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Protein precipitation and denaturation by dimethyl sulfoxide

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Abstract

Solvent conditions play a major role in a wide range of physical properties of proteins in solution. Organic solvents, including dimethyl sulfoxide (DMSO), have been used to precipitate, crystallize and denature proteins. We have studied here the interactions of DMSO with proteins by differential refractometry and amino acid solubility measurements. The proteins used, i.e., ribonuclease, lysozyme, β-lactoglobulin and chymotrypsinogen, all showed negative preferential DMSO binding, or preferential hydration, at low DMSO concentrations, where they are in the native state. As the DMSO concentration was increased, the preferential interaction changed from preferential hydration to preferential DMSO binding, except for ribonuclease. The preferential DMSO binding correlated with structural changes and unfolding of these proteins observed at higher DMSO concentrations. Amino acid solubility measurements showed that the interactions between glycine and DMSO are highly unfavorable, while the interactions of DMSO with aromatic and hydrophobic side chains are favorable. The observed preferential hydration of the native protein may be explained from a combination of the excluded volume effects of DMSO and the unfavorable interaction of DMSO with a polar surface, as manifested by the unfavorable interactions of DMSO with the polar uncharged glycine molecule. Such an unfavorable interaction of DMSO with the native protein correlates with the enhanced self-association and precipitation of proteins by DMSO. Conversely, the observed conformational changes at higher DMSO concentration are due to increased binding of DMSO to hydrophobic and aromatic side chains, which had been newly exposed on protein unfolding.

Keywords: Dimethyl sulfoxide; Amino acid solubility; Preferential interaction; Protein denaturation; Self-association

1. Introduction

A variety of solvent additives, or co-solvents, are used to purify, refold, stabilize (or destabilize and unfold), solubilize (or precipitate) and characterize proteins [1,2]. Technologies utilizing solvent additives, including certain organic solvents, are increasingly important in the proteomic research fields, in particular in the production of recombinant proteins for pharmaceutical applications [3–5]. Therefore, understanding the mechanism by which the solvent additives exert their effects on proteins is a key to their applications. The solvent additives are often used on a trial-and-error basis. Dimethyl sulfoxide (DMSO) is one of the most versatile solvents in biological science [6]. DMSO readily penetrates and diffuses through biological membranes and tissues and protects mammalian or bacterial cells from various damages during freezing and frozen storage, although it is a protein-denaturant at high concentrations or at high temperatures [6–8]. DMSO is also used to crystallize proteins and to study the mechanism of enzyme function and conformational stability of proteins [6,9–12]. DMSO has been shown to induce tubulin polymerization [13,14]. However, the mechanism of these effects of DMSO on protein solubility and conformational stability is not well understood; its effects on water structure have been proposed [6].

Preferential interactions have explained the effects of various solvent additives, including some organic solvents such as 2-chloroethanol, ethanol and 2-methy-2,4-pentanediol, on protein solubility, denaturation and stability [15–29]. Amino acid solubility data have revealed the interactions between solvent additives and amino acids, amino acid side chains and peptide groups [30–37]. This paper reports the protein preferential interactions and amino acid solubility in aqueous DMSO solution in an effort to obtain a mechanistic understanding of the effects of DMSO on protein denaturation and solubility.

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2. Materials and methods

The proteins: ribonuclease (RNase), lysozyme (Lyso), β-lactoglobulin (β-LG) and chymotrypsinogen (CTG) and the various reagents were obtained from Sigma.

Preferential interactions were studied by differential refractometry according to Pittz et al. [38–40]. The refractive index increment of proteins was measured at 20 °C using a photoelectric differential refractometer [38] as described by Pittz et al. [40]. For a given solvent, protein solutions at different concentrations were prepared at constant chemical potential (dialysis equilibrium) and molality (direct dissolution of dry protein powder) of the solvent components. The protein solution and the corresponding solvent were placed in the sample and reference compartments of the differential refractometer cell and the differential refractive index, Δn, was determined as a function of protein concentration in grams per ml (C_2); here components 1, 2 and 3 correspond respectively to water, protein and dialyzable component, i.e., DMSO. The refractive index increment of the protein, $(\partial n/\partial C_2)$, was determined by plotting $(\Delta n/C_2)$ vs. C_2. There was little protein concentration dependence of $(\Delta n/C_2)$. As described above, $(\partial n/\partial C_2)$ was determined at constant molality, $(\partial n/\partial C_2)_{T,\ P,\ m_3}$, and constant chemical potential, $(\partial n/\partial C_2)_{T,\ \mu_1,\ \mu_3}$, of diffusible components at 20 °C, where n is the refractive index, μ_1, μ_2, and μ_3 are the chemical potentials of water, protein and DMSO, T is the Kelvin temperature and P is the pressure. The refractive index, n, of DMSO solutions was determined using a Bausch and Lomb precision refractometer at 20 °C. From the plot of n vs. DMSO concentration in grams per ml, C_3, the refractive index increment of DMSO, $(\partial n/\partial C_3)$, was obtained. The partial specific volume of DMSO, υ_3, was determined in a similar manner using an Anton Paar precision density meter DMA-02 (17).

From these refractive index increments of the proteins, the preferential interaction parameters were calculated according to Pittz et al. [38,40] and Pittz and Timasheff [39], i.e., the preferential interaction of DMSO with the proteins, $(\partial g_3/\partial g_2)_{T,\ \mu_1,\ \mu_3}$, was obtained from

$$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} = 1/(1 - C_3\upsilon_3)\Big\{[(\partial n/\partial C_2)_{T,\mu_1,\mu_3} - (\partial n/\partial C_2)_{T,P,m_3}]/(\partial n/\partial C_3)_{T,P,m_3}\Big\} \quad (1)$$

where g_i is the concentration of component i in grams per gram of water.

Table 1
Thermodynamic properties of DMSO in aqueous solution at 20 °C

DMSO %	g_3, g DMSO/g water	m_3, mol DMSO/kg water	υ_3, ml/g	$(\partial n/\partial C_3)_{T,\ P,\ m_2}$
10	0.1097	1.404	0.8552	0.1444
20	0.2420	3.097	0.8564	0.1456
30	0.4047	5.180	0.8577	0.1479
40	0.6104	7.813	0.8615	0.1507
50	0.8783	11.24	0.8695	0.1540
60	1.244	15.92	0.8740	0.1479
70	1.785	22.85	0.8833	0.1418
80	2.680	34.30	0.8941	0.1342

Radius of water=0.14 nm. Radius of DMSO=0.3 nm (using υ_3=0.86 ml/g).

Table 2
Preferential interaction parameters of RNase with DMSO at pH 6.0

DMSO %	$(\partial g_3/\partial g_2)_{T,\ m_1,\ m_3}$	$(\partial g_1/\partial g_2)_{T,\ \mu_1,\ m_3}$	DMSO binding	
			A_1=0.277 g/g (native)	
			g/g	mol/mol
20	−0.0423±0.0157	0.175±0.065	0.0247	4
40	−0.153±0.042	0.251±0.068	0.0160	3
50	−0.0727±0.0195	0.083±0.022	0.170	30
60	−0.0810±0.0583	0.065±0.047	0.263	46

A_1 calculated from the excluded volume should be ml per gram protein. Here it is expressed as grams per gram with the assumption of water density of 1 g/ml. A_1=0.490 g/g (unfolded).

Solubility measurements of amino acids in aqueous DMSO solutions were carried out according to Arakawa and Timasheff [37]. Amino acids were thoroughly dried in a vacuum oven and weighed into test tubes. The solvents were added to the test tubes and the total weights were measured. These test tubes were incubated at 20 °C for over 72 h with frequent mixing; the amino acid–solvent mixtures were filtered to remove insoluble materials. The density of the filtrate was determined by the density meter and plotted against the weight concentration of the amino acids in the mixture. The inflection point was taken as the solubility.

3. Results and discussion

Preferential interaction measurements were carried out in aqueous solution at 20 °C, at which temperature the proteins maintain their native structures in the absence of DMSO. The amino acid solubility was determined at the same temperature.

3.1. Preferential interaction

Preferential interactions of DMSO were examined for RNase in 20 mM phosphate, 10 mM NaCl, pH 6.0; for Lyso in 20 mM glycine–HCl, 10 mM NaCl, pH 2.0; for β-LG in 10 mM NaCl, pH 2.0 (pH adjusted with HCl); for CTG in 10 mM HCl, pH 2.0. Table 1 lists the thermodynamic parameters of aqueous DMSO solutions required for the calculation of the preferential interaction parameters. The concentration of DMSO in Table 1 is expressed on the volume basis (%), the weight basis, i.e., grams of DMSO per gram of water (g_3) and the molal basis, moles of DMSO per kg of water (m_3). Both the partial specific volume,

Table 3
Preferential interaction parameters of Lyso with DMSO at pH 3.0

DMSO %	$(\partial g_3/\partial g_2)_{T,\ \mu_1,\ \mu_3}$	$(\partial g_1/\partial g_2)_{T,\ m_1,\ m_3}$	DMSO binding			
			A_1=0.273 g/g (native)		A_1=0.486 g/g (unfolded)	
			g/g	mol/mol	g/g	mol/mol
20	−0.0589±0.0365	0.244±0.151	0.00716	1	–	–
40	−0.112±0.017	0.184±0.028	0.0546	10	–	–
60	0.0060±0.0526	−0.005±0.042	0.345	63	–	–
80	0.130±0.078	−0.049±0.029	–	–	1.43	262

υ_3, and refractive index increment, $(\partial n/\partial C_3)_{T,\,P,\,m_2}$, of DMSO vary little over the wide range of DMSO concentration used, i.e., between 10 and 80%. The preferential interactions of DMSO with the four proteins are summarized in Tables 2 (RNase), 3 (Lyso), 4 (β-LG) and 5 (CTG). The preferential interactions of the proteins with DMSO are plotted in Fig. 1 as a function of DMSO concentration. Although the preferential interactions of DMSO with the proteins contain considerable experimental errors (see tables), the preferential binding is found to be significantly negative for all four proteins at low DMSO concentration and becomes positive at higher DMSO concentration for three of the proteins. For RNase, it remains negative even at 60% DMSO, the highest concentration measured for this protein (see Fig. 1, ○ and Table 2). Spectral analysis (data not shown) has shown that these proteins are in the native state at low DMSO concentrations and 20 °C, namely, CTG assumes the native structure below 40% DMSO, β-LG below 50%, Lyso below 60% and RNase below 70%.

The negative preferential binding of DMSO, observed for all the proteins at low DMSO concentrations, means a higher affinity of the proteins for water than for DMSO. Hence there is a deficiency of DMSO in the vicinity of the protein surface relative to its concentration in the bulk phase, which leads to preferential hydration and is related to preferential binding by [17,18,40]

$$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3} = -(1/g_3)(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}. \quad (2)$$

Eq. (2) states that when $(\partial g_3/\partial g_2)_{T,\,\mu_1,\,\mu_3}$ is negative, $(\partial g_1/\partial g_2)_{T,\,\mu_1,\,\mu_3}$ is positive which means that there is an excess of water around the protein surface. All four proteins are preferentially hydrated in the native state. Fig. 1 and Tables 2–5 show that the preferential binding of DMSO is increasingly negative and, hence, the preferential hydration increases in the order: CTG (●)<β-LG (□)<Lyso (Δ)<RNase (○).

At the higher DMSO concentrations, these proteins, except for RNase (for which the measurement was not performed above 60%), showed a positive value of preferential binding, i.e., they had a higher affinity for DMSO than for water (Fig. 1 and Tables 3–5). Preferential DMSO binding at high DMSO concentration increases in the order of Lyso<β-LG<CTG, i.e., the reverse of the order of preferential hydration at low DMSO concentration. This order is consistent with the calculated polarity parameter [41], namely, as shown in Table 6, this parameter decreases in the order, RNase>Lyso>β-LG>CTG. Thus, it is evident that the less polar proteins (more hydrophobic proteins, e.g., CTG) bind more DMSO and are less preferentially hydrated, which suggests the importance of the hydrophobicity of the proteins in binding DMSO. At high DMSO concentration, there is a sharp increase in preferential DMSO binding for CTG, β-LG and Lyso, as shown in Fig. 1. This parallels with the DMSO concentration of the onset of protein unfolding, shown by the arrows on the data point for each protein (except RNase). The conformational changes occur at 40–60% DMSO for CTG, 60–70% for β-LG, 60–70% for Lyso and 70–90% for RNase (data not shown).[1]

Table 4
Preferential interaction parameters of β-LG with DMSO at pH 2.0

DMSO %	$(\partial g_3/\partial g_2)_{T,\,\mu_1,\,\mu_3}$	$(\partial g_1/\partial g_2)_{T,\,\mu_1,\,\mu_3}$	DMSO binding			
			A_1=0.257 g/g (native)		A_1=0.460 g/g (unfolded)	
			g/g	mol/mol	g/g	mol/mol
20	−0.0034±0.0243	0.014±0.099	0.0588	14	–	–
40	−0.0512±0.0172	0.084±0.028	0.105	25	–	–
50	−0.0189±0.0276	0.022±0.031	0.206	48	–	–
60	−0.0099±0.0455	0.008±0.037	–	–	0.562	132
80	0.176±0.128	−0.066±0.048	–	–	1.408	332

Table 5
Preferential interaction parameters of CTG with DMSO at pH 2.0

DMSO %	$(\partial g_3/\partial g_2)_{T,\,\mu_1,\,\mu_3}$	$(\partial g_1/\partial g_2)_{T,\,\mu_1,\,\mu_3}$	DMSO binding			
			A_1=0.221 g/g (native)		A_1=0.397 g/g (unfolded)	
			g/g	mol/mol	g/g	mol/mol
10	−0.0030±0.0189	0.028±0.173	0.0212	7	–	–
20	−0.0092±0.0108	0.038±0.045	0.0440	14	–	–
30	−0.0302±0.0146	0.075±0.036	0.0592	19	–	–
40	−0.0331±0.0223	0.054±0.037	0.192	33	–	–
50	0.0349±0.0184	−0.040±0.021	0.229	75	0.383	126
60	0.108±0.016	−0.087±0.013	–	–	0.602	198
70	0.245±0.079	−0.137±0.045	–	–	0.954	314
80	0.478±0.165	−0.179±0.062	–	–	1.54	507

The preferential binding of DMSO at higher DMSO concentrations suggests that DMSO binds to sites on the proteins, because the preferential binding parameter, $(\partial g_3/\partial g_2)_{T,\,\mu_1,\,\mu_3}$, is related to the actual binding by

$$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} = A_3 - g_3 A_1 \quad (3)$$

where A_3 is the actual binding of DMSO, i.e., the total number of DMSO molecules that interact with the protein and A_1 is the hydration, expressed as grams per gram protein. Eq. (3) indicates that the preferential binding of DMSO has contributions from both the DMSO and water bindings, as well as the bulk concentration of DMSO. This can be readily viewed in the schematic representation of Fig. 2 [42], in which the bulk phase and the protein vicinity are separated by a hypothetical dialysis membrane. In dialysis equilibrium, what is measured is the difference in additive concentration between the bulk phase and the protein solution. The left side panel shows the familiar situation, in which the concentration of the additive is greater on the protein side than in the bulk phase, i.e., $(\partial g_3/\partial g_2)_{T,\,\mu_1,\,\mu_3}>0$, while the right side panel indicates a deficiency of the additive in the vicinity of the protein relative to the bulk phase, i.e. the situation observed at the lower DMSO concentrations. A calculation of A_3 requires knowledge of the hydration value, i.e. the amount of water in the layer at the protein surface from which

[1] The spectral data were not of sufficient quality to draw transition curves. Nevertheless, the character of the CD spectra changed drastically over the cited concentration spans.

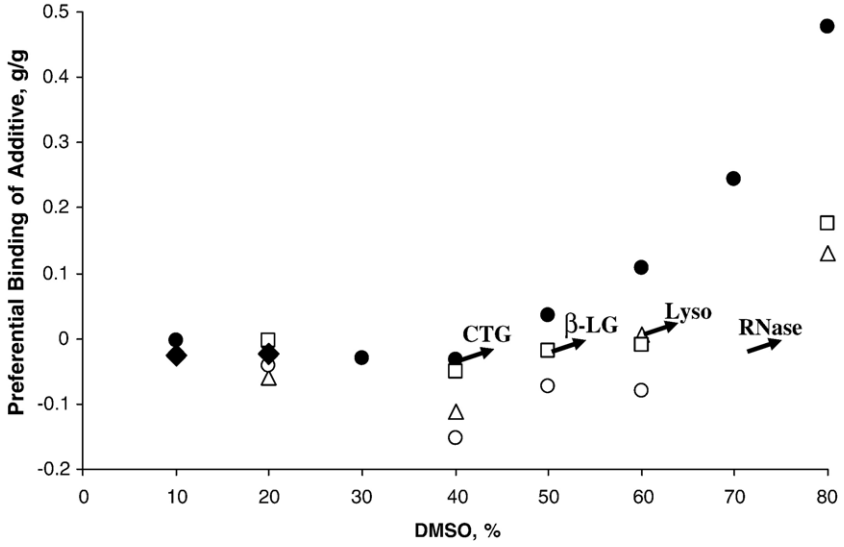

Fig. 1. Preferential interactions of DMSO with various proteins CTG (solid circle), β-LG (open square), Lyso (open triangle), RNase (open circle) and tubulin (solid diamond).

the additive is excluded. One of the universal causes of preferential hydration in the presence of additives is the excluded volume [43]. If the hydrodynamic size of the additive (here DMSO) is larger than the size of water, there must be a layer of water at the protein surface from which the additive is physically excluded. Such volume has been calculated based on the difference in the radii of the protein and the additive [44,45]. However, water is also physically excluded from the protein surface. In fact, such exclusion of water molecules has been correlated with protein folding in aqueous solution [46,47]. The exclusion of water molecules from the protein surface is thermodynamically unfavorable, which forces the protein molecule to assume a more compact structure and favors protein folding, because compact structures exclude fewer water molecules. With this in consideration, in the three component systems the effective excluded volume of DMSO can be calculated from the difference in the excluded volumes of DMSO and water (Prakash, Xie and Timasheff, in preparation), as

$$A_1 = 4\pi Av\left\{\left(R_{\text{Protein}} + R_{\text{DMSO}}\right)^3 - \left(R_{\text{Protein}} + R_{\text{water}}\right)^3\right\}/3M_2 \quad (4)$$

where R_{Protein} is the radius of the protein, R_{DMSO} is the radius of DMSO and R_{water} is the radius of water, Av is Avogadro's number and M_2 is the molecular weight of the protein. The radius of water used here was 0.14 nm [47] and the radius of DMSO was calculated from the partial specific volume (Table 1) to be 0.30 nm. The radii of the proteins were taken from Arakawa and Timasheff [48] (Table 6). The values of A_1 thus obtained for each protein are shown in Tables 2–5. Using the value of A_1, the DMSO binding in g/g was calculated for each protein at different DMSO concentrations and summarized in Table 2–5 and plotted in Fig. 3. DMSO bindings in mol/mol were also calculated from the molecular weight of the proteins and DMSO and given in Tables 2–5. As stated above, these proteins, except for RNase, unfold at higher DMSO concentrations, leading to a more expanded structure. The hydrodynamic radius of the unfolded structure was assumed to be 1.38 times larger than that of the native protein [43]. The preferential hydration contribution from the excluded volume of the unfolded protein thus calculated is also given in each table.[2] The values of DMSO binding for Lyso at 80%, for β-LG at 60 and 80% and for CTG at 60–80%, where the proteins are fully unfolded, were obtained using the A_1 value based on the radius of the unfolded proteins. For CTG, values using A_1 for both the native and unfolded structures are given at 50% DMSO, as this protein unfolds between 40 and 60% DMSO.[3] The actual DMSO binding values shown in Fig. 3 reflect binding to the native protein at lower DMSO concentrations and binding to the unfolded protein at higher DMSO concentrations. The range of DMSO binding of CTG at 50% DMSO is shown by a bar above the data point calculated for the native protein. As shown in Fig. 3, it is evident that binding of DMSO is small at low DMSO concentrations and sharply increases above 40–60% DMSO, depending on the protein. This sharp increase in DMSO binding correlates with the onset of DMSO-induced conformational changes.

The A_1 values calculated from the excluded volume depend on the radius of the protein and are larger for smaller proteins according to Eq. (4) and as shown in Tables 2–5. The A_1 value increases in the order of CTG<β-LG<Lyso<RNase, which is identical to the order of the preferential hydration observed at lower DMSO concentrations. Therefore, it may be concluded that the observed differences in preferential hydration between the four proteins at lower DMSO concentrations are, at least in part, due to the excluded volume effect and hydrodynamic size

[2] For RNase, there is no unfolding even at 60% DMSO and hence the value of A_1 (=0.490) for the unfolded state was not needed in the analysis.

[3] We do not have sufficient structure transition data to estimate the ratio of the native to unfolded as a function of DMSO concentration, which is required to calculate the DMSO binding of CTG at 50%.

Table 6
Molecular parameter of the proteins

Protein	Polarity parameter[a]	Radius	Molecular weight
Ribonuclease	1.73	1.55 nm	13,700
Lysozyme	1.18	1.58	14,300
b-Lactoglobulin	0.96	1.76	18,400
Chymotrypsinogen	0.83	1.95	25,700

[a] Calculated according to Bigelow [41].

of the proteins. The observed scatter in the preferential interaction data does not permit the quantitative analysis of DMSO binding based on the surface property of the protein.

3.2. Amino acid solubility

In order to enhance further the understanding of DMSO interactions with the proteins, the solubility of glycine in DMSO solutions was examined; i.e., polar electrically-neutral glycine may represent the hydrophilic surface of the native proteins. Table 7 shows the solubility of glycine, DL-alanine, L-leucine and L-tryptophan as a function of DMSO concentration. Although substantial experimental errors occur when the solubility becomes low, the errors do not affect the trend of the solubility changes with DMSO concentration. The solubility decreases 100–1000 fold at higher DMSO concentrations. Hence it was calculated as log solubility ratio, which is the ratio of the solubility of glycine in DMSO solutions to its solubility in water. Fig. 4 plots the solubility ratio for glycine. For comparison, the data for aqueous ethanol and dioxane at 25 °C are also shown [33]. The solubility of glycine sharply decreases as the DMSO concentration is increased; e.g., the solubility decreases 100-fold in 50% DMSO (white bar). A similar trend of glycine solubility is seen in aqueous ethanol and dioxane solutions; a 100-fold decrease occurs in 70% ethanol solution. This clearly indicates that the interactions of these organic solvents with the electrically-neutral polar glycine molecule are highly unfavorable and this unfavorable interaction increases sharply at higher organic solvent concentrations (Fig. 4). The effects are similar in the three organic solvents, suggesting a common mechanism of their interactions with glycine. Namely, the hydrophobic nature of these organic solvents makes the interactions with the polar glycine molecule highly unfavorable.

The effects of DMSO on amino acid solubility are a function of the side chains. Fig. 5 shows the solubility of glycine, alanine, leucine and tryptophan in aqueous DMSO solutions as a function of concentration. The solubility of both alanine and leucine decreases in the presence of DMSO, but to a smaller extent than that of glycine. The solubility of tryptophan even increases (positive log solubility ratio in Fig. 5), although slightly, in aqueous DMSO, indicating favorable interactions between DMSO and the tryptophan molecule. Since the interaction between glycine and DMSO is highly unfavorable, the observed favorable interaction for tryptophan implies that the favorable interaction between the tryptophan side chain and DMSO overcomes the unfavorable interaction between DMSO and the charges of carboxyl and amino groups in the tryptophan molecule (as deduced from the unfavorable interaction with glycine). The interaction free energy of the side chains can be obtained by subtracting the contribution of glycine, as described below. The solubility of leucine and alanine, being higher than that of glycine at the same DMSO concentration, is indicative of favorable interactions of DMSO with these side chains as well.

The transfer energy of the amino acids was calculated from the solubility difference in DMSO and water by

$$\Delta G_t = \mu^{\circ}_{\mathrm{DMSO}} - \mu^{\circ}_{\mathrm{water}} = \mathrm{RTln}(N_{\mathrm{water}}/N_{\mathrm{DMSO}}) + \mathrm{RTln}\,(\gamma_{\mathrm{water}}/\gamma_{\mathrm{DMSO}}) \quad (5)$$

where ΔG_t is the transfer free energy of the amino acid from water to a DMSO solution, $\mu^{\circ}_{\mathrm{DMSO}}$ is the chemical potential of the amino acid in DMSO, $\mu^{\circ}_{\mathrm{water}}$ is the chemical potential of the amino acid in water, N_{water} and N_{DMSO} are the solubility of the

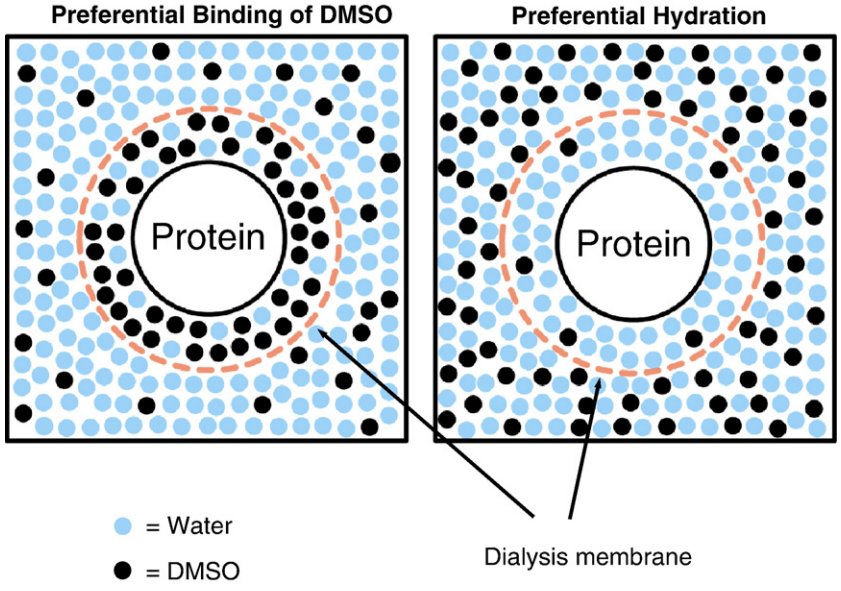

Fig. 2. Schematic presentation of preferential DMSO binding and hydration in dialysis equilibrium. Taken from Na and Timasheff [42].

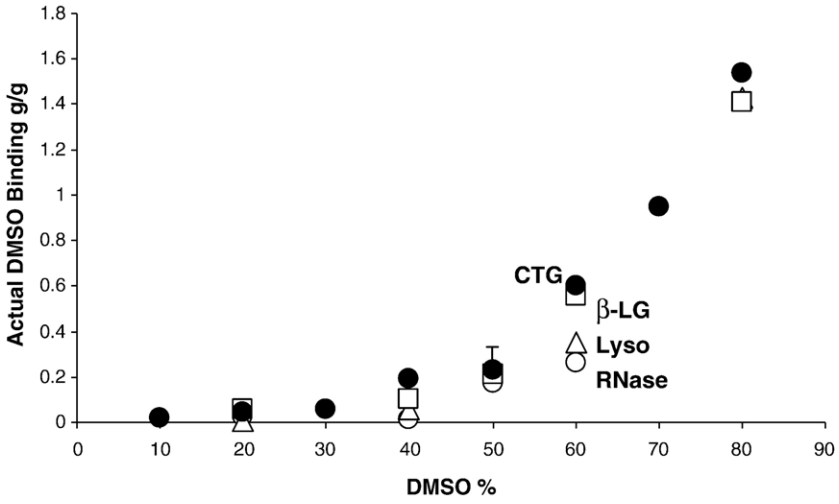

Fig. 3. Actual binding of DMSO to the proteins CTG (solid circle), β-LG (open square), Lyso (open triangle), RNase (open circle).

amino acid in water and DMSO expressed as mole fraction and the RTln ($\gamma_{water}/\gamma_{DMSO}$) is the self-interaction free energy of the amino acid. The self-interaction term was neglected. The transfer free energy of side chains (and hence the side chain interaction free energy) was calculated by subtracting the transfer free energy of glycine from the transfer free energies of alanine, leucine and tryptophan using Eq. (6).

$$\Delta G_t(\text{side chain}) = \Delta G_t(\text{amino acid}) - \Delta G_t(\text{glycine}) \quad (6)$$

where ΔG_t (side chain) is the transfer free energy of the side chain, ΔG_t (amino acid) is the transfer free energy of the amino acid and ΔG_t (glycine) is the transfer free energy of glycine. The transfer free energy of these side chains is plotted in Fig. 6. It is negative, indicating favorable interactions between these side chains and DMSO. The favorable interaction increases in the order of alanine < leucine < tryptophan. The interaction free energy is −4500 cal/mol for the tryptophan side chain in 80% DMSO solution, which means that the interaction between the tryptophan side chain and DMSO is extremely favorable. It is interesting to note that the interaction of the leucine side chain with DMSO is only slightly more favorable than that of the alanine side chain, indicating a marginal contribution of the increase in aliphatic chain length to the hydrophobic interaction with DMSO.

3.3. Mechanism of DMSO effects

DMSO has a broad range of effect on proteins. It is a strong cryoprotective agent. Although the mechanism of cryoprotection by DMSO is not fully understood, it has been proposed that the preferential hydration of DMSO may be involved [7]. As shown in this paper, at lower DMSO concentrations, proteins are preferentially hydrated in aqueous DMSO solutions and maintain the native state. Negative binding, or preferential hydration [15,23], can stabilize the native structure, provided that the binding of the additives does not increase upon unfolding. It is possible that at subzero temperatures, where DMSO exerts cryoprotection, favorable interactions of DMSO with hydrophobic side chains, as demonstrated by amino acid solubility measurements (see Fig. 6) and increasing preferential DMSO binding upon protein unfolding (Fig. 1), diminish due to the temperature dependence of hydrophobic interactions; hydrophobic interactions are weaker at lower temperatures. Thus, protein stabilization by the preferential hydration mechanism may dominate during freezing process and confer the cryoprotection to the proteins.

DMSO has been shown to enhance the self-association of tubulin to form microtubules [13,14]. As is true for several proteins, tubulin is also preferentially hydrated in aqueous DMSO solutions at 10–20% (Fig. 1, solid diamond). The preferential hydration of a protein (or negative preferential binding of solvent additive) has as a consequence that the solubility of the protein is decreased in the presence of the additives. The observed preferential hydration should thus be able to explain the enhanced self-assembly of tubulin. How does the observed preferential interaction relate to the effect of DMSO on tubulin self-association? A detailed description of the relation between preferential hydration (or exclusion of additive) and protein solubility is available in the literatures

Table 7
Solubility of amino acids in aqueous DMSO solutions at 20 °C

Amino acid	Solubility in g/100 g solvent at DMSO concentration of						
	0%	20%	40%	60%	70%	80%	100%
Glycine	22.7	9.33	2.50	0.239±0.012	0.106±0.020	0.039±0.017	
DL-Alanine	15.2	7.29	2.63	0.747			0.030±0.005
L-Leucine	2.14	1.15	0.472±0.007	0.168±0.003		0.078±0.004	
L-Tryptophan	1.23	1.73	1.86			2.68	

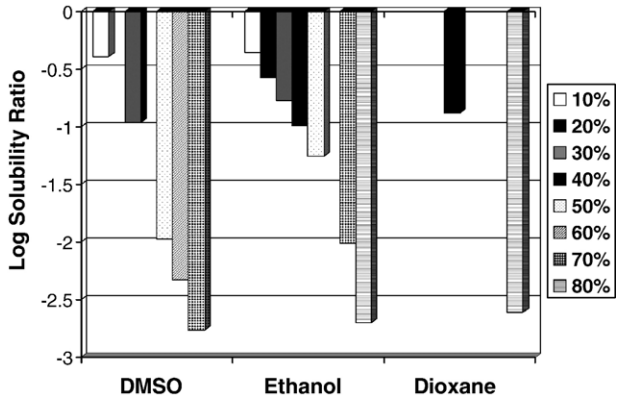

Fig. 4. Solubility of glycine in aqueous DMSO, ethanol and dioxane solutions at the indicated concentrations. The data for ethanol and dioxane are taken from Nozaki and Tanford [33].

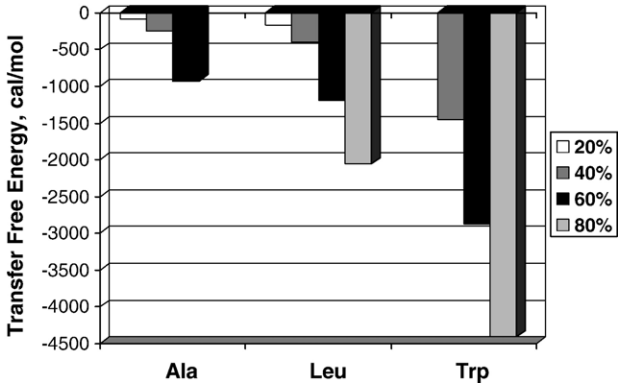

Fig. 6. Transfer free energy of amino acid side chains from water to aqueous DMSO solution of the indicated concentrations.

[e.g., 15,49,50] and hence only a brief statement is given here. The negative preferential binding of solvent additives to a protein surface is thermodynamically unfavorable, as [7,18,40]

$$(\partial\mu_2/\partial m_3)_{T,P,m_2} = -(\partial\mu_3/\partial m_3)_{T,P,m_2}(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3} \quad (7)$$

where $(\partial m_3/\partial m_2)$ is related to $(\partial g_3/\partial g_2)_{T,\ \mu_1,\ \mu_3}$ by

$$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3} = (M_2/M_3)(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}. \quad (8)$$

The value of $(\partial g_3/\partial g_2)_{T,\ \mu_1,\ \mu_3}$ and hence $(\partial m_3/\partial m_2)_{T,\ \mu_1,\ \mu_3}$ for tubulin and other proteins is negative at low DMSO concentration (Tables 2–5 and Fig. 1). When multiplied by the self-interaction term, $(\partial\mu_3/\partial m_3) = \mathrm{RT}/m_3 > 0$, the chemical potential change becomes positive for the negatively interacting solvent additives, such as DMSO at lower concentrations, with $(\partial m_3/\partial m_2) < 0$, i.e., $(\partial g_3/\partial g_2) < 0$. This is schematically depicted in Fig. 7, i.e., the negatively interacting DMSO (i.e., preferentially excluded DMSO) makes the native state of tubulin (monomeric form) more unfavorable. On the other hand, the associated state (i.e., microtubule) should have a less solvent-exposed surface area per monomer (as depicted in the bottom panel of Fig. 7) and hence a less negative interaction. This should lead to a lower free energy of association in DMSO solution, with a consequence that the addition of DMSO should enhance the self-association of tubulin.

DMSO is also used to decrease the solubility of proteins, i.e. to induce precipitation and crystallization [6]. The same argument that was used to explain the effects of DMSO on tubulin self-association should apply to DMSO-induced protein precipitation. Unfavorable interactions between the proteins and DMSO, observed at lower DMSO concentrations, should be less for the associated state of the proteins and hence the free energy of protein association is lower in the presence of DMSO. The amino acid solubility measurements also support this argument. DMSO interaction with the glycine molecule is highly unfavorable. Provided that the polar glycine molecule can represent the native state of the proteins, the addition of DMSO should decrease the solubility of the proteins, just as it decreases the solubility of glycine. Dioxane and ethanol have also been used to precipitate and crystallize proteins [51–57]. These solvent additives also decrease the solubility of glycine (Fig. 4), suggesting that the unfavorable interaction plays a role in the precipitation and crystallization of proteins by dioxane and ethanol, as well.

At higher DMSO concentrations, DMSO showed preferential binding to the proteins. This can be related to the protein unfolding (Fig. 1, arrows). As clearly seen, the preferential

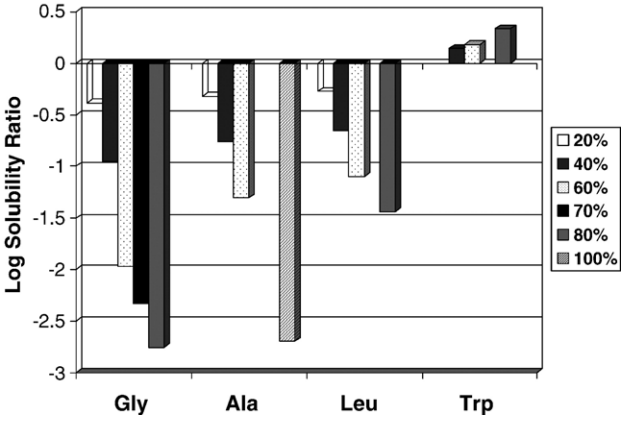

Fig. 5. Solubility of glycine, alanine, leucine and tryptophan in aqueous DMSO solution at the indicated concentrations.

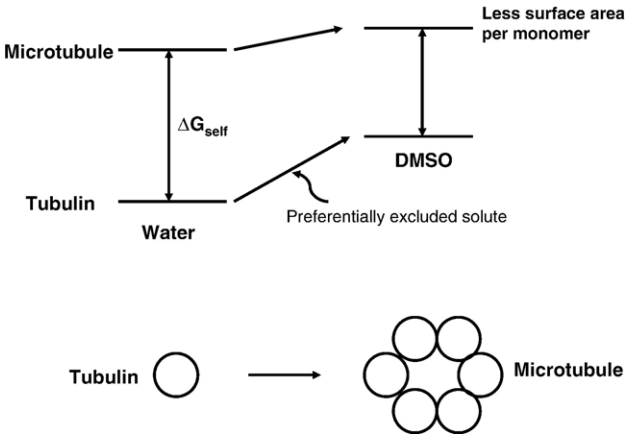

Fig. 7. Schematic illustration of the effect of DMSO on protein self-association.

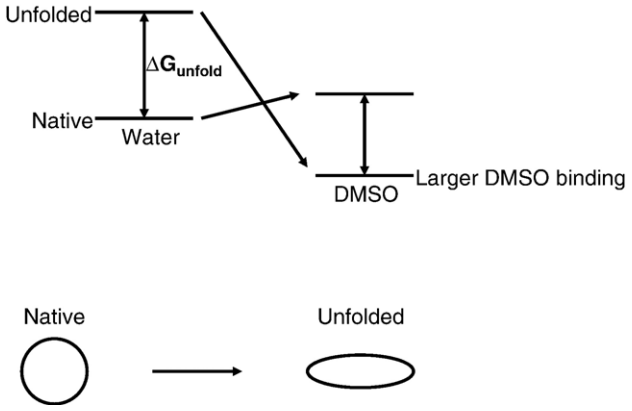

Fig. 8. Schematic illustration of the effect of DMSO on protein unfolding.

DMSO binding is closely associated with the onset of protein unfolding. How does preferential DMSO binding enhance protein unfolding? The amino acid solubility measurements showed the favorable interaction free energy of hydrophobic side chains, i.e., those of alanine, leucine and tryptophan. Thus, DMSO binding must be greatly enhanced upon unfolding, which exposes the non-polar surface of the proteins. As depicted in Figs. 7 and 8, the interaction between DMSO and the native protein is unfavorable. This unfavorable DMSO interaction is caused at least in part by the excluded volume of DMSO (which makes $A_1 > 0$). As the protein unfolds, the DMSO should be more strongly excluded as indicated by a larger A_1 value for the unfolded state, i.e., the excluded volume effects of DMSO stabilize the protein against unfolding. So why does the protein unfold at high DMSO concentrations? When the proteins unfold at higher DMSO concentrations, non-polar side chains became exposed to contact with the solvent and the unfolded structure binds more DMSO. Such binding should decrease the free energy of the unfolded state in DMSO solutions, as has been observed in the transfer free energy of the hydrophobic side chains (Fig. 6). Such a case is depicted in Fig. 8, where extensive DMSO binding can make the unfolded state the lower free energy state relative to the native state. This leads to DMSO-induced protein unfolding.

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