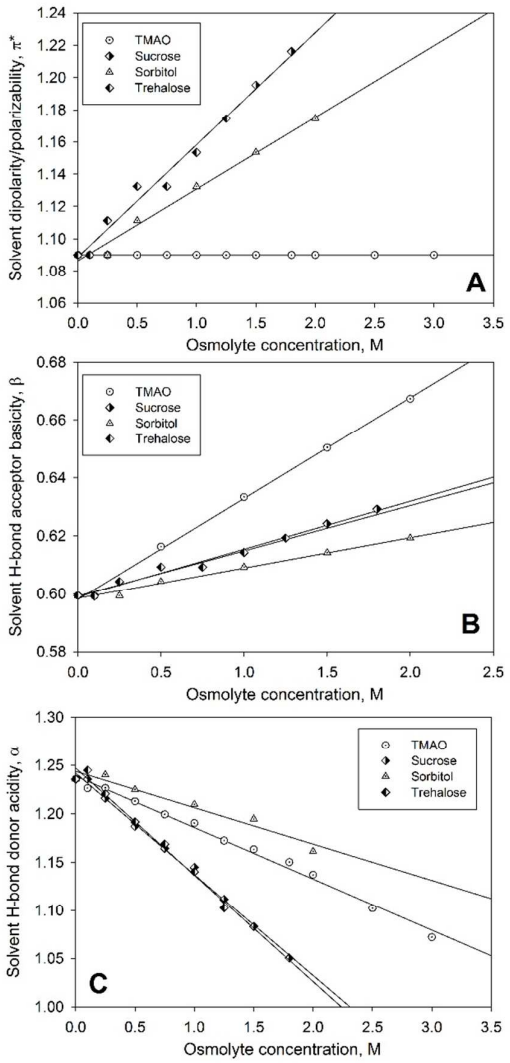

Figure 1. Solvent properties of water in aqueous solutions of osmolytes.
A. Solvent dipolarity/polarizability (π*) of water in aqueous solutions as a function of osmolyte concentration (lines are added for eye-guidance only).
B. Solvent hydrogen bond acceptor (HBA) basicity (β) of water in aqueous solutions as a function of osmolyte concentration (lines are added for eye-guidance only).
C. Solvent hydrogen bond donor (HBD) acidity (α) of water in aqueous solutions as a function of osmolyte concentration (lines are added for eye-guidance only).

For the analysis of the other compounds partitioning, aliquots of 50 - 120 µL from both phases were diluted up to 600 µL in 1.2 mL microtubes.

Water was used as diluent for all except phenol, p-nitrophenol, 3-hydroxybenzaldehyde and vanillin. 20 mM universal buffer with pH 12.4 was used as diluent (Universal buffer is composed of 0.01 M each of phosphoric, boric, and acetic acids adjusted to pH 12.4 with NaOH). Following vortexing and a short centrifugation (12 min), aliquots of 250 - 300 µL were transferred into microplate wells, and the UV-VIS plate reader was used to measure optical absorbance at wavelengths previously determined to correspond to maximum absorption. The maximum absorption wavelength for each compound was determined in separate experiments by analysis of the absorption spectrum over the 240–500 nm range. In the case of the four aforementioned compounds the maximum absorption was found to be more concentration sensitive in the presence of the universal buffer at pH 12.4. In all measurements the same dilution factor was used for the upper and lower phases and correspondingly diluted pure phases were used as blank solutions.

The partition coefficient, *K*, is defined as the ratio of the sample solute concentration in the top phase to that in the bottom phase. The *K*-value for each solute was determined as the slope of the concentration (fluorescence intensity or absorbance depending on the compound) in the top phase plotted as a function of the concentration in the bottom phase averaged over the results obtained from two to four partition experiments carried out at the specified composition of the system.[38] The deviation from the average *K* value was always less than 3 % and in most cases lower than 1 %.

Computational methods. The linear regression analyses were performed using the SigmaPlot, version 12 software package (SSI, San Jose, CA, USA). The multiple linear regression analysis was performed using fitting code developed with the Matlab software, version R2010b MathWorks (Natick, MA, USA).

Results and Discussion

Solvent properties of water in osmolyte solutions

The solvent dipolarity/polarizability, π^*, representing the ability of water to participate in dipole-dipole interactions increases with osmolyte concentration in aqueous solutions of sorbitol, sucrose, and trehalose, and is not affected by the presence of TMAO as shown graphically in Figure 1A. The observed effects are similar for sucrose and trehalose, and these effects are quite significant. In trehalose or sucrose solution at the concentration of 1.5 M, the dipolarity/ polarizability of water exceeds that observed in solutions of macromolecular crowding agents, such as dextran-75, Ficoll-70 or PEG-10,000 at concentrations of 40% wt.[39] Sorbitol effect is not as pronounced but is quite noticeable too, since the solvent dipolarity/polarizability of water in its solution at concentration of 2.0 M is similar to that of dextran-75 and Ficoll-70 at concentrations of 40% wt.[39]

All osmolytes increase the water HBA basicity, β, as shown in Figure 1B. The effects are quite noticeable and decrease in the sequence: TMAO > sucrose=trehalose > sorbitol. The TMAO effect at concentration of 2.0 M is comparable to those of macromolecular crowding agents, such as dextran-75 and Ficoll-70 at concentrations of 40% wt.[39] Even smaller effect of trehalose (sucrose) at 1.8 M exceeds slightly that of dextran-75 at 40% wt.[39]

Table 1. Differences between the solvent properties of the phases and partition coefficients for simple organic compounds and free amino acids in Dex-PEG-0.01M K/NaPB, pH 7.4 and Dex-PEG-0.5M osmolyte-0.01M K/NaPB, pH 7.4 ATPS (K/NaPB – sodium/ potassium phosphate buffer)

Solvent properties[c]	0.01M K/NaPB[a]	0.5M Sorbitol	0.5M Sucrose[b]	0.5M Trehalose[b]	0.5M TMAO[a]
ΔG(CH2), cal/mole	-45±1.2	-43±1.1	-39.4±0.44	-47.7±0.6	-40.9±0.6
E	0.033 ±0.001	0.032 ±0.002	0.029 ±0.001	0.035 ±0.001	0.028 ±0.001
C	0.058 ±0.003	0.090 ±0.003	0.110 ±0.002	0.113 ±0.002	0.083 ±0.002
*Δπ**	-0.042 ±0.002	-0.042 ±0.004	-0.073 ±0.004	-0.042 ±0.003	-0.031 ±0.002
Δα	-0.051 ±0.003	-0.066 ±0.003	-0.046 ±0.005	-0.081 ±0.003	-0.074 ±0.003
Δβ	0.006 ±0.004	0.006 ±0.005	0.023 ±0.006	0.006 ±0.005	0.009 ±0.008
Compound	Partition coefficient				
Adenine	1.220 ±0.006	1.311 ±0.007	1.271 ±0.009	1.367 ±0.009	1.264 ±0.005
Adenosine	1.128 ±0.004	1.210 ±0.005	1.215 ±0.003	1.256 ±0.007	1.192 ±0.007
Benzyl alcohol	1.409 ±0.009	1.52 ±0.015	1.607 ±0.009	1.697 ±0.007	1.454 ±0.008
Caffeine	1.154 ±0.009	1.178 ±0.003	1.160 ±0.004	1.186 ±0.006	1.147 ±0.008
Coumarin	1.490 ±0.009	1.684 ±0.005	1.697 ±0.006	1.780 ±0.006	1.590 ±0.008
Glucopyranoside[d]	1.232 ±0.003	1.31 ±0.002	1.332 ±0.009	1.368 ±0.001	1.246 ±0.003
3-Hydroxybenz aldehyde	1.709 ±0.003	1.927 ±0.004	2.005 ±0.007	2.118 ±0.009	1.762 ±0.009
Methyl anthranilate	1.77 ±0.01	2.035 ±0.005	2.124 ±0.007	2.24 ± 0.011	1.847 ±0.007
p-Nitrophenol	1.486 ±0.006	1.688 ±0.005	1.724 ±0.002	1.796 ±0.009	1.568 ±0.004
Phenol	1.70 ±0.02	2.008 ±0.006	2.07 ±0.017	2.211 ±0.009	1.809 ±0.009
2-Phenylethanol	1.469 ±0.005	1.60 ±0.01	1.695 ±0.009	1.697 ±0.009	1.51 ±0.01
Vanillin	1.709 ±0.009	1.82 ±0.03	1.969 ±0.005	2.105 ±0.006	1.761 ±0.005
Gly	0.739 ±0.008	0.730 ±0.005	0.732 ±0.008	0.754 ±0.007	0.715 ±0.005
Ala	0.824 ±0.004	0.79 ±0.01	0.853 ±0.009	0.837 ±0.008	0.762 ±0.009
Val	0.833 ±0.007	0.847 ±0.004	0.856 ±0.009	0.87 ±0.012	0.826 ±0.003
Leu	0.872 ±0.009	0.894 ±0.005	0.913 ±0.006	0.919 ±0.008	0.837 ±0.008
Phe	0.884 ±0.007	0.901 ±0.004	0.920 ±0.006	0.932 ±0.009	0.856 ±0.007
Trp	0.905 ±0.008	0.891 ±0.005	1.043 ±0.005	1.074 ±0.009	0.906 ±0.004
Gln	0.781 ±0.004	0.745 ±0.008	0.796 ±0.009	0.796 ±0.009	0.768 ±0.004
Asn	0.715 ±0.009	0.713 ±0.005	0.718 ±0.006	0.716 ±0.004	0.720 ±0.005
Thr	0.782 ±0.004	0.745 ±0.007	0.814 ±0.007	0.803 ±0.009	0.757 ±0.009
Glu	0.765 ±0.009	0.754 ±0.003	0.781 ±0.009	0.782 ±0.006	0.763 ±0.002
Asp	0.759 ±0.008	0.748 ±0.005	0.761 ±0.005	0.75 ±0.011	0.785 ±0.009
Lys	0.584 ±0.003	0.565 ±0.006	0.556 ±0.006	0.56 ±0.022	0.547 ±0.007
Arg HCl	0.590 ±0.005	0.591 ±0.006	0.60 ± 0.01	0.573 ±0.006	0.566 ±0.004

[a] – Data for osmolyte-free ATPS and ATPS with 0.5M TMAO in 0.01M K/NaPB, pH 7.4 are from ref.[21]; [b] – data for ATPS with 0.5M sucrose and 0.5M trehalose in 0.01M K/NaPB, pH 7.4 are from ref.; [c] parameters *E* and *ΔG(CH2)* characterize the difference between the relative hydrophobicities of the coexisting phases of a given ATPS, parameter *C* value characterizes the difference between the electrostatic properties of the phases (for explanation see text), *Δπ** characterizes the difference between the solvent dipolarity/polarizability of the phases, *Δα* characterizes the difference between the solvent hydrogen bond donor acidity of the phases, *Δβ* characterizes the difference between the solvent hydrogen bond acceptor basicity of the phases; [d] p-nitrophenyl-α-D-glucopyranoside.

The HBD acidity of water, *α*, is also affected by osmolytes, as illustrated by Figure 1C. All osmolytes reduce the H-bonding acidity of water with the effects increasing as: sucrose=trehalose > TMAO > sorbitol.

The effects of the osmolytes on the water H-bonding acidity are, however, much less pronounced than the effects observed for nonionic polymers.[39]

The above data indicate that all the osmolytes under consideration alter solvent properties of water in their solutions in the osmolyte-specific manner (except identical effects displayed by sucrose and trehalose). It has been shown recently that the solvent properties of aqueous media in solutions of macromolecular crowding agents may explain to a large degree the crowding effects on protein folding,[39] but it is premature as of yet to speculate if these solvent properties in osmolytes solutions may be at the root of the stabilization effects of osmolytes on proteins.

Solvent properties of the ATPS phases

Solvatochromic solvent properties of the phases. Each of the solvent parameters *π**, *α*, and *β*, in each ATPS phase were determined using a set of single solvatochromic probes as previously described.[26-28, 30] The solvatochromic parameters measured in each phase of the ATPS are presented in Table S1. The differences between the values found for the top phases and those for the corresponding bottom phases are shown in Table 1.

The changes in the differences between solvatochromic parameters characterizing the coexisting phases due to the osmolyte presence may be examined using those obtained for the ATPS with 0.01 M K/NaPB, pH 7.4 as reference. This analysis shows that the changes under consideration are both solvent property- and osmolyte additive-specific. It is also of interest to consider the osmolytes additives effects in comparison with those of different salt additives.[28]

The osmolytes effects on the absolute difference between the solvent dipolarity/polarizability, *Δπ**, of the two phases decrease in the sequence: sucrose >> trehalose = sorbitol > TMAO. If we consider the osmolyte additives effects together with those of salt additives,[28] the sequence (in the absolute values of *Δπ**) is: 0.11 M NaPB, pH 7.4 >> 0.01 M NaPB, pH 7.4 > 0.5 M sucrose > 0.15 M NaCl > 0.15 M NaBr ≥ 0.15 M KCl > 0.10 M Li_2SO_4 > 0.15 M KBr = 0.01 M K/NaPB, pH 7.4 = 0.5 M trehalose = 0.5 M sorbitol > 0.10 M Na_2SO_4 > 0.5 M TMAO, where NaPB is sodium phosphate buffer. It is important to notice that the effects of 0.01M NaPB and 0.01 M K/NaPB in the dextran-PEG ATPS of the same polymer composition are very significant: *Δπ** amounts to -0.109 in the presence of 0.01 M NaPB and it is -0.042 in the presence of 0.01 M K/NaPB (both at pH 7.4).

The effects of osmolytes on the difference in the solvent hydrogen bond donor acidity, *Δα*, between the two phases decrease in the sequence: trehalose > TMAO > sorbitol > sucrose. When we consider these effects together with those reported for different salt additives,[28] the sequence (in the absolute values of *Δα*) is: 0.5M trehalose > 0.10 M Na_2SO_4 ≥

0.5 M TMAO > 0.5 M sorbitol > 0.10 M Li_2SO_4 ≥ 0.01 M K/NaPB > 0.15 M KBr ≥ 0.5 M sucrose ≥ 0.11 M NaPB > 0.15 M NaBr > 0.15 M KCl > 0.15 M NaCl > 0.01 M NaPB. Here again the effects of 0.01 M K/NaPB and 0.01M NaPB are quite different.

The osmolytes effects on the difference in the solvent hydrogen bond acceptor basicity, $\Delta\beta$, between the two phases decrease in the sequence: sucrose > TMAO > sorbitol > sucrose = sorbitol. The more general sequence (in the absolute $\Delta\beta$ values) is:

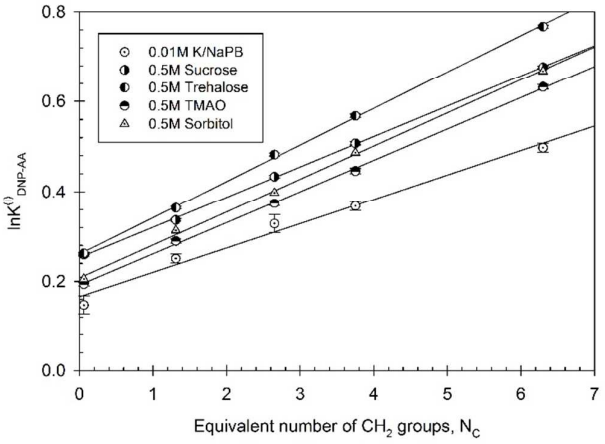

Figure 2. Logarithm of the partition coefficient value, $lnK_{DNP\text{-}AA}$, for sodium salts of DNP-amino acids with aliphatic side-chains in aqueous dextran–PEG two-phase systems as a function of equivalent length of the side-chain, N_C, expressed in terms of equivalent number of CH_2 units: in dextran-PEG-0.01 M potassium/sodium phosphate buffer (K/NaPB), pH7.4 ATPS,[21] in dextran-PEG-0.5 M TMAO-0.01 M K/NaPB ATPS,[21] in dextran-PEG-0.5 M sucrose-0.01 M K/NaPB ATPS,[22] in dextran-PEG-0.5 M trehalose-0.01 M K/NaPB ATPS,[23] and in dextran-PEG-0.5 M sorbitol-0.01 M K/NaPB ATPS.

0.01 M NaPB > 0.5 M sucrose > 0.15 M KCl = 0.15 M KBr > 0.10 M Na_2SO_4 > 0.11 M NaPB > 0.10 M Li_2SO_4 > 0.5 M TMAO ≥ 0.15 M NaCl ≥ 0.5 M trehalose = 0.5 M sorbitol = 0.01 M K/NaPB > 0.15 M NaBr.

The above comparison of the effects of various additives on the differences between the solvent properties of the coexisting phases in dextran-PEG ATPS shows that osmolytes additives used in this study (at the concentration of 0.5 M) affect the solvent hydrogen-bond donor acidity more than most of the salt additives. Furthermore, the 0.5 M sucrose additive affects the differences between the solvent dipolarity/polarizability and hydrogen bond acceptor basicity of the phases more than most of the salt additives used (at concentrations of 0.10-0.15 M).[28] It should also be noted that the effects of 0.01M NaPB and 0.01M K/NaPB are very different.

Partitioning of DNP-amino acids. Figure 2 shows the dependence of logarithms of partition coefficients $K^{(i)}_{DNP\text{-}AA}$ for sodium salts of DNP-amino acids in dextran-PEG-0.01 M K/NaPB ATPS with and without 0.5 M osmolyte (sorbitol, sucrose, trehalose, and TMAO[21, 22]) on the length of the aliphatic side-chain of DNP-amino acid expressed in equivalent number of CH_2 groups, N_C. In each case, the observed dependence is linear and can be described as:

$$lnK^{(i)}_{DNP\text{-}AA} = C^{(i)} + E^{(i)}N_C \quad (6)$$

where superscript (*i*) denotes the particular *i*-th ATPS used for the partition experiments; *E* and *C* are constants, which can be determined from this plot (*E* is an average ln*K* increment per CH_2 group; *C* represents the total contribution of the non-alkyl part of the structure of a DNP-amino acid into $lnK_{DNP\text{-}AA}$ and may be used to characterize the difference between the electrostatic properties of the coexisting phases as described previously[25, 28, 29]).

The values of coefficients $E^{(i)}$ and $C^{(i)}$ determined for the ATPSs examined are presented in Table 1 in log units. As the standard free energy of transfer of a solute from the bottom phase to the top phase is described as:

$$\Delta G^0 = -RTlnK \quad (7)$$

where *R* is the universal gas constant and *T* is the absolute temperature in Kelvin, it follows that

$$\Delta G^0(CH_2) = -RTE \quad (8)$$

where $\Delta G^0(CH_2)$ is the standard free energy of transfer of a methylene group from one phase to another. The $\Delta G^0(CH_2)$ values calculated from the experimental data with Eqs. 6–8 are listed in Table 1.

The presence of 0.5 M osmolyte affects the difference between the relative hydrophobic character of the coexisting phases depending on the particular osmolyte present as: 0.5 M trehalose > 0.01 M K/NaPB ≥ 0.5 M sorbitol > 0.5 M TMAO ≥ 0.5 M sucrose (see the $\Delta G^0(CH_2)$ values in Table 1). The difference between the electrostatic properties of the phases characterized by the parameter *C* value (Table 1) increases in the presence of 0.5 M osmolyte relative to the osmolyte-free ATPS: 0.01 M K/NaPB < 0.5 M TMAO < 0.5 M sorbitol < 0.5 M sucrose = 0.5 M trehalose. The osmolyte-induced changes of the differences between the hydrophobic and electrostatic properties of the coexisting phases may occur due to the osmolyte effect on the polymer compositions of the two phases and/or on the osmolyte effect on the properties of water in the phases.

Partitioning of organic compounds. Partition coefficients of various organic compounds in dextran-PEG ATPS containing 0.5 M sorbitol and those in the presence of other osmolyte additives reported previously[21, 22] are listed in Table 1. The data in Table 1 show that for the most of the compounds examined, there are small but noticeable differences in their partition behavior in the ATPSs with different osmolytes.

It has been shown previously[25, 38, 40-42] that the partition coefficients for different compounds (including proteins) in ATPSs of different compositions are commonly interrelated in accordance with the so-called Collander solvent regression equation:[43-46]

$$logK_{ji} = a_{io}\, logK_{jo} + b_{io} \quad (9)$$

where K^j_i and K^j_o are partition coefficients for any given j^{th} solute in the i^{th} and o^{th} two phase systems; a_{io} and b_{io} are constants, the values of which depend upon the particular composition of the i^{th} and o^{th} two-phase systems under comparison and may depend on the type of the solutes being examined.

It has been shown[22, 25, 31] also that different organic compounds, proteins, and nucleic acids commonly fit the same linear relationship (Eq. 9) if the partition coefficients of solutes in two different ATPSs are compared.

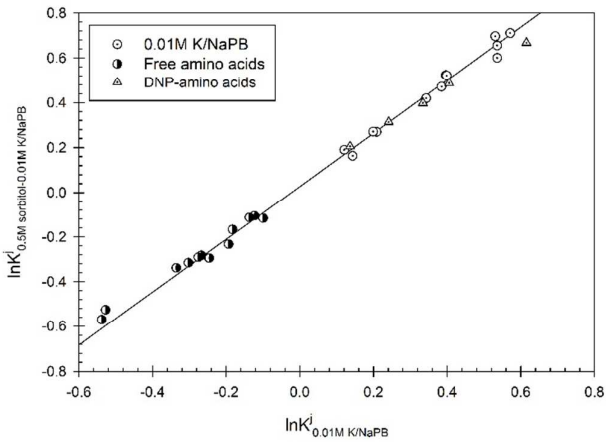

Figure 3. Logarithms of partition coefficients for organic compounds, free amino acids, and DNP-amino acids sodium salts in dextran-PEG-0.5 M sorbitol-0.01 M K/NaPB ATPS versus those for the same compounds in dextran-PEG-0.01 M K/NaPB ATPS. K/NaPB – potassium/sodium phosphate buffer, pH 7.4.

The linear relationship may not hold for a solute if an additive/ligand capable to associate directly with the solute is introduced into one of the two ATPSs. The reason is that the solute is modified in the presence of such an additive and its interactions with the solvent would differ from those in the absence of the additive. The partition coefficients for nonionic and ionizable organic compounds and zwitterionic free amino acids (Table 1) in the osmolyte-free and 0.5 M sorbitol-containing ATPS fit the linear relationship as shown in Figure 3. This relationship may be described as:

$$lnK^j_{0.5M\ sorbitol\text{-}0.01M\ K/NaPB} = 0.03_{\pm 0.006} + 1.15_{\pm 0.02} * lnK^j_{0.01M\ K/NaPB} \quad (10)$$

$N = 30$; $R^2 = 0.9946$; $SD = 0.030$; $F = 5163$

where $K^j_{0.5M\ sorbitol\text{-}0.01M\ K/NaPB}$ and $lnK^j_{0.01M\ K/NaPB}$ are partition coefficients for the same compound in the dextran-PEG-0.5 M sorbitol in 0.01 M K/NaPB ATPS and in dextran-PEG-0.01 M K/NaPB ATPS, respectively; N is the number of compounds examined; R^2 is the correlation coefficient; SD is the standard deviation; and F is the ratio of variance. It should be noted that similar relationships were previously reported[21, 22] in the presence of other osmolytes.

Solute-specific coefficients. All the data for each compound (listed in Table 1) were used to determine the coefficients (S_s, A_s, B_s and C_s) in Eq. 1 by the multiple linear regression analysis. It should be noted that we followed the procedure described by Ab Rani *et al.*[47] using the p-value as a test for significance for each solute-specific coefficient in Eq. 1 for a given compound. In view of the extremely small number of five ATPSs utilized, we have chosen to use the maximum statistical significance value of $p \leq 0.1$. If all four coefficients (S_s, A_s, B_s, and C_s) proved statistically significant ($p \leq 0.1$), then the correlation was accepted. If one or more values reveal a p-value > 0.1, then equations contained different combinations of coefficients were examined. The equation with a set of coefficients providing p-values for all parameters below or equal to 0.1 was accepted.

Table 2. Solute-specific coefficients[a] (see Eq. 1) for organic compounds in 0.01M K/NaPB (calculated by multiple linear regression analysis from data in Table 1). K/NaPB – sodium/potassium phosphate buffer, pH 7.4

Compound	S_s	A_s	B_s	C_s
Adenine	-0.8 ± 0.1	0[d]	1.12 ± 0.08	0[d]
p-values[b]	0.002		0.0002	
Adenosine	0[d]	-0.6 ± 0.1	-0.21 ± 0.05	0.81 ± 0.05
p-values[b]		0.02	0.03	0.0005
Benzyl alcohol	-2.0 ± 0.1	0[d]	-1.39 ± 0.08	0[d]
Caffeine	-1.1 ± 0.1	-1.3 ± 0.3	-0.45 ± 0.04	0[d]
p-values[b]	0.02	0.02	0.001	
Coumarin	-2.1 ± 0.4	0[d]	-1.6 ± 0.3	0[d]
p-values[b]	0.008		0.008	
Glucopyranoside[c]	-1.21 ± 0.03	-2.21 ± 0.08	-0.28 ± 0.02	0.68 ± 0.02
p-values[b]	0.0006	0.001	0.0006	0.001
3-Hydroxybenzaldehyde	-3.11 ± 0.05	-4.0 ± 0.1	-1.44 ± 0.04	0.92 ± 0.04
p-values[b]	0.0002	0.001	0.0006	0.002
Methyl anthranilate	-3.2 ± 0.2	-4.0 ± 0.4	-1.5 ± 0.1	1.0 ± 0.1
p-values[b]	0.003	0.01	0.006	0.01
p-Nitrophenol	-2.0 ± 0.3	-2.6 ± 0.6	-1.0 ± 0.2	1.0 ± 0.2
p-values[b]	0.03	0.1	0.05	0.06
Phenol	-2.9 ± 0.4	0[d]	-2.5 ± 0.3	0[d]
p-values[b]	0.002		0.0009	
2-Phenylethanol	-2.16 ± 0.05	-2.1 ± 0.1	-0.98 ± 0.04	0.67 ± 0.04
p-values[b]	0.0005	0.004	0.001	0.003
Vanillin	-2.5 ± 0.3	0[d]	-2.5 ± 0.2	0[d]
p-values[b]	0.0008		0.0003	
Gly	2.4 ± 0.5	0[d]	2.3 ± 0.4	1.3 ± 0.5
p-values[b]	0.02		0.009	0.06
Val	1.6 ± 0.3	0[d]	1.7 ± 0.2	1.2 ± 0.3
p-values[b]	0.01		0.004	0.02
Leu	0[d]	-4.0 ± 1.0	2.3 ± 0.4	1.5 ± 0.4
p-values[b]		0.05	0.01	0.03
Phe	0[d]	-3.7 ± 0.9	1.0 ± 0.3	1.1 ± 0.3
p-values[b]		0.03	0.01	0.04
Trp	0[d]	-4.9 ± 0.8	2.6 ± 0.3	2.3 ± 0.3
p-values[b]		0.01	0.003	0.004
Asn	2.4 ± 0.2	0[d]	2.3 ± 0.1	1.3 ± 0.1
p-values[b]	0.0007		0.0004	0.07
Glu	1.9 ± 0.4	0[d]	2.5 ± 0.3	1.5 ± 0.4
p-values[b]	0.02		0.004	0.03
Asp	2.5 ± 0.2	2.4 ± 0.6	1.3 ± 0.2	0.6 ± 0.2
p-values[b]	0.008	0.05	0.01	0.07
Lys	2.7 ± 0.1	-0.9 ± 0.3	3.54 ± 0.07	1.13 ± 0.07
p-values[b]	0.001	0.07	0.0004	0.004
Arg HCl	3.07 ± 0.06	-1.3 ± 0.2	4.10 ± 0.05	2.0 ± 0.05
p-values[b]	0.0004	0.02	0.0001	0.0006

[a] Solute specific coefficients represent the following solute-water interactions: S_s – dipole-dipole interactions; A_s – hydrogen bonding with solute as a donor; B_s – hydrogen bonding with solute as an acceptor; C_s – induced dipole-ion interactions; [b] Statistical significance p-value (not shown for $p < 0.0001$); [c] p-nitrophenyl-α-D-Glucopyranoside; [d] 0, solute-specific coefficients could not be reliably determined (with $p < 0.1$) and in subsequent calculations are taken as 0.

The solute-specific coefficients determined for each compound are presented in Table 2 together with the corresponding p-values (except the cases when $p < 0.001$).

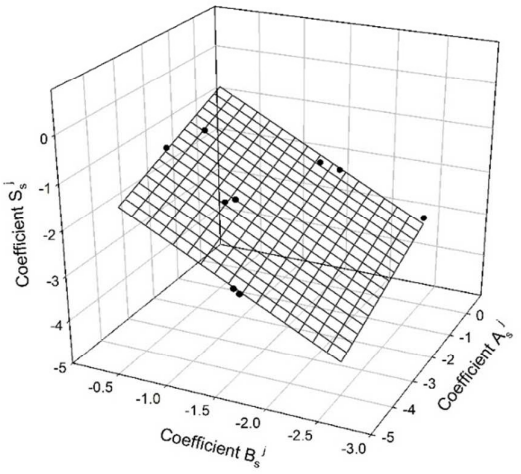

Figure 4. Solute-specific coefficients S_s^j determined for j^{th} nonionic organic compounds versus solute-specific coefficients A_s^j and B_s^j for the same compounds, all determined in the presence of 0.01 M K/NaPB. K/NaPB – sodium/potassium phosphate buffer, pH 7.4.

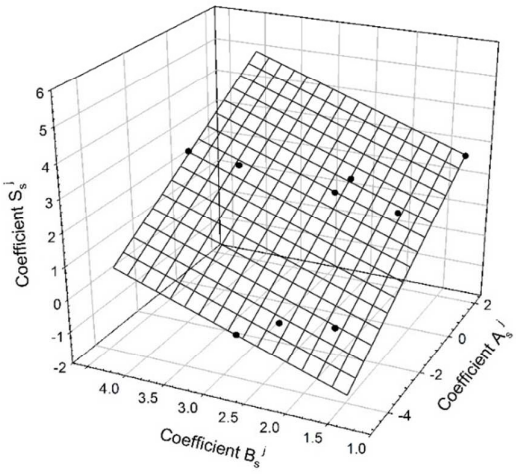

Figure 5. Solute-specific coefficients S_s^j determined for j^{th} free amino acids versus solute-specific coefficients A_s^j and B_s^j for the same amino acids, all determined in the presence of 0.01 M K/NaPB. K/NaPB – sodium/potassium phosphate buffer, pH 7.4.

The solute-specific coefficients for organic compounds differ from those reported previously[26, 29-32] for the same compounds in 0.15M NaCl in 0.01 M NaPB, pH 7.4. It has been reported [27] for proteins that different solute-specific coefficients for different proteins are linearly interrelated.

We explored the relationships between the different solute-specific coefficients for nonionic organic compounds and for zwitterionic free amino acids examined.

Both relationships are illustrated graphically in Figures 4 and 5. The relationship in Figure 4 for organic compounds may be described as:

$$S_s^j = -0.3_{\pm 0.14} + 0.30_{\pm 0.03} A_s^j + 1.1_{\pm 0.08} B_s^j \quad (11)$$

$N = 9$; $r^2 = 0.9733$; $SD = 0.14$; $F = 109.4$

where S_s^j, A_s^j and B_s^j are the jth solute specific coefficients as defined above; N is the number of compounds (adenosine, adenine, and vanillin do not fit the relationship); r^2, SD, and F as defined above.

For free amino acids the relationship show in Figure 5 may be described as:

$$S_s^j = 0.3_{\pm 0.2} + 0.49_{\pm 0.03} A_s^j + 0.82_{\pm 0.09} B_s^j \quad (12)$$

$N = 10$; $r^2 = 0.9722$; $SD = 0.23$; $F = 122.5$

where all the parameters are as defined above.

Thus, the solute specific coefficients evaluated using a minimal number of five ATPSs with different osmolytes additives are in agreement with the trend established[27] earlier for the same coefficients for proteins determined with 10 ATPSs of different polymer and same fixed ionic composition. This finding indicates that the solute partitioning in a given ATPS (dextran-PEG-0.01 M K/NaPB ATPS, in particular) is governed by solute-solvent interactions.

It can be concluded therefore that osmolytes change the solvent properties of the aqueous media in the phases and do not associate with organic compounds. It remains to be examined if osmolyte additives affect protein-solvent interactions in the similar manner. These studies are in progress in our laboratories.

In order to explore what structural properties of the compounds under study might govern different aspects of the solute-water interactions under the conditions employed, we analyzed different properties of the compounds using the ChemAxon software available at http://www.chemspider.com. The calculated properties of the compounds examined are listed in Table 3. Significant values for the solute-specific coefficients S_s and B_s were determined for most of the compounds (see Table 2), and hence we examined if these two coefficients might be described in terms of the structural properties of compounds.

The solute-specific coefficient S_s representing contribution of the dipole-dipole and induced dipole-dipole solute-solvent interactions into partition coefficient of the solute may be described as:

$$S_s^i = -1_{\pm 0.07} * logP^i - 0.064_{\pm 0.038} * k^i \quad (13)$$

$N = 18$; $r^2 = 0.9335$; $SD = 0.64$; $F = 105.2$

where $logP$ is the logarithm of the solute partition coefficient in octanol-water system; k is the molecular polarizability of the solute; superscript (i) denotes the i^{th} compound; all the other parameters are as defined above.

The solute-specific coefficient B_s representing contribution of the hydrogen bonding between solute and water with solute playing a role of H-bond acceptor into solute partition coefficient may be described as:

Table 2. Structural properties of compounds indicated calculated with ChemAxon software at http://www.chemspider.com

Compound	logP	PSA[a]	Polarizability	Pi energy
Adenine	-0.66	80.48	13.35	18.97
Adenosine	-2.09	139.54	24.55	35.76
Benzyl alcohol	1.21	20.23	12.79	12.18
Caffeine	-0.55	58.44	17.87	23.18
Coumarin	1.78	26.30	15.70	18.83
Glucopyranoside[b]	-0.66	142.52	26.65	44.99
3-Hydroxybenzaldehyde	1.38	37.30	12.83	16.06
Methyl anthranilate	1.80	52.32	15.89	19.21
p-Nitrophenol	1.61	63.37	12.86	24.09
Phenol	1.67	20.23	10.94	12.31
2-Phenylethanol	1.49	20.23	14.56	12.18
Vanillin	1.22	46.53	15.36	20.38
Gly	-2.04	63.19	5.54	6.06
Val	-1.95	63.32	12.0	10.44
Leu	-1.59	63.32	13.84	10.44
Phe	-1.18	63.32	17.89	18.44
Trp	-1.09	79.11	23.09	24.44
Asn	-4.29	106.41	11.51	16.99
Glu	-3.24	100.62	12.69	18.14
Asp	-3.50	100.62	10.90	18.14
Lys	-3.21	89.34	15.37	13.18
Arg HCl	-3.16	125.22	16.90	19.37

[a] PSA – polar surface area; [b] p-nitrophenyl-α-D-glucopyranoside

$$B_s^i = -1.4_{\pm 0.63} - 0.31_{\pm 0.04} * E_{pl}^i + 0.22_{\pm 0.07} * k^i + 0.062_{\pm 0.006} * PSA^i \quad (14)$$

$N = 22$; $r^2 = 0.8614$; $SD = 0.80$; $F = 37.2$

where E_{pl}^i is the Huckel Pi energy and PSA^i is the polar surface area of the i^{th} solute; all the other parameters are as defined above.

It seems reasonable that hydrophobicity (expressed as *logP*-value) and polarizability affect the ability of compound to participate in dipole-dipole solute-solvent interactions (Equation 13). Equation 14 indicates that the ability of a compound to serve as an acceptor of H-bond in solute-solvent interactions depends on the compound Pi energy, polarizability, and polar surface area. It seems to us that these results should not be viewed as conclusive. In fact, more extensive analyses are needed to better understand the contributions of the solute structural features and the different types of solute-water interactions (solute-specific coefficients) into partition coefficient of a solute. We are planning to explore these issues in detail in the near future.

Conclusions

Solvent properties of aqueous media (dipolarity/polarizability, hydrogen bond acidity; hydrogen bond basicity) are quantified and found to be altered in the presence of sorbitol, sucrose, trehalose, trimethylamine N-oxide in water. Solvent properties of media in aqueous dextran-polyethylene glycol two-phase systems containing 0.5 M osmolyte are characterized as well. Analysis of partition coefficients for 25 organic compounds in the two-phase systems with additives of different osmolytes in terms of the solvent properties of the phases shows that osmolytes affect partition behavior of compounds due to their effects on the solvent properties of the phases.

It should be emphasized here that in order to determine solute-specific coefficients we previously used multiple ATPSs of the same ionic composition formed by the different pairs of polymers or polymers with different molecular weights.[26-32] It has been established in this study that it is possible to use for the same purpose a set of the systems with the same polymer and ionic composition containing different non-ionic additives (osmolytes in this case) capable of affecting the solvent properties of aqueous media in the coexisting phases. There are two advantages of this approach: (i) it is more convenient to use an ATPS of a fixed polymer composition with different additives, and (ii) it was found that certain ATPSs, such as formed by polyethylene glycol and Ucon, for example, are poorly suitable for analysis of protein partitioning because of the protein precipitation at the interface or poor protein solubility and aggregation, and that these obstacles reduce the number of different polymer-polymer ATPSs suitable for this purpose. The possibility of using ATPSs of a fixed polymer composition suitable for a given protein with a number of non-ionic additives enables one to overcome the aforementioned difficulty.

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