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Effects of osmolytes on protein-solvent interactions in crowded environments: Study of sucrose and trehalose effects on different proteins by solvent interaction analysis

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Partitioning of 11 different proteins and 30 small organic compounds was examined in aqueous dextran-PEG-sodium/potassium phosphate buffer (0.01 M K/NaPB, pH 7.4) two-phase systems (ATPSs) containing 0.5 M sucrose or 0.5 M trehalose. The data obtained were compared to those reported previously for the same compounds and proteins in osmolyte-free ATPS and ATPS containing 0.5 M TMAO (Breydo *et al*. (2015) *Archives of Biochemistry and Biophysics*. in press), and analyzed in terms of the so-called Collander linear solvent regression relationship. It was found that the logarithms of the partition coefficients of proteins in the presence of 0.5 M sucrose and trehalose are linearly interrelated. The structural distances of protein 3D structures relative to that of ribonuclease B were estimated. These estimates were shown to be linearly related to the previously reported values determined for the same proteins based on their responses to different ionic environments.

Introduction

Naturally occurring disaccharides (monomeric polyols), sucrose (α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside) and trehalose (α-D-glucopyranosyl-(1→1)-α-D-glucopyranoside), belong to a family of the so-called protective osmolytes. They are used by multiple organisms to counteract the effects of environmental stresses, such as temperature and pH variations, high salinity, freezing, and dehydration. One of the important effects of protective osmolytes is their ability to stabilize proteins *in vitro* without substantial changes in protein structure and function.[1, 2] There are different views on the mechanisms of stabilizing effects of polyols on proteins in solution. The dominant view is based on the preferential solvation model, according to which osmolytes are excluded from protein surface and increase Gibbs free energy change associated with protein denaturation.[3-5] The destabilizing effects of osmolytes demonstrated for a variety of proteins at high osmolyte concentration and/or non-physiological pH ranges indicate that even stabilizing osmolytes may act as denaturants under certain conditions.[3] The mechanisms of osmolyte effects on proteins clearly depend on the protein-water, osmolyte-water, and protein-osmolyte interactions. The mechanisms of these interactions remain unclear, however.

We used here the solvent interaction analysis (SIA) method for studying effects of sucrose and trehalose on several proteins (trypsinogen, α-chymotrypsinogen A, ribonuclease A, ribonuclease B, β-lactoglobulin A, β-lactoglobulin B, papain, chymotrypsin, lysozyme, hemoglobin, and concanavalin A).[6-8] The SIA method is based on quantifying interactions of a protein with two aqueous media of different solvent properties. It is known that there is a strict correlation between the protein structure and peculiarities of the protein–water interactions.[6-8] This can be illustrated as well-known protein denaturation and/or conformational changes induced by the different additives, from urea to different salts in the Hofmeister series. Since protein–water interactions are intertwined with the active 3D-structure of ordered proteins, quantifying such interactions could enable detection of changes in the protein structure. SIA is conducted using partitioning of proteins in several aqueous two-phase systems.[6-8]

Aqueous two-phase systems (ATPS) naturally arise in aqueous mixtures of different water-soluble polymers or a single polymer and a specific salt. When two specific polymers, for example, dextran and Ficoll, are mixed in water above certain concentrations, the mixture separates into two 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 suitable for biological products.[9-11] These systems 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.[9-11] In ATPS, each phase provides a distinct solvent environment for proteins or other solutes. Differences in solute–solvent interactions in the two phases may lead to unequal solute distribution which is readily quantified by a partition coefficient, designated as K, and may be exploited for sensitive detection of changes in the solute structure.[9-11] The partition coefficient K of a protein is defined as the ratio of the protein concentrations in the two phases.[12]

Because of highly aqueous and therefore mild nature, which is compatible with the maintenance of macromolecular structure, ATPSs have been employed for the separation of biological macromolecules for over 50 years.[9, 13] Furthermore, various ATPS systems are also used in industrial biotechnology quality control for the detection of denaturation and degradation of proteins.[9] Overall, partitioning in ATPS has found applications in many different fields of science and technology due to the fact that ATPS media are nonvolatile, nontoxic, and non-flammable.[9, 14] It has been shown that the partition coefficient K-value may serve as a structural descriptor of a protein, and it is highly sensitive to structural changes in proteins (e.g., see refs.[15-22]). It has been established that the K-value reflects interactions between the solvent-exposed groups of the protein with the two aqueous solvent environments in ATPS.[9-11, 20, 21] It was shown in particular that the partition coefficient of the prostate-specific antigen (PSA) in Dextran–Ficoll ATPS is sensitive to the presence of other proteins, such as albumin, transferrin, and gamma-globulin due to non-specific PSA–protein interactions (formation of the PSA–protein encounter complexes) affecting the PSA conformation.[22]

It was also reported that the partition coefficients for different proteins can be used to characterize the differences between the proteins 3D structures. [6-8] The approach reported in refs. [6-8] was used here for analysis of osmolyte-protein interactions.

Results and Discussion

Partitioning of DNP-amino acids

Figure 1 shows the dependence of logarithms of partition coefficients $K^{(i)}_{DNP\text{-}AA}$ for DNP-amino acids in dextran-PEG-0.01M K/NaPB ATPS with and without 0.5 M osmolyte (sucrose, trehalose, and TMAO, see ref.[23]) on N_C, which is the equivalent number of CH_2 groups in the aliphatic side-chain of a given DNP-amino acid. In this analysis, each dependence is linear and can be described as:

$$lnK^{(i)}_{DNP\text{-}AA} = C^{(i)} + E^{(i)}N_C \qquad (1)$$

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 $\ln K_{DNP\text{-}AA}$ and may be used to characterize the difference between the electrostatic properties of the coexisting phases as described previously, see refs.[9-11]).

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

$$\Delta G^0 = -RTlnK \qquad (2)$$

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

$$\Delta G^0(CH_2) = -RTE \qquad (3)$$

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. 2–3 are listed in Table 1.

The presence of 0.5 M osmolyte decreases or slightly increases the difference between the relative hydrophobic character of the phases depending on the particular osmolyte present as indicated by the $\Delta G^0(CH_2)$ values listed 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 depending on the osmolyte present relative to the osmolyte-free ATPS.

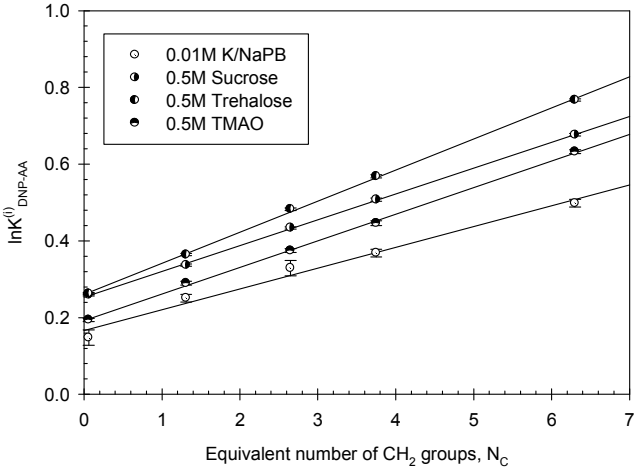

Figure 1. Logarithm of the partition coefficient value, lnKDNP-AA, for sodium salts of DNP-amino acids with aliphatic side-chains in aqueous dextran–PEG two-phase systems as a function of equivalent length of the side-chain, NC, expressed in terms of equivalent number of CH2 units: in dextran-PEG-0.01 M potassium/sodium phosphate buffer (K/NaPB), pH7.4 ATPS,[23] in dextran-PEG-0.5 M TMAO-0.01 M K/NaPB ATPS,[23] in dextran-PEG-0.5 M sucrose-0.01 M K/NaPB ATPS, and in dextran-PEG-0.5 M trehalose-0.01 M K/NaPB ATPS.

Table 1. Differences between the hydrophobic and electrostatic 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-05 M osmolyte-0.01M K/NaPB, pH 7.4 ATPS.

Compound	Partition coefficient			
	0.01M K/NaPB[a]	0.5M Sucrose	0.5M Trehalose	0.5M TMAO[a]
E*	0.076 ± 0.002	0.067 ± 0.0007	0.081 ± 0.001	0.069 ± 0.001
$\Delta G(CH_2)$*, cal/mole	-45 ± 1.2	-39.4 ± 0.44	-47.7 ± 0.6	-40.9 ± 0.6
C*	0.134 ± 0.007	0.253 ± 0.003	0.261 ± 0.004	0.192 ± 0.004
Adenine	1.220 ± 0.006	1.271 ± 0.009	1.367 ± 0.009	1.264 ± 0.005
Adenosine	1.128 ± 0.004	1.215 ± 0.003	1.256 ± 0.007	1.192 ± 0.007
Benzyl alcohol	1.409 ± 0.009	1.607 ± 0.009	1.697 ± 0.007	1.454 ± 0.008
Caffeine	1.154 ± 0.009	1.160 ± 0.004	1.186 ± 0.006	1.147 ± 0.008
Coumarin	1.490 ± 0.009	1.697 ± 0.006	1.780 ± 0.006	1.590 ± 0.008
Glucopyranoside[b]	1.232 ± 0.003	1.332 ± 0.009	1.368 ± 0.001	1.246 ± 0.003
3-Hydroxybenzaldehyde	1.709 ± 0.003	2.005 ± 0.007	2.118 ± 0.009	1.762 ± 0.009
Methyl anthranilate	1.77 ± 0.01	2.124 ± 0.007	2.24 ± 0.011	1.847 ± 0.007
p-Nitrophenol	1.486 ± 0.006	1.724 ± 0.002	1.796 ± 0.009	1.568 ± 0.004
Phenol	1.70 ± 0.02	2.07 ± 0.017	2.211 ± 0.009	1.809 ± 0.009
2-Phenylethanol	1.469 ± 0.005	1.695 ± 0.009	1.697 ± 0.009	1.51 ± 0.01
Vanillin	1.709 ± 0.009	1.969 ± 0.005	2.105 ± 0.006	1.761 ± 0.005
Gly	0.739 ± 0.008	0.732 ± 0.008	0.754 ± 0.007	0.715 ± 0.005
Ala	0.824 ± 0.004	0.853 ± 0.009	0.837 ± 0.008	0.762 ± 0.009
Val	0.833 ± 0.007	0.856 ± 0.009	0.87 ± 0.012	0.826 ± 0.003
Leu	0.872 ± 0.009	0.913 ± 0.006	0.919 ± 0.008	0.837 ± 0.008
Phe	0.884 ± 0.007	0.920 ± 0.006	0.932 ± 0.009	0.856 ± 0.007
Trp	0.905 ± 0.008	1.043 ± 0.005	1.074 ± 0.009	0.906 ± 0.004
Gln	0.781 ± 0.004	0.796 ± 0.009	0.796 ± 0.009	0.768 ± 0.004
Asn	0.715 ± 0.009	0.718 ± 0.006	0.716 ± 0.004	0.720 ± 0.005
Thr	0.782 ± 0.004	0.814 ± 0.007	0.803 ± 0.009	0.757 ± 0.009
Glu	0.765 ± 0.009	0.781 ± 0.009	0.782 ± 0.006	0.763 ± 0.002
Asp	0.759 ± 0.008	0.761 ± 0.005	0.75 ± 0.011	0.785 ± 0.009
Lys	0.584 ± 0.003	0.556 ± 0.006	0.56 ± 0.022	0.547 ± 0.007
Arg HCl	0.590 ± 0.005	0.6 ± 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.[23];

[b] – p-nitrophenyl-α-D-glucopyranoside

* Parameters E and $\Delta G(CH_2)$ values 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).

One of the likely reasons for an observed increase of the difference between the hydrophobic and electrostatic properties of the coexisting phases in the presence of osmolytes under consideration may be the different osmolyte-induced changes in the polymer compositions of the two phases.

Partitioning of different compounds

It has been shown previously[9, 24-27] 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:[28-31]

$$logK_{ji} = a_{io}\, logK_{jo} + b_{io} \qquad (4)$$

where K_{ji} and K_{jo} 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 ith and oth two-phase systems under comparison and generally may depend on the type of the solutes being examined.

The Collander equation[28-31] describes a linear relationship between partition coefficients of solutes of the same chemical nature in different organic solvent-water systems. The coefficients of the relationship depend on the particular systems under comparison as well as on the type of the solutes being examined. In practice it describes especially well partitioning of different compounds in different alkanol-water systems. It is emphasized in ref.[32] that the intercept (coefficient b_{io}) in Collander equation would differ for solutes with different hydrogen bond donor and acceptor abilities.

It has been established[9, 25, 33] that different organic compounds, proteins, and nucleic acids commonly fit the same linear relationship (Eq. 4) if the partition coefficients of solutes in two different ATPS are compared. The linear relationship may not hold for a solute (protein, organic compound) if an additive/ligand capable to bind or directly interact with the solute is introduced into one of the two ATPS. The reason is that the solute is modified in the presence of a ligand and its interactions with the solvent differ from those in the absence of the ligand. It was suggested that both coefficients (a_{io} and b_{io}) represent the distinctive features of the interactions of the solute with the solvents being compared.[9, 33]

Table 2. Partition coefficients for proteins in Dex-PEG-0.01M K/NaPB, pH 7.4 and Dex-PEG-05 M osmolyte-0.01 M K/NaPB, pH 7.4 ATPS.

Protein	Partition coefficient			
	0.01M K/NaPB[a]	0.5M Sucrose	0.5M Trehalose	0.5M TMAO[a]
α-Chymotrypsin	0.42 ± 0.01	0.42 ± 0.01	0.41 ± 0.01	0.42 ± 0.01
α-Chymotrypsinogen A	1.00 ± 0.01	1.78 ± 0.02	1.93 ± 0.01	1.37 ± 0.02
Concanavalin A	0.236 ± 0.003	0.242 ± 0.003	0.226 ± 0.003	0.233 ± 0.004
Hemoglobin human	0.129 ± 0.005	0.118 ± 0.003	0.091 ± 0.002	0.208 ± 0.002
β-Lactoglobulin A	0.46 ± 0.01	0.309 ± 0.004	0.255 ± 0.003	0.505 ± 0.005
β-Lactoglobulin B	0.33 ± 0.01	0.211 ± 0.003	0.151 ± 0.003	0.27 ± 0.007
Lysozyme	0.23 ± 0.003	0.325 ± 0.004	0.318 ± 0.002	0.255 ± 0.009
Papain	1.05 ± 0.01	1.27 ± 0.01	1.37 ± 0.01	1.21 ± 0.02
Ribonuclease A	0.313 ± 0.005	0.332 ± 0.006	0.311 ± 0.003	0.304 ± 0.006
Ribonuclease B	0.781 ± 0.004	0.347 ± 0.005	0.318 ± 0.004	0.768 ± 0.004
Trypsinogen	0.357 ± 0.005	0.463 ± 0.008	0.413 ± 0.006	0.431 ± 0.004

[a] – Data reported in ref.[23] and presented for comparison;

It has also been shown[27] that the Collander relationship may exist for comparison of partition coefficients for proteins in PEG-Na_2SO_4 ATPSs with different salt additives. It was suggested in particular[27] that proteins engaged in specific interactions with the salt additives present in the ATPS do not fit the linear relationship. We used this approach here to explore if there may be specific protein-osmolyte interactions.

Partitioning of simple organic compounds and free amino acids

Partition coefficients of simple organic compounds and free amino acids in the absence and presence of sucrose and trehalose are presented in Table 1. The data in Table 1 clearly show that for the most of the compounds examined, there are noticeable differences in their partition behavior in the ATPS with different osmolytes.

The partition coefficients for nonionic and ionizable organic compounds and zwitterionic free amino acids (Table 1) in the osmolyte-free and 0.5 M osmolyte-containing ATPS fit the linear relationships as shown in Figures 2a and 2b, respectively. These relationships may be described as:

$$lnK^{0.5M\ sucrose\text{-}0.01M\ K/NaPB} = 0.063_{\pm 0.007} + 1.15_{\pm 0.02} * lnK^{0.01M\ K/NaPB} \quad (5)$$
$$N = 29;\ R^2 = 0.9932;\ SD = 0.034;\ F = 3953$$

where $lnK^{0.5M\ sucrose\text{-}0.01M\ K/NaPB}$ and $K^{0.01M\ K/NaPB}$ are partition coefficients for the same compound in the dextran-PEG-0.5 M sucrose 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; adenine does not fit the relationship; and

$$lnK^{0.5M\ Trehalose\text{-}0.01M\ K/NaPB} = 0.081_{\pm 0.007} + 1.23_{\pm 0.02} * lnK^{0.01M\ K/NaP} \quad (6)$$
$$N = 30;\ R^2 = 0.9937;\ SD = 0.035;\ F = 4413$$

where $K^{0.5M\ trehalose\text{-}0.01M\ K/NaPB}$ is the partition coefficient for the compound in the dextran-PEG-0.5 M trehalose in 0.01 M K/NaPB ATPS; $K^{0.01M\ K/NaPB}$, N, R^2, SD, and F are as defined above.

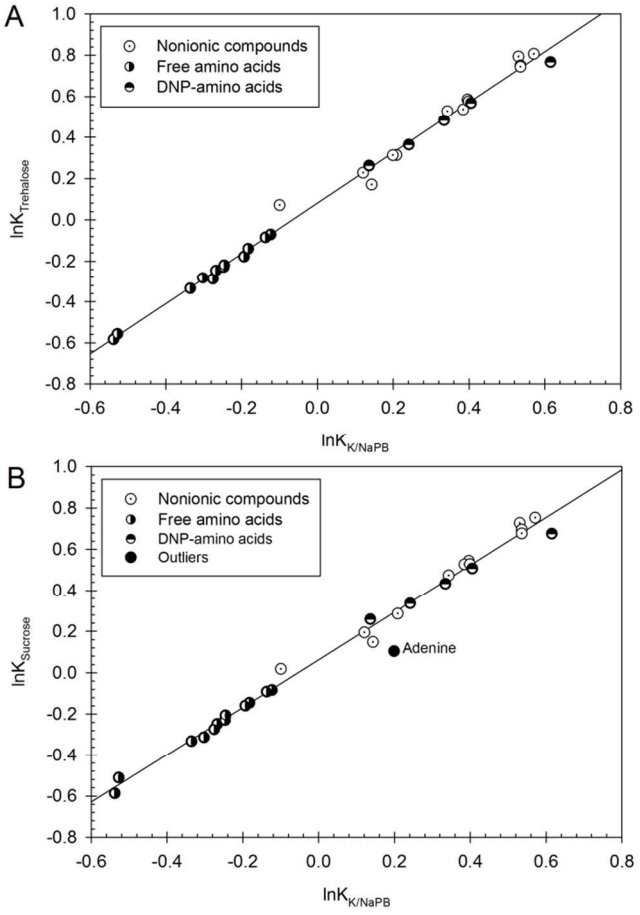

Figure 2. A. Logarithms of partition coefficients for nonionic organic compounds, free amino acids, and DNP-amino acids sodium salts in dextran-PEG-0.5 M sucrose-0.01 M K/NaPB ATPS versus those for the same compounds in dextran-PEG-0.01 M K/NaPB ATPS. K/NaPB – potassium/sodium phosphate buffer, pH 7.4. **B**. Logarithms of partition coefficients for nonionic organic compounds, free amino acids, and DNP-amino acids sodium salts in dextran-PEG-0.5 M trehalose-0.01 M K/NaPB ATPS versus those for the same compounds in dextran-PEG-0.01 M K/NaPB ATPS.[23] K/NaPB – potassium/sodium phosphate buffer, pH 7.4.

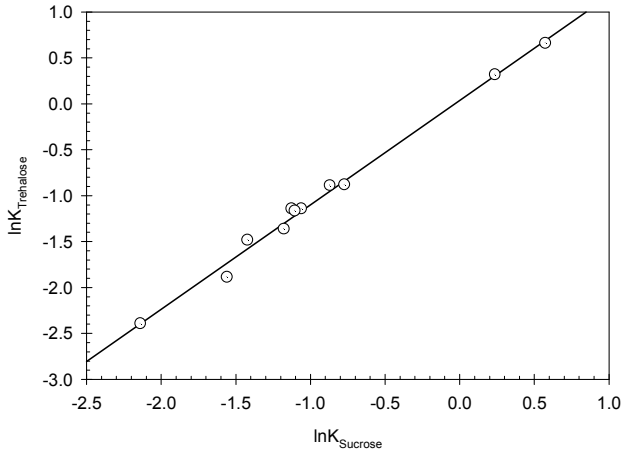

Figure 3. Logarithms of partition coefficients for proteins in dextran-PEG-0.5 M trehalose-0.01 M K/NaPB ATPS versus those for the same proteins in dextran-PEG-0.5 M sucrose-0.01 M K/NaPB ATPS. K/NaPB – potassium/sodium phosphate buffer, pH 7.4.

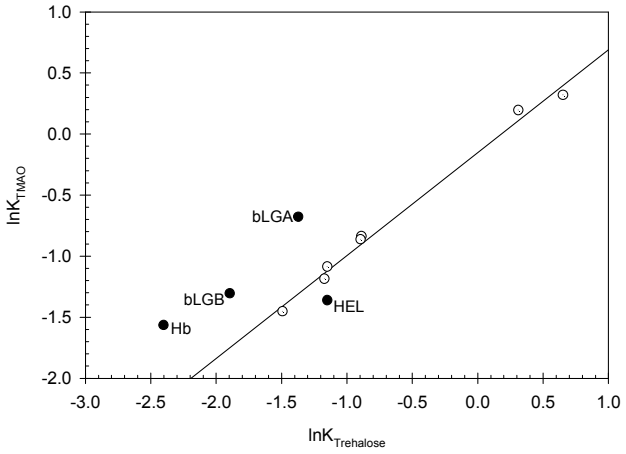

Figure 4. Logarithms of partition coefficients for proteins in dextran-PEG-0.5 M TMAO-0.01 M K/NaPB ATPS[23] versus those for the same proteins in dextran-PEG-0.5 M trehalose -0.01 M K/NaPB ATPS. K/NaPB – potassium/sodium phosphate buffer, pH 7.4.

Partition of proteins in osmolyte-free and osmolyte-containing ATPSs

Partition coefficients of the proteins in various ATPSs examined here in the absence and presence of osmolytes are presented in Table 2. The data in Table 2 indicate that for the most of the proteins studied, there are noticeable differences in their partition behavior in the ATPS employed. Analysis of the relationship between the proteins partition coefficients in the presence of 0.5M sucrose and 0.5M trehalose illustrated in Figure 3, shows that it may be described as:

$$lnK_{protein}^{0.5M\ trehalose\text{-}0.01M\ K/NaPB} = 0.04_{\pm 0.04} + 1.14_{\pm 0.03} * lnK_{protein}^{0.5M\ sucrose\text{-}0.01M\ K/NaPB} \quad (7)$$

$$N = 11;\ R^2 = 0.9927;\ SD = 0.08;\ F = 1219$$

where $K_{protein}^{0.5M\ trehalose\text{-}0.01M\ K/NaPB}$ and $K_{protein}^{0.5M\ sucrose\text{-}0.01M\ K/NaPB}$ are partition coefficients for the same protein in the dextran-PEG-0.5M trehalose-0.01M K/NaPB ATPS and in dextran-PEG-0.5M sucrose-0.01M K/NaPB ATPS, respectively; all the other parameters are as defined above.

Analysis of the data obtained for the same proteins in the same ATPS with 0.5M TMAO[23] as illustrated in Figure 4 show similar linear relationship described as:

$$lnK_{protein}^{0.5M\ TMAO\text{-}0.01M\ K/NaPB} = -0.15_{\pm 0.03} + 0.84_{\pm 0.03} * lnK_{protein}^{0.5M\ trehalose\text{-}0.01M\ K/NaPB} \quad (8)$$

$$N = 7;\ R^2 = 0.9914;\ SD = 0.07;\ F = 579$$

where $K_{protein}^{0.5M\ TMAO\text{-}0.01M\ K/NaPB}$ is the partition coefficient for a given protein in the dextran-PEG-0.5M TMAO-0.01M K/NaPB ATPS; all the parameters are defined above. It should be noted that hemoglobin, β-lactoglobulins A and B, and lysozyme do not fit the relationship.

There is no need in performing the similar correlation study for the protein partitioning in TMAO and sucrose, since there is a solid linear correlation between the partitioning of target proteins in TMAO and trehalose (Eq. 8), and since the partition coefficients of these proteins in trehalose and sucrose are also linearly interrelated (Eq. 7).

It has been established by us recently that various properties of small organic compounds and proteins in aqueous solutions, such as solubility,[34] lipophilicity (expressed as *logD* values in octanol-water system),[35] and partition coefficients in ATPS[8] in the presence of different salt additives are linearly interrelated as:

$$logSP_{salt\text{-}1} = k_1 + k_2 * logSP_{salt\text{-}2} + k_3 * logSP_{salt\text{-}3} \quad (9)$$

where SP_{salt} is the property of a solute in the presence of a given salt additive, k_1, k_2, and k_3 are constants depending on the solute property under consideration and the salt compositions employed.

It has been reported[8] in particular that the logarithms of partition coefficients of proteins in dextran-PEG ATPS containing 0.01M sodium phosphate buffer and different salts additives (CsCl, Na_2SO_4, $NaClO_4$, and NaSCN) at the slightly various concentrations in the range of 0.09-0.17M are linearly interrelated. Hence we examined the data obtained here and reported earlier in regard to applicability of Eq. 9 to partition coefficients of the proteins in presence of different osmolyte additives.[23] We intentionally examined the ln*K*-values for proteins in the ATPS with largest number of outliers in the Collander relationships between *K*-values for the proteins in each two ATPS. The data presented in Figure 5 show that for the proteins studied in the ATPS employed the linear relationship does exist, and it may be described as:

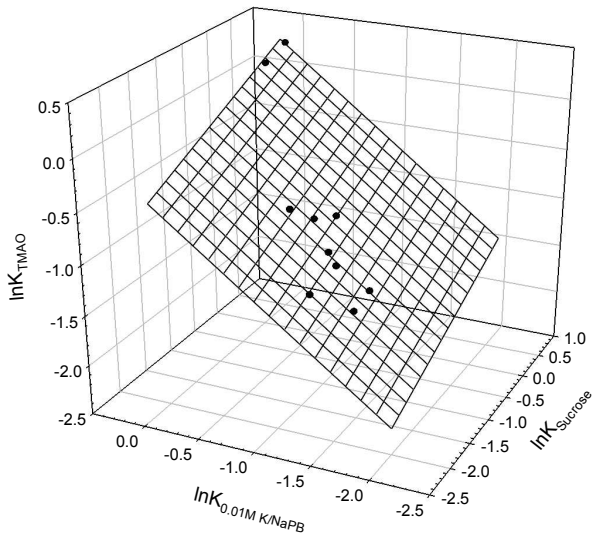

Figure 5. Logarithms of partition coefficients for proteins in dextran-PEG-0.5 M TMAO-0.01 M K/NaPB ATPS[23] versus those for the same proteins in dextran-PEG-0.5 M sucrose -0.01 M K/NaPB ATPS and in dextran-PEG-0.01 M K/NaPB ATPS.[23] K/NaPB – potassium/sodium phosphate buffer, pH 7.4.

$$lnK_{protein}^{0.5M\ TMAO\text{-}0.01M\ K/NaPB} = 0.13_{\pm 0.06} + 0.29_{\pm 0.095} * lnK_{protein}^{0.5M\ sucrose\text{-}0.01M\ K/NaPB} + 0.8_{\pm 0.13} * lnK_{protein}^{0.01M\ K/NaPB} \quad (10)$$

$N = 10$; $R^2 = 0.9856$; $SD = 0.08$; $F = 239$

where all the parameters are defined above. It should be noted that only data for hemoglobin do not fit the above relationship.

It should be noted that for essentially the same proteins there is no linear relationship between the ln*K*-values in ATPS containing 0.01M NaPB and in ATPS containing different salts additives in the same buffer, presumably because the protein-salt interactions at the low ionic strength of 0.026 M in 0.01M NaPB differ from those at 0.12-0.54 M ionic strength (in the presence of salt additives in 0.01 M or 0.11 M NaPB).[8] It seems to follow from the relationship described by Eq. 10 that the effects of the osmolytes examined here and TMAO[23] do not involve direct osmolyte-protein interactions even though the responses of different proteins to the presence of various osmolytes are clearly different.

It was shown[8, 27] that the 3D structure of a native protein in solution may be represented as a vector comprised of the protein partition coefficients in several (four or more) ATPSs of the same polymer and different ionic compositions. These vectors can then be used to estimate the differences between the structures of different proteins, but only after we have chosen a reference sample. Originally cytochrome *c* was used[8] as the reference, and the partition coefficients for all proteins were normalized against the partition coefficient for cytochrome *c* in each ATPS chosen to characterize the proteins structures (see below).

The normalized Euclidian distance between the normalized structural signatures in the 4-dimensional space represented by *K*-values in ATPSs with four different salt additives for each protein and cytochrome *c* was then evaluated. This distance was calculated as:

$$d_{i,o} = \left(\sum_j \left(\frac{K_i - K_o}{K_o} \right)^2 \right)^{0.5} \quad (11)$$

where $d_{i,o}$ is the distance between the structural signature of protein sample *i* to that of the reference protein, K_{ij} and K_{oj} are the partition coefficients for protein *i* and the reference protein *o* in system *j*, correspondingly. The structural distances for all the proteins examined were calculated[8] using Eq. 11 and *K*-values measured in ATPSs with different salt additives.

Since cytochrome *c* was not used in this study, we selected ribonuclease B (RNase B), which was examined earlier[8] and in this work, as a reference protein. The distance values determined previously were re-calculated using RNase B as a reference protein, and these values are listed in Table 3. These distances characterize the differences between the structures of the proteins examined in the presence of different salts in 0.01M NaPB. Similarly, we used the *K*-values determined for the proteins in ATPS with and without different osmolyte additives (see Table 2) and RNase B as a reference protein and estimated the structural distances between the 3D structures of the proteins with Eq. 11. Table 3 lists the resulting structural distances.

Table 3. Structural distances (d_{io}) for the proteins examined relative to RNase B.

Protein	d_{io}^{Salts*}	$d_{io}^{Osmolytes**}$
α-Chymotrypsin	1.09 ± 0.02	0.49 ± 0.01
α-Chymotrypsinogen A	7.4 ± 0.2	7.5 ± 0.2
Concanavalin A	1.46 ± 0.02	0.60 ± 0.01
Hemoglobin human	1.21 ± 0.02	1.22 ± 0.02
β-Lactoglobulin A	1.50 ± 0.02	0.65 ± 0.01
β-Lactoglobulin B	1.21 ± 0.02	0.68 ± 0.01
Lysozyme	26.0 ± 0.5	0.41 ± 0.01
Papain	7.4 ± 0.2	5.4 ± 0.2
Ribonuclease A	0.72 ± 0.01	0.13 ± 0.01
Ribonuclease B[a]	0	0
Trypsinogen	1.03 ± 0.02	0.53 ± 0.01

[a] Structural distances are determined with Eq. 11 (see text) and ribonuclease B (RNase B) as the reference protein.

* d_{io}^{Salts} determined based on partition coefficients for the proteins in dextran-PEG-salt-0.01M NaPB, pH 7.4 (salts: CsCl, $NaClO_4$, Na_2SO_4, NaSCN).[8]

** $d_{io}^{Osmolytes}$ determined based on partition coefficients for the proteins in dextran-PEG-0.5 M osmolyte-0.01 M K/NaPB, pH 7.4 (osmolytes: TMAO, trehalose and sucrose) and in dextran-PEG-0.01M K/NaPB, pH 7.4. NaPB – sodium phosphate buffer; K/NaPB – potassium/sodium phosphate buffer.

Figure 6 illustrates the concept of structural distances by showing partition coefficients of four proteins in three dextran-PEG ATPSs with the different additives with the ribonuclease B (RNase B) being used as a reference point. Although Figure 6 represents the relationship of structural distances to different

ionic environments, this representation is an obvious oversimplification, since only 3-D illustration is possible whereas structural signatures are determined in the 4-dimensional space represented by *K*-values in ATPSs with four different salt additives for each protein.

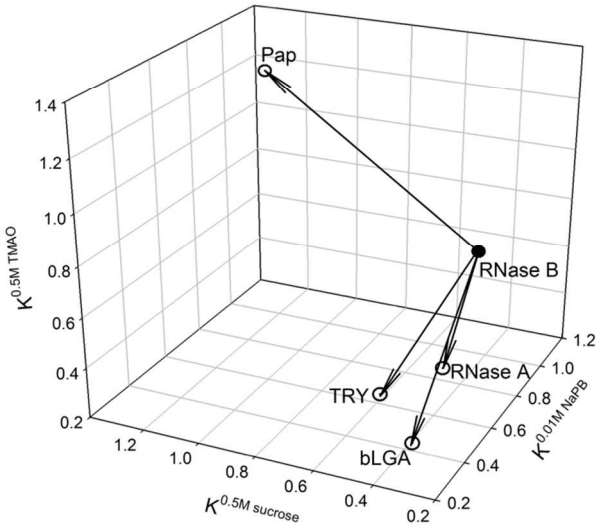

Figure 6. Illustration (simplified) of the concept of the structural distance. Partition coefficients of proteins in three dextran-PEG ATPSs with the additives indicated. Ribonuclease B (RNase B) is used as a reference point. The distances between the points for indicate proteins: ribonuclease A (RNase A), β-lactoglobulin A (bLGA), trypsinogen (TRY), and papain (Pap) and the point for RNase B shown by the arrows correspond to the structural distances.

Analysis of the structural distances estimated for proteins on the basis of their responses to the presence of different salt additives and to the presence of different osmolytes shows that there is a linear relationship between the two.

The relationship is illustrated in Figure 7 and it may be described as:

$$d_{io}^{\ Osmolytes} = -0.2_{\pm 0.12} + 0.75_{\pm 0.04} * d_{io}^{\ Salts} \qquad (12)$$
$$N = 9;\ R^2 = 0.9761;\ SD = 0.27;\ F = 286$$

where $d_{io}^{\ Salts}$ is the structural distance calculated for i^{th} protein with RNase B as the reference based on *K*-values determined in four ATPSs containing different salt additives in 0.01M sodium phosphate buffer, pH 7.4;[8] $d_{io}^{\ Osmolytes}$ is the structural distance calculated for the same i^{th} protein with RNase B as the reference based on *K*-values in four ATPSs listed in Table 2; all the parameters are as defined above. Lysozyme and α-chymotrypsinogen are the two proteins not fitting the relationship.

It should be mentioned that the structural distance $d_{io}^{\ Osmolytes}$ values listed in Table 3 should be considered as the preliminary estimates only. This is because we used the *K*-values for proteins in ATPS containing 0.5M trehalose and 0.5M sucrose as separate values, whereas the corresponding ln*K*-values are linearly interrelated (see Figure 3).

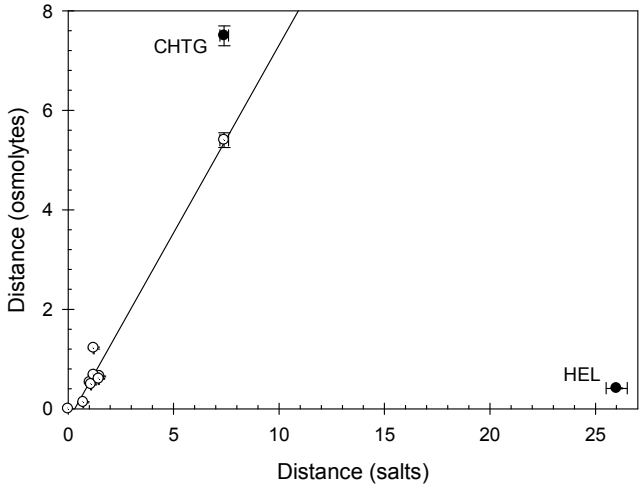

Figure 7. Structural distances determined for proteins relative to RNase B in the presence of different osmolytes, $d_{io}^{\ Osmolytes}$, versus those determined for the same proteins relative to RNase B in the presence of different salt additives, $d_{io}^{\ Salts}$ (Table 3).

Our study shows that osmolytes can bring noticeable changes to protein-solvent interactions in crowded environments. Here the crowding is generated by synthetic polymers. It would be interesting to perform similar studies under conditions of biomolecular crowding. Since our analyses are based on the investigation of the partitioning of small molecules and proteins in ATPSs, the challenge here is in finding appropriate biological polymers that would be able to undergo phase transitions to form ATPSs suitable for such partition analysis. Also, so far we used strongly stabilizing osmolytes, such as TMAO, sucrose, and trehalose. However, osmolytes, being natural solutions against osmotic stress inside various cells, are known to be of very different nature. Further studies with different types of osmolytes (such as proline, betaine, or denaturant urea) are needed before any general conclusion could be made. These studies are in progress in our laboratories.

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).

PROTEINS. α-Chymotrypsin from bovine pancreas, α -chymotrypsinogen A from bovine pancreas, concanavalin A from *Canavalia ensiformis* (jack beans), hemoglobin human, β-lactoglobulin A from bovine milk (>90%), β-lactoglobulin B

from bovine milk (>90%), lysozyme from chicken egg white, papain from papaya latex, ribonuclease B from bovine pancreas and trypsinogen from bovine pancreas were purchased from Sigma–Aldrich, ribonuclease A from bovine pancreas was from MB Biomedicals (Solon, OH, USA). All proteins and the corresponding abbreviations used throughout the text are listed in Table 4.

Table 4. Proteins used in this study

Protein	Abbreviation	Molecular weight, kDa	pI
α-Chymotrypsin	CHY	25.0	8.75
α-Chymotrypsinogen A	CHTG	25.7	8.97
Concanavalin A	ConA	104.0	4.5-5.5
Hemoglobin human	HHb	64.5	6.8
β-Lactoglobulin A	bLGA	18.3	5.3
β-Lactoglobulin B	bLGB	18.3	5.1
Lysozyme	HEL	14.3	11.0
Papain	Pap	23.4	8.75-9.55
Ribonuclease A	RNase A	13.7	9.63
Ribonuclease B	RNase B	17.0	8.88
Trypsinogen	TRY	24.0	8.7; 9.3

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, 2-phenyl ethanol, vanillin, p-nitrophenol, p-nitrophenyl-α-D-glucopyranoside, tryptophan, glutamic acid, lysine, and trehalose were from Sigma; sucrose was from USB (Cleveland, OH, USA); phenylalanine, leucine, arginine, aspartic acid, asparagine, valine, threonine, glycine, alanine were from MB Biomedicals, and glutamine was provided by Bachem (King of Prussia, PA, USA) and used without further purification.

Other chemicals. *o*-Phthaldialdehyde (OPA) reagent solution (complete) was purchased from Sigma. All salts and other chemicals used were of analytical-reagent grade and used without further purification.

Methods

Aqueous two-phase systems (ATPSs). Stock solutions of PEG 8000 (50 wt.%), Dex-75 (~42 wt.%), and sucrose (1.8 M) and trehalose (1.25 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[12] 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 sucrose or trehalose, stock buffer solutions, and water were added to give the ionic, polymer, and smolyte composition required for the final system (after the sample addition – see below) with total weight of 0.5g (total volume 440±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 sucrose (or 0.5 M trehalose).

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 including proteins 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 sucrose or trehalose. 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.

For the analysis of the proteins (with exception of hemoglobin) and 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.

For the analysis of the other compounds (including hemoglobin) 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 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.[12] The deviation from the average *K* value was always less than 3 % and in most cases lower than 1 %.

ELECTROPHORESIS. All protein samples were characterized by SDS-PAGE electrophoresis in a microfluidic chip using Bioanalyzer 2100, Protein 200 Plus Assay (Agilent Technologies, USA) under non-reduced conditions. All proteins were observed as single bands in the electrophoregrams.

Conclusions

We examined partitioning of 30 organic compounds and 11 proteins in aqueous dexran-PEG two-phase systems containing 0.5M sucrose and 0.5M trehalose additives. The results were analyzed together with those obtained in the presence of 0.5M TMAO reported previously. The analysis implies that proteins responses to the presence of different osmolytes are governed by the proteins structures. These responses are less pronounced than those observed in the presence of different salts additives but appear to be governed by the same structural features of the proteins.

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Notes and references

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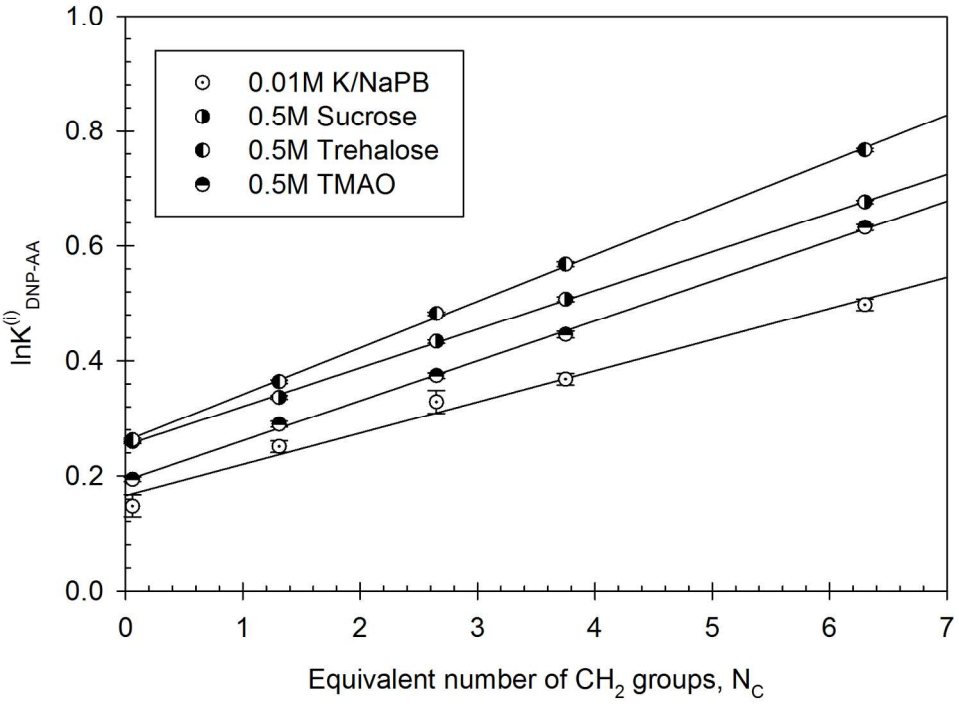

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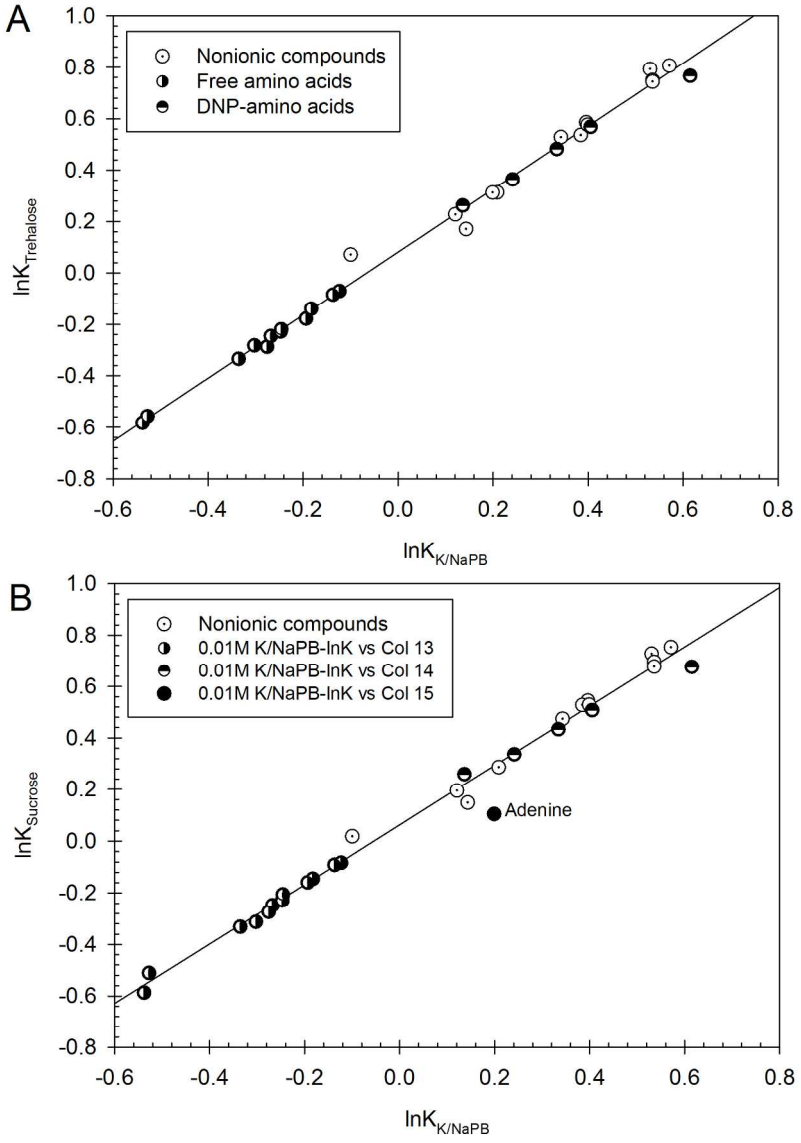

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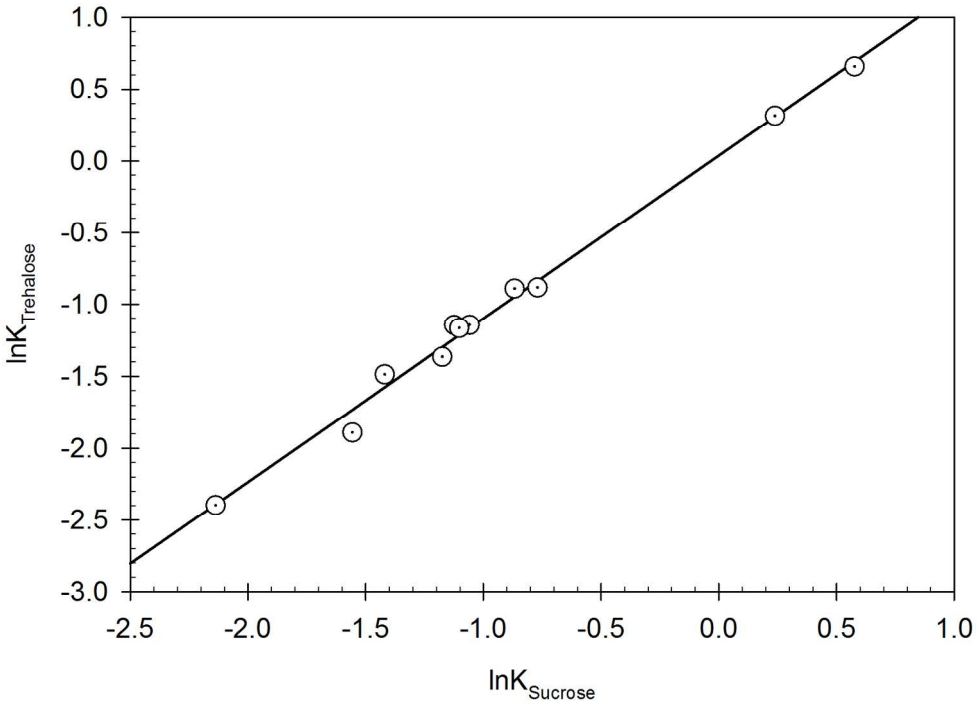

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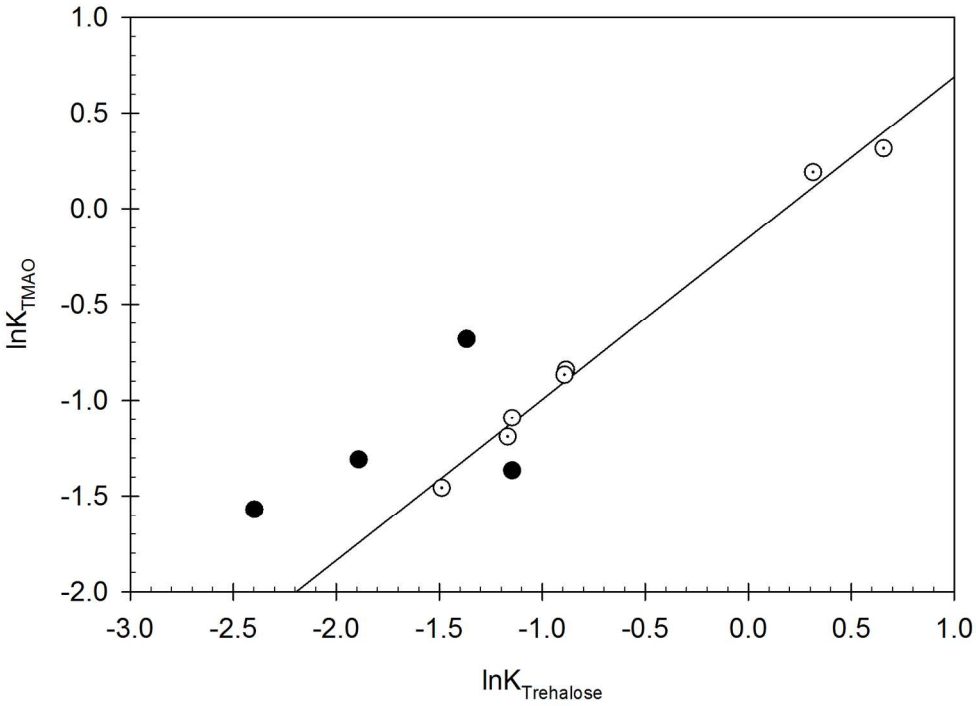

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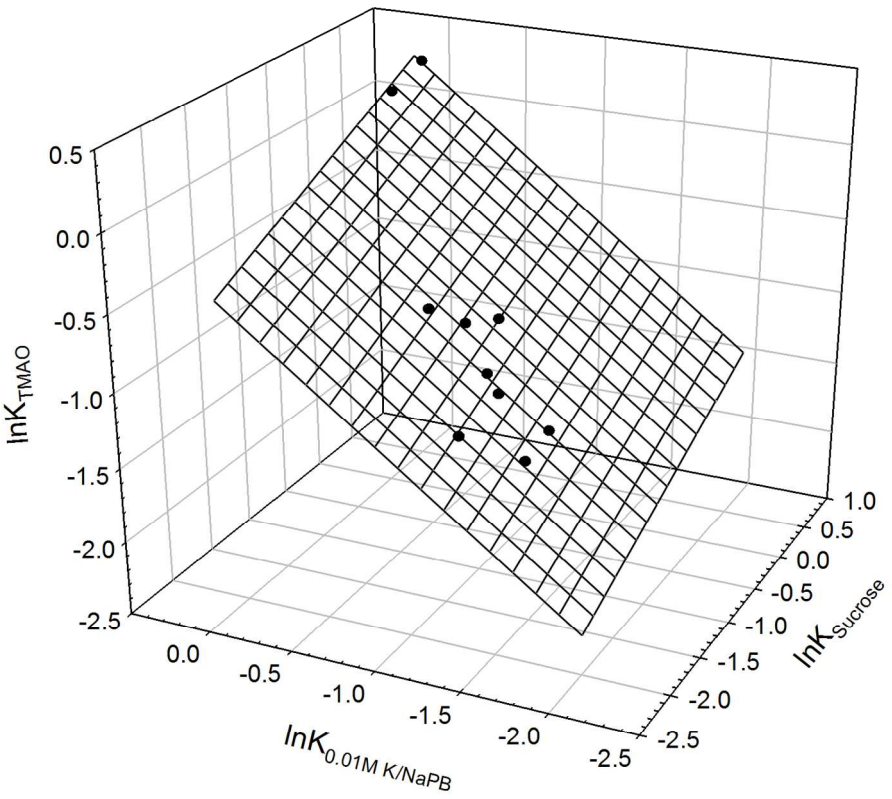

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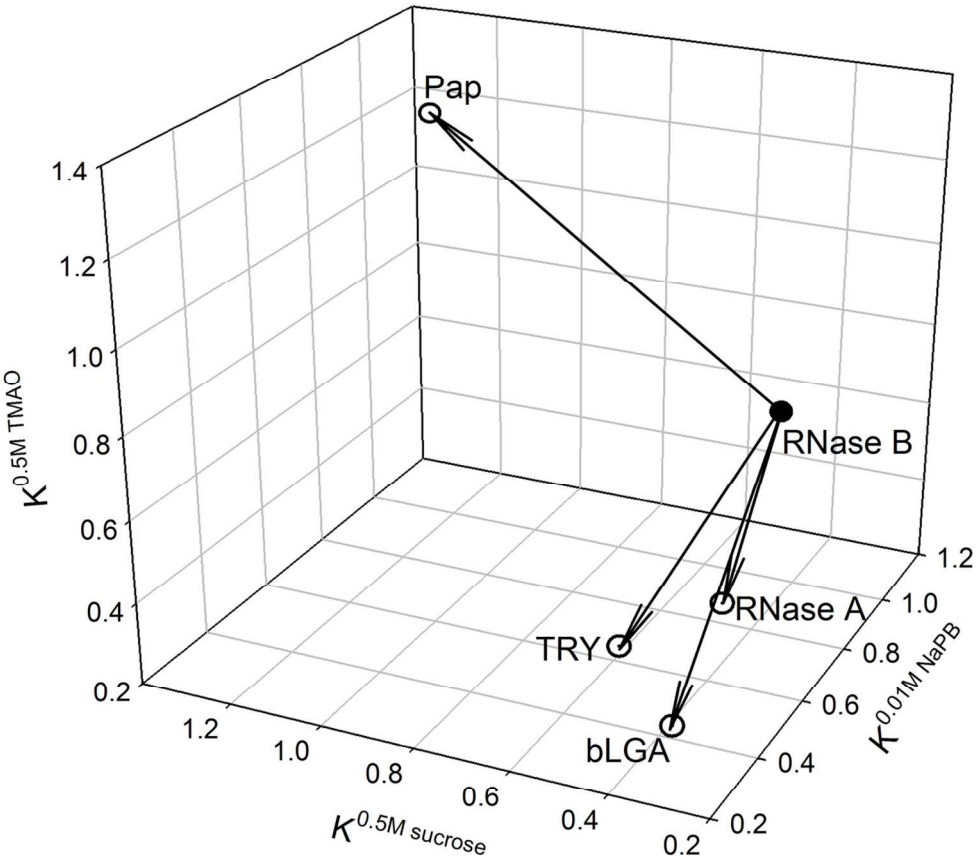

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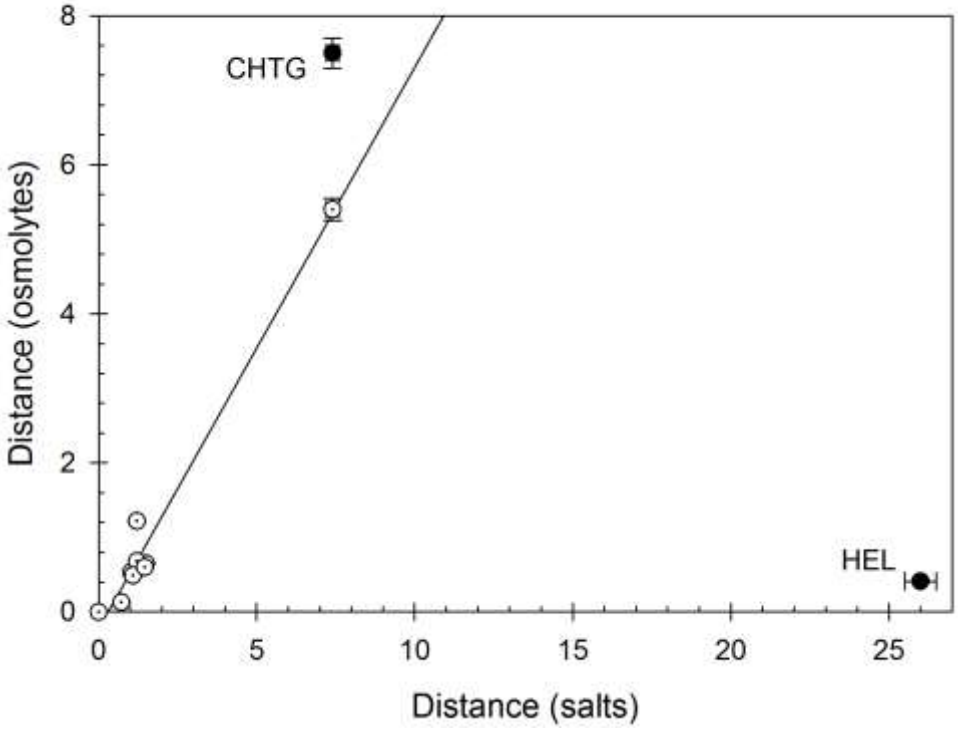

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