Preferential Solvation Changes upon Lysozyme Heat Denaturation in Mixed Solvents

Evgenii L. Kovrigin and Sergey A. Potekhin*

Institute of Protein Research, Russian Academy of Sciences, Pushchino, Moscow Region, 142292 Russia

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ABSTRACT: On the basis of scanning microcalorimetry data from literature and our own measurements, we have calculated the changes in preferential solvation of lysozyme upon heat denaturation in six solvent systems: water + methanol, ethanol, propanol [data from Velicelebi, G., & Sturtevant, J. M. (1979) *Biochemistry 18*, 1180], acetone, *p*-dioxane [data from Fujita, Y., & Noda, Y. (1983) *Bull. Chem. Soc. Jpn. 56*, 233], and dimethylsulfoxide [our data Kovrigin, E. L., Kirkitadze, M. D., & Potekhin, S. A. (1996) *Biofizika 41*, 549−553; Kovrigin, E. L., & Potekhin, S. A. (1996) *Biofizika 41*, 1201−1206]. These preferential solvation changes are (in effect) the numbers of cosolvent molecules entering or leaving the solvation shell of the protein upon denaturation. It has been shown that for a group of five substances in the initial activity range (approximately up to 0.3) the denaturational changes of preferential solvation of lysozyme do not depend on the nature of the solvent and depend only on its activity. This suggests that lysozyme does not distinguish these substances in the initial activity range and preferential solvation has a nonspecific character. It has been shown also that preferential solvation $\Delta\Gamma_{23}$ does not depend on the pH value at least for dimethylsulfoxide−water solutions. This indicates that the chargeable groups exposed on denaturation do not contribute significantly to preferential interaction of the protein surface with the solution components.

One of the most interesting and important issues of macromolecular physics (Ben-Naim, 1990a; Timasheff, 1992, 1993; Fu & Freire, 1992) and biotechnology (Zaks & Klibanov, 1988; Klibanov, 1989; Gupta, 1992) is to understand how various solvents influence protein structure and its stability. The protein structure thermodynamics has been extensively studied [e.g., Privalov (1979), Privalov and Gill (1988), and Dill (1990)]. An objective of these studies is to clarify the role of the solvent and its components in maintaining native structure of proteins. Numerous works have been published on the effects on proteins of salts, acids, bases, urea, guanidinium hydrochloride, alcohols, and some other solvents [for review, see, Timasheff (1992, 1993) and Baldwin (1996)]. Various theoretical models have been developed describing solvation of protein surface in mixed solvents [e.g., Tanford (1970), Schellman (1975, 1978), and Ben-Naim (1990a,b)]. The solvent exchange model elaborated by Schellman (1990) has proved to be the most applicable when the solution concentration of added cosolvent reaches a high level. Nevertheless, until now there is no common quantitative theory which could predict the effect of solution components on the protein structure stability in such solutions on the basis of their intrinsic physicochemical properties.

The most direct technique to obtain thermodynamic information on protein stability is scanning microcalorimetry (Privalov & Potekhin, 1986). There is a number of calorimetry studies of globular proteins in mixed solvents, but in most of them researchers have restricted themselves to reporting primary calorimetry data and no common models have been suggested. It was noted (Timasheff, 1992, 1993) that, to refine the understanding of the role of the solution environment in protein stability, we have to analyze as a greater variety of substances as possible, and finally we will be able to find common rules and quantitative relations between properties of the solvent and protein energetics in the solution.

In this paper, we are analyzing the thermodynamic quantity that is not often used in calorimetry, namely, a preferential solvation change on protein denaturation. Preferential solvation is the most relevant parameter reflecting the protein−solvent interaction, and therefore it can be used as a basic tool to associate thermodynamic properties of protein structure in a mixed solvent with intrinsic physical properties of solution components. This is a purely thermodynamic measure of relative affinity of a solvated surface to the solution components (Schellman, 1990). Preferential solvation depends on the nature of a solvated surface and, hence, is conformation dependent for proteins. The change of preferential solvation upon cooperative protein unfolding is the main parameter characterizing stability of the native protein structure in mixtures of water with another solvent. On the molecular scale, the denaturational change of preferential solvation of a protein is the number of additional solvent molecules entering/leaving the solvation shell of a denaturing macromolecule. This microscopic description is true when the concentration of the added non-water solvent is not high, otherwise preferential solvation includes a contribution of events in the bulk solution (Schellman, 1990).

We have calculated and analyzed changes of preferential solvation upon heat denaturation of hen egg white lysozyme in aqueous solutions of six various solvents: dimethylsulphoxide (DMSO) [our data, Kovrigin and Potekhin (1996)

* Address correspondence to this author at the Institute of Protein Research, Pushchino, Moscow Region, 142292, Russia. Phone, +7-(095)-924-04-93. Fax, +7(095)-924-04-93. E-mail, spot@sun.ipr.serpukhov.su.

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and Kovrigin et al. (1996)], methanol, ethanol, propanol [data from Velicelebi and Sturtevant (1979)], acetone, and *p*-dioxane [data from Fujita and Noda (1983)].

THEORY

We briefly summarize here some thermodynamic definitions and results which we used in our analysis. We employ the convention of Scatchard (1946) and use subscripts 1, 2, and 3 to designate water, the protein, and the cosolvent, respectively; thus, x_1 and x_3 are the water and cosolvent (solvents 1 and 3) mole fractions. Following Schellman (1990), we define preferential solvation by the quantity Γ_{23}, which is read as "the preferential solvation of component 2 by 3":

$$\Gamma_{23} \equiv (\partial \nu_3/\partial \nu_2)_{T,\mu_3} \tag{1}$$

It represents the number of molecules of component 3, ν_3, which should be added to the solvent to restore the chemical potential of component 3 μ_3, when a molecule of component 2, the macromolecule, has been added to the solution. This value can be shown to be the excess number of component 3 molecules which are present in the solvation shell of the macromolecule relative to the bulk solvent when the total content of component 3 is not high (Schellman, 1978).

Preferential solvation [or "selective interaction", (Schellman, 1990)] is a purely thermodynamic measure of the relative interaction of solvent i ($i = 1$ or 3) with macromolecules. It can be measured quantitatively with no assumptions about underlying molecular events (Robinson & Stokes, 1955). This quantity is relevant if one is interested in the change of the chemical potential of a macromolecule caused by addition of denaturants, stabilizers, acids, bases, substrates, etc. When the interaction is strong, it is identical to the usual molecular definitions of binding. When interactions are weak (relative to another bulk component) it can differ significantly from the molecular description (Schellman, 1990).

Since, in principle, solvent 1 (water) and solvent 3 (cosolvent) are interchangeable, we can write corresponding equations for Γ_{21} which is commonly named as "preferential hydration". However, in a water-rich region, Γ_{21} *cannot* be directly associated with the excess number of water molecules in the solvation shell; when the component is present at a high concentration, Γ_{21} has a complex structure as shown elsewhere (Schellman, 1978, 1990).

Γ_{21} and Γ_{23} are not independent quantities since they correspond to the same event in the solution. As it was shown (Eisenberg, 1976), they are fundamentally related by $\Gamma_{23}/\Gamma_{21} = -x_3/x_1$. This formula is a general relation which is independent of the model and serves for checking the thermodynamic consistency of the results.

Preferential solvation parameters originate from dialysis equilibrium studies; however, thermodynamics allows us to obtain such information by means of an entirely different technique provided the data we use are true thermodynamic quantities. Here, we intend to apply the preferential solvation analysis to protein denaturation studies, particularly to the data from scanning microcalorimetry of protein solutions.

When the native molecule denatures, its surface changes its character (e.g., hydrophobicity) and we could expect a corresponding change in preferential solvation of this macromolecule by components of the surrounding solution: $\Delta\Gamma_{23} = \Gamma_{D3} - \Gamma_{N3}$. Schellman (1975, 1990) and Plaza del Pino and Sanchez-Ruiz (1995) developed eq 1 to use experimental data available from scanning microcalorimetry for practical calculations of $\Delta\Gamma_{23}$. With a slight transformation of their results (Supporting Information, section 1), we arrive at

$$\Delta\Gamma_{23} = -\frac{\Delta H\,(\partial T_m/\partial x_3)_{pH}}{RT_m^{\,2}\left(\dfrac{\partial \ln a_3}{\partial x_3}\right)_{T_m}} \tag{2}$$

This formula is an exact result for a reversible transition between two states and gives a denaturational change of preferential solvation of component 2 (protein) by 3 at the transition temperature T_m. ΔH is the enthalpy absorbed upon transition under these conditions, R is the gas constant, $(\partial T_m/\partial x_3)_{pH}$ is the rate of variation in T_m on increasing x_3 at constant pH. The term $[\partial \ln(a_3)/\partial x_3]_T$ in the denominator gives the contribution of solution nonideality and contains a_3 which is the activity of component 3. Details on the evaluation and taking into account the nonideal character of real solutions are given in Supporting Information, section 2.

RESULTS AND DISCUSSION

Lysozyme in DMSO−Water Solutions. The first question which arises from the preferential solvation study of proteins is the pH dependence of this parameter. It is known that about four chargeable groups are exposed and recharged at acid pH on lysozyme heat denaturation (Pfeil & Privalov, 1979). Is such a number of charges exposed upon unfolding able to contribute significantly to the total value of the preferential solvation change? To clarify this question, we used our own data. Recently, we determined thermodynamic parameters for lysozyme heat denaturation in aqueous DMSO solutions using differential scanning microcalorimetry (Kovrigin & Potekhin, 1996; Kovrigin et. al., 1996). We examined the entire range of DMSO concentrations at four pH values (from 2.5 to 9.0). The concentration of buffer salts was about 20 mM. Under all experimental conditions, the protein denaturation was shown to be reversible, equilibrium, and conforming to the "two-state" mechanism. Using these data, we performed calculations of the denaturational changes in preferential solvation according to eq 2. The activity coefficients for DMSO−water mixtures were taken from Lam and Benoit (1974) and assumed to be independent of the temperature (for reasoning, see Supporting Information, section 3).

The results of these calculations are presented in Figure 1, where we plotted the preferential solvation change $\Delta\Gamma_{23}$ for lysozyme in DMSO−water solutions. The meaning of these quantities is simple: if we carry out the dialysis equilibrium experiment at a certain solution composition and at the temperature equal to the midpoint of denaturational transition, then $\Delta\Gamma_{23}$ will be a number of molecules of component 3 which cross the dialysis membrane when one molecule of lysozyme denatures. The positive sign of $\Delta\Gamma_{23}$ indicates that DMSO favors the denatured protein conformation relative to the native one.

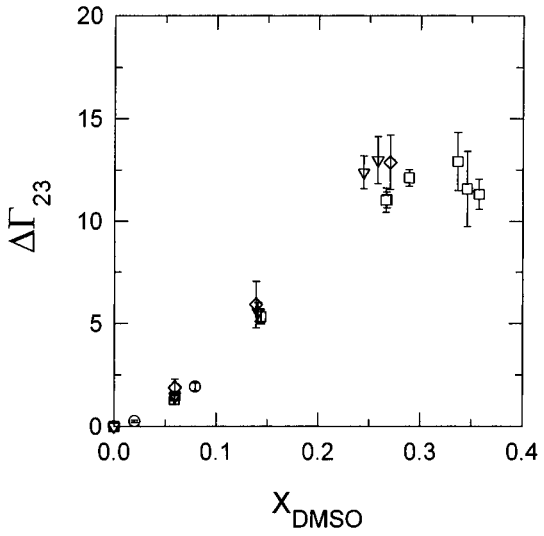

FIGURE 1: Denaturational change in preferential solvation $\Delta\Gamma_{23}$ of hen lysozyme in DMSO−water solutions at four pH values as a function of the DMSO mole fraction. Markers: ○, pH 2.5; □, 4.5; ▽, 6.0; ◇, 9.0.

The data presented in Figure 1 were obtained at four pH values (2.5, 4.5, 6.0, 9.0); the corresponding series are shown by different markers. It is clearly seen that the pH variation at constant x_3 causes insignificant changes of $\Delta\Gamma_{23}$ as compared to the experimental uncertainty. This suggests that the chargeable groups exposed upon denaturation do not play a noticeable role in the process of lysozyme preferential solvation.

Five Other Solvents. In the available literature, we have found several works on microcalorimetry of lysozyme heat denaturation in the mixtures of water with various organic solvents. Fortunately, for some of them the thermodynamic parameters characterizing solution nonideality were available in the full range of the solution composition. Thus, we were able to calculate the denaturational changes of lysozyme preferential solvation in the five solvent systems: mixtures of water with methanol, ethanol, propanol, (Velicelebi & Sturtevant, 1979), *p*-dioxane, and acetone (Fujita & Noda, 1983). These calorimetric data are given in Tables 1 and 2. In all cases, the denaturational transitions were reported to be fully reversible, equilibrium, and conforming to the "two-state" mechanism. This allowed the data analysis by eq 2 to be applied. Ionic conditions were at approximately the same level, and the salt concentration did not exceed 10−20 mM. As in the previous case, we neglected the presence of buffer salts assuming water and solvent 3 to be the only bulk components. For the first three systems, the original calorimetric data corresponded to pH 2.0; the last two systems were studied at pH 3.0. However, on the basis of our results for DMSO we assume that preferential solvation does not depend on pH in these solvents either. Having this extrapolation in mind, we will compare values of $\Delta\Gamma_{23}$ calculated for these solvent systems. The activity values of the corresponding compounds in water solutions were calculated from their partial pressures in binary systems by the conventional procedure (Supporting Information, section 2). Primary data were critically examined for self-consistency as described in Kogan et al. (1966) and the best sets of data were used for our calculations: methanol (Kogan et al., 1966, ref 414), ethanol (Kogan & Fridman, 1957, refs 65 and 219), propanol (Kogan et al., 1966, refs 34 and 414),

Table 1. Calorimetric Data on Hen Lysozyme Heat Denaturation in Water Solutions of Methanol, Ethanol, and Propanol at pH 2.0 (Velicelebi & Sturtevant, 1979)[a]

C (M)	T_d (K)	ΔH (kJ/mol)	C (M)	T_d (K)	ΔH (kJ/mol)
	methanol			propanol	
0.00	325.2	380	0.00	325.2	380
1.20	324.0	395	0.64	320.4	389
2.44	322.4	402	1.40	314.8	400
3.69	319.6	404	2.04	306.4	398
4.95	317.2	408	2.64	298.4	307
7.44	311.6	383	3.34	288.8	245
9.90	305.2	353			
	ethanol				
0.00	325.2	380			
0.84	325.2	423			
1.70	322.0	432			
2.54	318.8	441			
3.50	315.2	436			
5.15	307.6	406			
6.89	298.8	325			

[a] Data were read from figures by a digitizer. Column headings: C (M), the solvent molar concentration; T_d (K), the temperature of denaturation; ΔH (kJ/mol), the enthalpy of denaturation.

Table 2. Calorimetric Data on Hen Lysozyme Heat Denaturation in Water Solutions of Acetone and Dioxane at pH 3.0 (Fujita & Noda, 1983)[a]

C (M)	T_d (K)	ΔH (kJ/mol)	C (M)	T_d (K)	ΔH (kJ/mol)
	acetone			dioxane	
0.00	344.4	488	0.00	344.4	488
1.26	338.0	512	0.94	336.5	440
2.21	334.1	520	1.89	330.1	408
3.47	327.7	536	2.97	323.0	360
4.73	320.6	504	3.97	315.8	304
6.00	314.2	448	4.86	307.9	216

[a] Data were read from figures by a digitizer. Column headings are the same as in Table 1.

acetone (Kogan & Fridman, 1957, refs 345 and 376), and dioxane (Kogan et al., 1966, refs 615 and 685; Kogan & Fridman, 1957, ref 389). The temperature effect on activities of these substances was found to be negligible (this issue is discussed in Supporting Information, section 3). The protein concentrations in the experiments were small enough; therefore, we could take activities of solvents as those in binary systems. The values of the preferential solvation changes calculated on the basis of these data are shown in Figure 2. Our results on DMSO−water system are given as well.

As in the previous case, a positive sign of $\Delta\Gamma_{23}$ for all these solvents indicates that they have greater affinity to the protein surface exposed upon denaturation than that of water. This agrees with microcalorimetry evidence that these substances decrease the thermal stability of lysozyme when adding them to the medium.

A homologous series of alcohols (open symbols) shows clear correlation between the hydrophobic residue length and the values of preferential solvation. However, this rule does not always hold true: one can see that the dioxane curve (filled squares) crosses the acetone and ethanol curves (filled circles and open squares, respectively). The maxima would possibly appear for every solvent as well as for dioxane and DMSO; however, the concentration ranges of the data

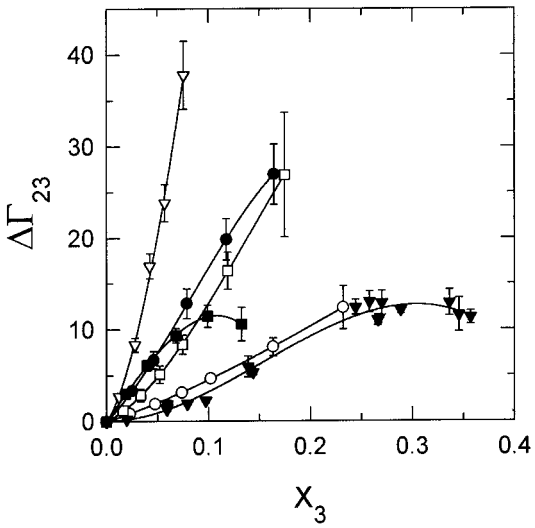

FIGURE 2: Comparative plot of denaturational changes in preferential solvation $\Delta\Gamma_{23}$ of hen lysozyme in solutions of six organic solvents as a function of the solvent mole fraction: ○, methanol; □, ethanol; ▽, propanol (calculated from the data of Velicelebi and Sturtevant, 1979); ●, acetone; ■, dioxane (calculated from the data of Fujita and Noda, 1983); ▼, DMSO (our data).

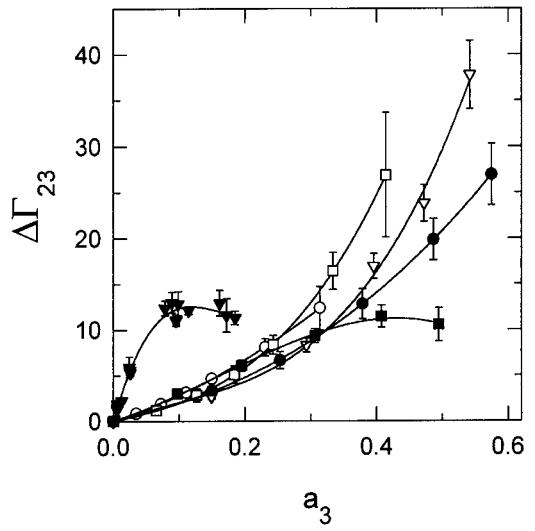

FIGURE 3: Comparative plot of denaturational changes in preferential solvation $\Delta\Gamma_{23}$ of hen lysozyme in solutions of six organic solvents as a function of activities of corresponding solvents. Series markers are the same as in Figure 2.

examined were constrained by their origin: points of the highest concentration in each data set corresponded to the conditions under which the temperature of denaturation dropped down to the lower temperature limit of a scanning microcalorimeter.

Notice that Figure 2 represents $\Delta\Gamma_{23}$ values plotted versus the mole fraction of the solvent. However, it is well-known that concentrated solutions usually show considerable deviations from ideality. Therefore, when analyzing data on various nonideal systems, it is more correct and physically meaningful to use corresponding activities instead of concentrations of components. This should equalize solutions of differing substances by taking into account their intrinsic nonideal properties. In Figure 3 we plotted $\Delta\Gamma_{23}$ values versus activities of corresponding solvents. It is easy to see that this transformation reveals a remarkable phenomenon: homologous alcohols, acetone, and dioxane have now very close values of preferential solvation changes within the initial activity range (up to 0.3). The proximity of $\Delta\Gamma$ values for these solvents means that when one lysozyme molecule denatures, the protein solvation shell will include the same number of additional molecules of any solvent at the same activities in the solution. This is the main finding of our work. In other words, lysozyme does not distinguish these substances in the low activity region.

There is one sharply deviating case. This is a curve for the DMSO−water system. While general physical properties of DMSO are similar to those for other five substances, there is still a feature sharply distinguishing DMSO from others. This is the character of its interaction with water. It is intriguing that the five solvents which give close $\Delta\Gamma$ values have activity coefficients in water solutions above unity. DMSO is the only substance whose activity coefficient is less than unity. This is a noticeable correlation and a further work should elucidate its real physical nature.

In Figure 3 one can see that the preferential solvation nonspecificity does not take place above 0.3 activity unit. This indicates that the denaturational preferential solvation change should consist of at least two major contributions. The first should result from some solvation mechanism common for the five cosolvents and determine the value of $\Delta\Gamma$ at low solvent activities. At higher activities, another contribution becomes dominating; it should be strongly dependent on the molecular parameters of the cosolvent to account for the divergence of $\Delta\Gamma$ curves observed in our experiments.

It is noteworthy that our calculated $\Delta\Gamma_{23}$ values are not *isothermal*: by definition, all the changes of preferential solvation correspond to the experimental transition temperatures. Therefore, to make a correct comparison, one should extrapolate all $\Delta\Gamma_{23}$ values to a common temperature using the values of heat capacity increment ΔC_p for protein denaturation. Unfortunately, in water−organic solutions, these values are highly unreliable. Nevertheless, to illustrate how the temperature can affect the $\Delta\Gamma_{23}$ curves, we estimated preferential solvation changes for lysozyme denaturation in methanolic solutions [data from Velicelebi and Sturtevant (1979)] at three constant temperatures. The result is shown in Figure 4. Extrapolated $\Delta\Gamma_{23}$ are shown by lines; symbols correspond to experimental values of $\Delta\Gamma_{23}$ found at these temperatures (they are shown to check the correctness of extrapolation). We should note that this is a rough qualitative estimate so it does not matter here what units (concentrations or activities) are plotted on the abscissa. In this figure, one can see that the temperature contribution to the preferential solvation sharply increases when the methanol content exceeds some value. Therefore, it can be expected that at higher solvent concentrations nonisothermal $\Delta\Gamma_{23}$ values for various substances *should* diverge.

An additional cause, which could exaggerate this divergence, is the progressive slowing down of the unfolding kinetics on increasing the cosolvent concentration. We observed this phenomenon in DMSO−water solutions (Kovrigin et al., 1996) and found that it had immediate consequences for the microcalorimetry results: the apparent transition temperatures (peak maxima) became overestimated and this error grew up on increasing the DMSO concentration. Equation 2 shows that $\Delta\Gamma_{23}$ is not very sensitive to the error introduced through the absolute values of T_m, but the numerator contains $\partial T_m/\partial x_3$; this is the term which can be greatly affected by the error due to overestimation of the

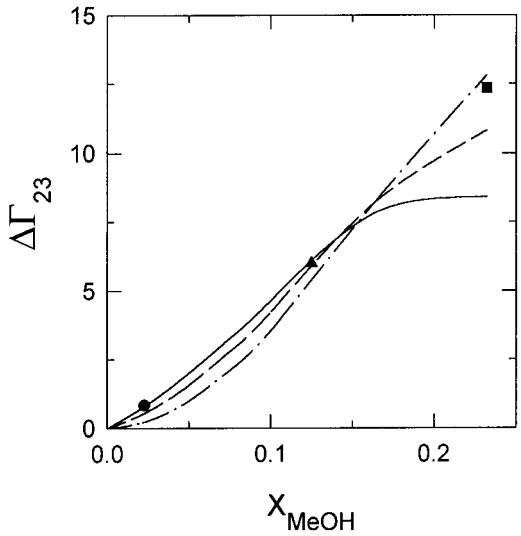

FIGURE 4: Extrapolated isothermal denaturational changes in preferential solvation $\Delta\Gamma_{23}$ of hen lysozyme in methanol−water solutions at three temperatures. Primary calorimetry data from (Velicelebi & Sturtevant, 1979). $\Delta\Gamma_{23}$ values are calculated at 30 °C (−·−), 40 °C (− − −), 50 °C (—). Markers show experimental $\Delta\Gamma_{23}$ at certain methanol concentrations and corresponding denaturation temperatures (●, T_d = 50.8 °C; ▲, 38.4 °C; ■, 32.0 °C).

transition temperature. However, all authors reported their melting experiments to be carried out at *equilibrium* (sufficiently low scan rates). Therefore, we supposed the influence of slow kinetics to be excluded.

In summary, the most interesting result of this work is that the change of preferential solvation upon protein denaturation by organic solvents does not appear to be too specific: in our consideration, lysozyme did not distinguish five substances in the initial activity range. We believe that this phenomenon is well worth being considered further and is possibly a manifestation of a common physical mechanism governing stability of the protein structure in aqueous media when the water structure is perturbed by the presence of another solvent.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

Mathematical derivation of the equation for $\Delta\Gamma_{23}$ used in the calculations; details of evaluation of the activity coefficients; discussion of their temperature dependence (5 pages). Ordering information is given on any current masthead page.

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