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ARTICLE *in* PHYSICAL CHEMISTRY CHEMICAL PHYSICS · MARCH 2000

Impact Factor: 4.49 · DOI: 10.1039/B001176M

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NMR studies on hydrophobic interactions in solution

Part 5.† Effect of urea on the hydrophobic self-association of *tert*-butanol in water at different temperatures

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Received 11th February 2000, Accepted 20th March 2000

An NMR A_{22} parameter study of the effect of urea on the hydrophobic self-association of *tert*-butanol in aqueous solutions is presented. One aim of the present paper is to contribute to the understanding of the urea effect in protein denaturation. The experimental data used for the computation of the A_{22} parameters, namely the self-diffusion coefficients and the intermolecular ^{1}H relaxation rates of *tert*-butanol in the ternary system, together with the additionally measured self-diffusion coefficients of water, are also examined with respect to a possible structure-breaking effect of urea on the aqueous phase which has been discussed in the literature. We obtain the remarkable result that in a certain mixture range addition of urea can increase the translational mobility of the solute *tert*-butanol, whereas it always decreases the mobility of bulk water. Thus, we could not detect a structure-breaking effect of urea on the aqueous phase. The urea content was varied up to 12 mol% in the temperature range 10–40 °C. It is found that low urea concentrations enhance the hydrophobic self-association in the water-rich region in the whole temperature range. With low *tert*-butanol concentrations this enhancement effect reaches a pronounced maximum. At higher urea concentrations a destabilisation of the hydrophobic interaction is observed. This latter result is in agreement with the need for relatively high urea concentrations to achieve protein denaturation. Obviously, the same basic mechanism controls the effect of urea not only on the stability of hydrophobic association of comparatively small molecules but also on the conformation of large biomolecules.

1 Introduction

We have recently published an experimental NMR study on the temperature and concentration dependence of the hydrophobic self-association of *tert*-butanol (*t*-BuOH) in pure water,[1] as part of a series[2–4] we devoted to hydrophobic interactions in solution.[5] The NMR method applied in this series is the so-called NMR association parameter (*A* parameter) method. It was reported there that *t*-BuOH exhibits a self-association behaviour characterised by a maximum association tendency at about 2–3 mol% in aqueous solution at 10 and 25 °C and that this maximum was shifted to lower *t*-BuOH concentrations as the temperature was raised to 40 °C. In the present paper we extend our studies to the investigation of the urea effect on the association of *t*-BuOH under the same conditions as we had for the binary system. The addition of a third component is a frequently applied method for the modification of the aggregation behaviour of particles in liquid media. However, in this way polynary liquid mixtures arise and hence the understanding of processes on a molecular level becomes very difficult. This difficulty even increases with increasing complexity of the molecules involved in the system as *e.g.* in biochemical systems. For example the influence of urea as denaturant of protein conformations in aqueous solutions, an effect of high scientific and technical interest,[6] is not yet satisfactorily understood.[7] Numerous attempts have been made to elucidate the denaturing molecular mechanism of urea. The most favoured proposition, supported by a number of thermodynamic studies,[8] treats urea as a water structure-breaker and thus describes an *indirect* mechanism.[9] However, results of molecular dynamic simulations are inconsistent with this mechanism, since it is found that urea has only a small effect on the water structure and that a direct substitution of water molecules in the hydration sphere of the dissolved particles occurs,[10,11] which means a support for a *direct* mechanism. Now assuming that a native protein conformation is essentially determined by hydrophobic interactions of the side chains, it seems obvious that investigations of the urea effect on the hydrophobic self-association of model molecules such as small alcohols can help in the understanding of the denaturation phenomenon. Therefore in the present series of work, studies on aqueous solutions of propanol[2] and ethanol[3] were performed revealing that the presence of relatively small quantities of urea enhanced the hydrophobic self-association, a fact which was unexpected within the generally accepted biochemical and biophysical point of view. However, this result corroborated the findings of Matteoli and Lepori[12] from their Kirkwood–Buff integral (KBI) investigations of the effect of urea on the self-association of propanol in water. Ben-Naim and Yaacobi[13] also reported in their thermodynamic studies on the effect of various solutes on the strength of hydrophobic interaction in aqueous solutions of methane and ethane that urea concentrations up to 7 M enhanced the hydrophobic interaction. All the mentioned results mean that urea stabilises the hydrophobic interaction and would therefore also stabilise a native protein conformation instead of denaturing it.

Now, as pointed out in our previous paper,[1] one should expect more pronounced effects in aqueous solutions of *t*-BuOH, owing to its greater hydrophobicity compared with the lower alcohols and alkanes. Furthermore, for the investi-

† For Part 4 see ref. 1.

DOI: 10.1039/b001176m

gation of a possible transition from stabilisation to destabilisation we wanted to vary systematically the urea concentrations from low values up to those values reported in the literature as typical of those at which protein denaturation occurs.[14–16]

As a contribution to the understanding of the mechanism of the urea influence on the hydrophobic interaction we will also examine the self-diffusion results for *t*-BuOH, obtained in the *A* parameter procedure, in combination with results from additional water self-diffusion measurements. The aim of this part of the work is the investigation of the question if urea really acts as a structure-breaking agent in aqueous solution as supposed in the literature in connection with the indirect denaturing mechanism.[8a] Here, as in previous NMR studies,[17–19] an increase or decrease of the self-diffusion coefficient of the solvent upon the addition of small amounts of a solute, is used for the detection of a "structure-breaking" or "structure-making" effect of the solute, respectively. (However, we should keep in mind that in this way a dynamic quantity serves as a measure for structural changes.)

2 Theoretical

The NMR A_{22} parameter method and the kind of measurements involved were described in previous papers[1–4] and more theoretical details can be found elsewhere.[20,21] The definition of the A_{22} parameter is given by the following equation:

$$A_{22} = \frac{1}{2}\frac{\gamma^4\hbar^2}{a^4}\int_a^\infty \left(\frac{a}{r}\right)^6 g_{22}(r)4\pi r^2\,\mathrm{d}r \qquad (1)$$

where $g_{22}(r)$ is the atom–atom pair distribution function of the atoms carrying the interacting nuclei. The subscripts 2 refer here to the solute of interest (*t*-BuOH). The lower integration limit "*a*" is the closest distance of approach between the interacting nuclei. Thus from the A_{22} parameter we can get information on the modification of a weighted integral over the pair distribution function consecutive to a variation of the system composition and/or temperature.

The measurable quantity related to the NMR A_{22} parameter is the ^{1}H intermolecular magnetic dipole–dipole relaxation rate $(1/T_1)_{\mathrm{inter}}$ which can be extracted from the total spin–lattice relaxation rate $(1/T_1)_{\mathrm{tot}}$ by means of isotopic dilution experiments.[22] The relationship between A_{22} and $(1/T_1)_{\mathrm{inter}}$ is given by:

$$A_{22} = \left(\frac{1}{T_1}\right)_{\mathrm{inter}}\frac{D}{c'} \qquad (2)$$

where D and c' are the self-diffusion coefficient and the spin number density of component 2, respectively. An increase of the A_{22} parameter with dilution of component 2 reflects the self-association of this component, whereas a decrease is related to a deassociation tendency.

3 Experimental

Ternary mixtures of D_2O (component 1), *t*-BuOD (component 2) and urea-d_4 (component 3) were investigated at 10, 25 and 40 °C. For consistency in the comparison with previously measured systems we expressed the *t*-BuOD concentration by $x_2' = x_2/(x_1 + x_2)$ where x_1 and x_2 are the mole fractions of D_2O and *t*-BuOD, respectively. The studied *t*-BuOD concentrations were limited to a range where significant hydrophobic self-association of the alcohol in water occurs, namely $0.01 \leqslant x_2' \leqslant 0.5$. Two sets of measurements were performed: (i) we determined the A_{22} parameter in the whole *t*-BuOD concentration range at all three temperatures, keeping the urea concentration constant at mole fractions $x_3 = 0.05$ and 0.12, respectively, and (ii) at some chosen fixed concentrations of *t*-BuOD ($x_2' = 0.02$, 0.04 and 0.2) we varied the urea concentration from $x_3 = 0$ to 0.15, but at 25 °C only.

The required ^{1}H intermolecular relaxation rates $(1/T_1)_{\mathrm{inter}}$ of *t*-BuOD methyl protons were obtained from measurements of ^{1}H spin–lattice relaxation rates with the standard inversion–recovery technique. The ^{1}H-NMR signals were also used in spin–echo measurements for the determination of the self-diffusion coefficients D of *t*-BuOD, by applying the pulsed gradient spin–echo (PGSE) technique. The deuterated compounds D_2O and urea-d_4 served to avoid undesired ^{1}H signal contributions from water and urea. For the measurement of the self-diffusion coefficients of water the ^{1}H signal from normal water was used while the other mixture components were then fully deuterated. For the isotopic dilution experiments we have utilised the fully deuterated *t*-BuOD-d_{10}. All measurements were performed at 90 MHz using a Bruker SXP 4-100 spectrometer system combined with a laboratory-made pulsed field gradient unit. The density measurements were performed with an Anton Paar densimeter. All samples were prepared by weighing and for T_1 measurements they were degassed by several freeze–pump–thaw cycles in order to remove paramagnetic oxygen. The temperature instability was less than 0.5 °C for the NMR measurements and 0.005 °C for the density measurements. The experimental error was less than 2% in both T_1 and D measurements and of the order of 10^{-5} for the density.

t-BuOD (99% D), *t*-BuOD-d_{10} (99% D) and D_2O (99.9% D) from Cambridge Isotope Laboratories were used as supplied. Urea-d_4 (98% D) purchased from Aldrich Chemical Co. was dried before use at 110 °C under vacuum. H_2O was doubly distilled.

4 Results and discussion

4.1 Self-diffusion

4.1.1 Urea effect on the translational dynamics of *tert*-butanol. The behaviour of the self-diffusion coefficient D of *t*-BuOH at different urea concentrations and temperatures is presented in Fig. 1. The experimental results used are listed in Tables S1 to S6.‡ For the purpose of comparison we included in Fig. 1 also the curves for the binary system water + *t*-BuOH as taken from ref. 1. The self-diffusion measurements, although carried out for the later determination of A_{22} parameters, are of interest in themselves since they delivered the first self-diffusion coefficients of *t*-BuOH in these ternary mixtures and hence important information about the translational dynamics of this component in the mixtures.

Fig. 1a and b show from bottom to top the experimental results at 10, 25, and 40 °C when 5 and 12 mol% urea are added to the aqueous *t*-BuOH solutions, respectively. We first consider Fig. 1a, where one can see that at 10 °C the curve of the ternary system lies above the curve of the binary system up to a mole fraction $x_2' \approx 0.23$ (intersection point of the curves). This means that at 10 °C the addition of 5 mol% urea increases the translational mobility of the solute *t*-BuOH in the concentration range $0 < x_2' < 0.23$. Above this concentration, 5 mol% of urea slows down the translational dynamics of *t*-BuOH. As we can recognise from Fig. 1a, the raise of temperature to 25 °C moves the mentioned intersection point to slightly smaller x_2' values and at 40 °C the two curves do not intersect anymore. Thus at 40 °C the addition of urea causes a slowing down of the translational motions of *t*-BuOH in the whole concentration range measured.

In Fig. 1b, where the results with 12 mol% urea (corresponding to 7 m on the molality scale in the water-rich

‡ Available as electronic supplementary information. See http://www/rsc.org/suppdata/cp/b0/b001176m

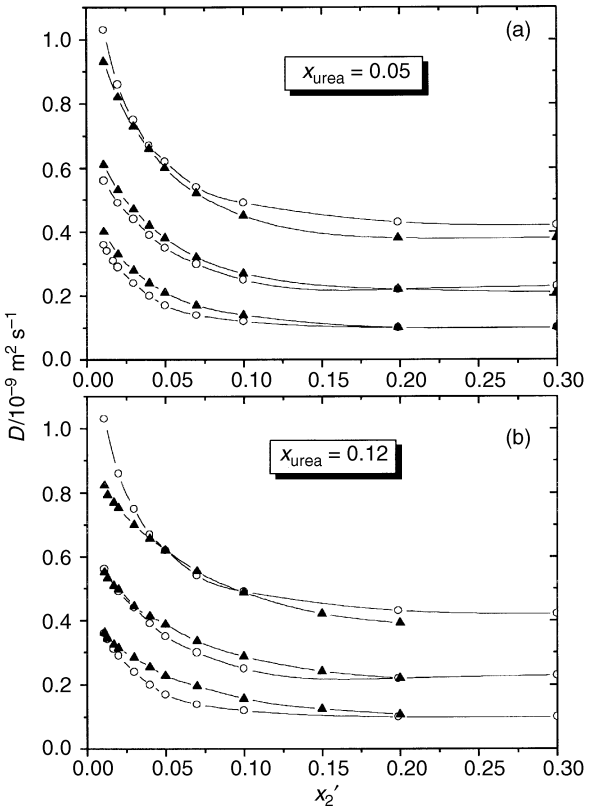

Fig. 1 (a) Comparison of the self-diffusion coefficients, D, of t-BuOD: for D_2O + t-BuOD + urea-d_4 [x_3 = 0.05 (▲)] and D_2O + t-BuOD (○) [from ref. 1] mixtures at different temperatures (from bottom to top: 10, 25 and 40 °C) as a function of $x_2' = x_2/(x_1 + x_2)$. (b) As (a), but with x_3 = 0.12 (▲).

region) are shown, one sees that the qualitative behaviour is a little more complicated. First we consider the range $0 < x_2' < 0.03$. At 10 and 25 °C there is almost no difference between the diffusion coefficients of the binary and the ternary system, which means that compared to Fig. 1a the self-diffusion coefficient in the ternary system decreased. This decrease with increasing urea content is even more pronounced at 40 °C. In the range $0.03 < x_2' < 0.2$ we observe at 10 and 25 °C a clear increase of the translational mobility of t-BuOH in the ternary mixture. However, at 40 °C this increase is not present anymore. Thus, if we also relate an increase of the *solute* translational mobility with a structure-breaking effect, as often performed for solvent water in the literature,[17–19,23] then we can summarise the results of Fig. 1 by stating that urea has in the ternary system both a structure-breaking and a structure-making effect. Thus the two qualifications can here obviously only be used in conjunction with solution composition and/or temperature range specifications. Moreover, it should be emphasised that we observed here solely the mobility behaviour of *one* component at relatively low concentration in a ternary system, which might be related to only a *local* structural change in the complex mixture. At this point we don't know whether the solvent water molecules experience in the ternary mixture the same dynamic changes as induced by urea on t-BuOH. This led us to measurements of the self-diffusion coefficients of water in these mixtures.

4.1.2 Urea effect on the translational dynamics of water. In the literature experimental results can be found regarding water dynamics in the *binary* system water + urea. Easteal[24] showed by tracer diffusion measurements that there is *no* increased *translational* mobility of water molecules, *i.e.* no structure-breaking effect when urea is added; the experiments even indicate a weak structure-forming property of urea. The same qualitative result has also been obtained for the *rotational* motion of water. Yoshida *et al.*[25] found in their NMR studies on aqueous solutions containing up to 8 mol% urea, namely that at normal pressure the reorientational correlation time of water molecules was longer in the hydration sphere of urea than in bulk water, a behaviour which is typical for structure-making solutes. Baring in mind these results, we asked the question if urea could perhaps act as a *local* structure-breaker in the ternary system, namely locally in the hydrophobic hydration sphere. To address this question we decided to measure the self-diffusion coefficients of water in the ternary water + t-BuOH + urea system. However, in such a mixture rapid exchange takes place between the hydroxy protons or hydroxy deuterons of the alcohol and water. Thus the observed water self-diffusion would be biased through the self-diffusion of t-BuOH carrying the exchangeable hydrogen atom. However, if the self-diffusion coefficient of the alcohol is measured independently *via* the CH_3-protons, the self-diffusion coefficient of water can be easily corrected for the exchange influence. Taking into account that water has two exchangeable hydrogen atoms and t-BuOD only one, we can express the observed self-diffusion coefficient of water as follows:

$$D_{H_2O,\,obs} = \frac{2x_{H_2O}}{2x_{H_2O} + x_{t\text{-BuOD}}} D_{H_2O} + \frac{x_{t\text{-BuOD}}}{2x_{H_2O} + x_{t\text{-BuOD}}} D_{t\text{-BuOD}} \quad (3)$$

where x is the mole fraction of the indexed mixture component. Thus if we measure the observed self-diffusion coefficient of water $D_{H_2O,\,obs}$ in the usual manner, we can compute the corrected D_{H_2O} from eqn. (3). The results obtained at 25 °C are presented in Fig. 2 and in Table S7.‡

From Fig. 2 it is obvious that the addition of urea both to pure water ($x'_{t\text{-BuOD}} = 0$, uppermost curve) and to aqueous t-BuOD solutions (lower curves) does *not* increase the diffusion coefficients of water molecules. For the binary and ternary mixtures we even find a decrease of the water diffusion coefficients with increasing urea concentration. (For the two highest $x'_{t\text{-BuOD}}$ values, within the error limits, also an independence of D_{H_2O} from the urea concentration might be stated.) Thus, firstly the results in Fig. 2 mean that our NMR measurements in the *binary* system confirm the above mentioned qualitative results by Easteal[24] and Yoshida *et al.*[25] and secondly that also in the ternary system there is no hint of a structure-breaking effect of urea on the bulk water.

In order to get quantitative information about the translational dynamics of water in the *direct neighbourhood* of a t-BuOH molecule we further evaluate our results using the

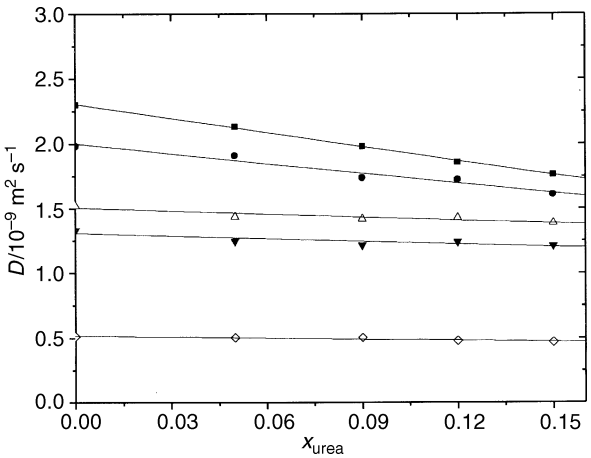

Fig. 2 Self-diffusion coefficients, D, of water: for H_2O + t-BuOD-d_{10} + urea-d_4 at 25 °C with t-BuOD-d_{10} concentrations x_2' = 0 (■), 0.01 (●), 0.03 (△), 0.05 (▼) and 0.25 (◇) as a function of the urea concentration.

so-called B coefficient concept,[17,19] here applied with the self-diffusion B_D coefficient.[18,26] In brief, the B_D coefficient is obtained from the concentration dependence of the water self-diffusion coefficient in diluted aqueous solutions *via* the following expression:[18,23]

$$\frac{1/D}{1/D_0} = 1 + B_D \bar{m} \tag{4}$$

where D_0 is the self-diffusion coefficients of water in the absence of the solute of interest and D is the self-diffusion coefficient of water in the presence of this solute, whose concentration, expressed in the aquamolality scale $\bar{m}$ (mol solute/55.5 mol solvent), is varied. In our case we varied each time the *t*-BuOD concentration at a fixed urea concentration. We did this in the urea concentration range from $x_3 = 0$ to $x_3 = 0.15$. B_D is then obtained as the slope of the function $D_0/D(\bar{m})$ in the limit $\bar{m} \to 0$. The sign of the B_D coefficient gives the direction, the absolute value the strength of the dynamic change induced by the solute in its vicinity. If these *local* changes in the translational mobility are again related to solvent structure changes, a positive sign of B_D means a local solvent structure enhancement and a negative sign a weakening of the solvent structure in the solvation sphere of the solute particle. By determination of the B_D coefficient for *t*-BuOD at different urea concentrations, we obtain information about the translational mobility of the local water in the hydration sphere of *t*-BuOD compared to the bulk water in the presence of urea at the given concentrations. In Fig. 3 the B_D coefficients are shown which are derived from the experimental results in Fig. 2. (Numerical values obtained are summarised in Table 1).

It can be seen from Fig. 3 that the B_D coefficient for *t*-BuOD is positive in the binary mixture ($x_3 = 0$) and also over the whole urea concentration range examined. The first result reflects the well-known fact that the mobility of water is slowed down in hydrophobic hydration spheres,[27] a fact which is related to an enhanced structure of water near hydrophobic solutes.[8b,28] However, an interesting feature in Fig. 3 is the decrease of the absolute B_D values with increasing urea concentration, which means that under the influence of urea the diffusivity of water in the hydrophobic hydration sphere of *t*-BuOD is less reduced relative to the corresponding aqueous bulk phase than it is in the binary system. Thus, we observe an influence of urea on the strength of the hydrophobic slowing down effect, although, as we can see, urea concentrations up to 15 mol% are not enough to invert the sign of the B_D coefficient.

More quantitative information about the translational dynamics of water in the hydrophobic hydration sphere can

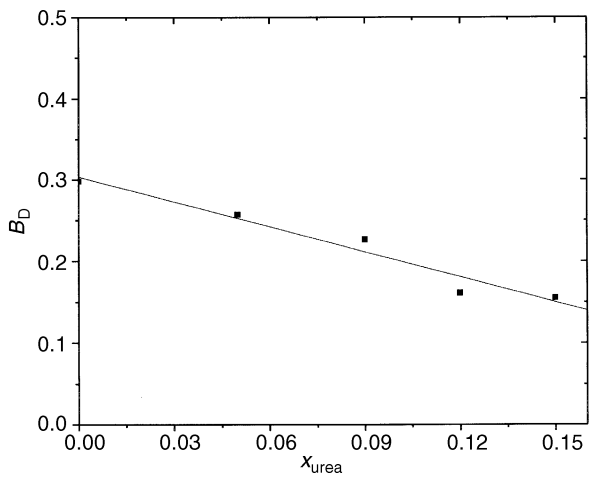

Fig. 3 B_D coefficients for water: for in H_2O + *t*-BuOD-d_{10} + urea-d_4 mixtures at 25 °C as a function of urea concentration.

Table 1 B_D coefficients at fixed urea concentrations in the system H_2O + *t*-BuOD-d_9 + urea-d_4 and derived ratios of self-diffusion coefficients of bulk water and of water in the hydration sphere of *t*-BuOD

x_{urea}	B_D	$D_{H_2O}^{bulk}/D_{H_2O}^{hydrat}$	$D_{H_2O}^{hydrat}$ [a]/10^{-9} m^2 s^{-1}
0	0.298	1.66	1.38
0.05	0.257	1.57	1.37
0.09	0.226	1.50	1.35
0.12	0.161	1.36	1.33
0.15	0.155	1.34	1.32

[a] $D_{H_2O}^{hydrat}$ has been determined as described in the text.

be obtained from the NMR B_D coefficient by applying a "two-state model" with fast exchange.[19] This model permits the evaluation of the self-diffusion coefficient of hydration water $D_{H_2O}^{hydrat}$ relatively to that of bulk water $D_{H_2O}^{bulk}$ using the following expression:

$$\frac{1}{D_{H_2O}} = (1 - x)\frac{1}{D_{H_2O}^{bulk}} + x\frac{1}{D_{H_2O}^{hydrat}} \tag{5}$$

where D_{H_2O} is the measured average self-diffusion coefficient and x is the mole fraction of water located in the hydration sphere. Knowing that the number of water molecules in the hydration sphere is given by the product of the hydration number n_h and the number of the solute molecules, the following expression can be derived:[17,18,23]

$$\frac{D_{H_2O}^{bulk}}{D_{H_2O}^{hydrat}} = 1 + \frac{55.5}{n_h} B_D \tag{6}$$

which gives the ratio of the diffusion coefficient of bulk water to the diffusion coefficient of hydration water *via* the B_D coefficient if n_h is known. Molecular dynamics simulations[29] and X-ray small angle scattering studies[30] assessed the hydration number of *t*-BuOD to be approximately $n_h = 25$. Assuming in a first approximation that n_h is independent of urea concentration, we obtain the ratio $D_{H_2O}^{bulk}/D_{H_2O}^{hydrat}$ as a function of urea concentration from eqn. (6). The results are presented in Table 1.

It can be seen from Table 1 that for *t*-BuOH in water ($x_{urea} = 0$), $D_{H_2O}^{bulk}/D_{H_2O}^{hydrat} = 1.66$ is obtained. This means that in the binary mixture the translational mobility of water in the hydration sphere of *t*-BuOH is reduced by about 40% relative to that of bulk water. As Table 1 shows, with addition of urea $D_{H_2O}^{bulk}/D_{H_2O}^{hydrat}$ decreases, which means a weakening of the retardation effect. At 15 mol% urea the remaining retardation effect is about 25%. The absolute values of $D_{H_2O}^{bulk}$ for the binary system water + urea are given in Table S7‡ ($x_2' = 0$). These are the corresponding values entering in the ratio $D_{H_2O}^{bulk}/D_{H_2O}^{hydrat}$ in Table 1 whose best fits can be used for the determination of $D_{H_2O}^{hydrat}$ (given in Table 1) *via* eqn. (6). One can recognise a weak decrease of this quantity with increasing x_{urea}. An unambiguous interpretation of this behaviour can not be given at the present stage, since there are two possibilities. First, one could argue that the addition of urea decreases more strongly the bulk water mobility than that of hydration water near the hydrophobic group, which could be an indication that urea is only present in the bulk water. This interpretation would partly support the "indirect mechanism" in the denaturing process. However, we have to keep in mind that a structure-breaking effect on water could *not* be observed. A second interpretation of the data in Table 1 could be as follows: the small decrease of $D_{H_2O}^{hydrat}$ compared with the strong decrease of $D_{H_2O}^{bulk}$, that is the reduced retardation effect, is caused by a *local* structure-breaking effect of urea in the hydrophobic hydration sphere. Urea would then slow down the bulk water dynamics and enhance the hydration water mobility. Urea would then likely enter the hydrophobic

hydration sphere. Summarising we can state: if there is really a structure-breaking effect by urea, as found by Wetlaufer *et al.*[31] and reported by Frank and Franks[9] and Castronuovo *et al.*,[32] then, in the light of our above results, it could only be a local effect on the structure of the hydrophobic hydration sphere. This interpretation would then support the above mentioned "direct mechanism" in denaturing processes by urea. In order to decide which of the above two interpretations is most plausible, further experiments are required. These experiments, namely the study of the A_{23} parameter showing the approach of urea towards *t*-BuOH, are in progress in our laboratory.

4.2 A_{22} parameter

Now let us examine the influence of urea on the hydrophobic self-association behaviour of *t*-BuOD in water as monitored through the A_{22} parameter at 10, 25 and 40 °C (Fig. 4). The experimental results used for the computation of A_{22} are summarised in Tables S1 to S6.‡ For a discussion of the results we will use as a reference (open squares in Fig. 4) the concentration dependence of A_{22} in the binary water + *t*-BuOD system.[1]

We first consider the addition of 5 mol% urea to the *t*-BuOD aqueous solution (solid circles in Fig. 4), which leads at all measured temperatures to an increase of the A_{22} parameter relative to the binary system *i.e.* urea promotes the self-association of *t*-BuOH. One sees further that at 10 °C, as in the binary system, the A_{22} parameter of the ternary system also passes through a maximum at $x_2' \approx 0.02$. At 25 and 40 °C the A_{22} parameter increases continuously with increasing dilution and no maximum is observed. However, as already mentioned in ref. 1, the maximum could well have moved to smaller *t*-BuOD concentrations. It can also be seen that in the water-rich region at 40 °C the *absolute* difference to the binary system is smaller than at 25 °C. This means that with a gradual raise of the temperature the urea effect will gradually become smaller or in other words, the higher the self-association in the binary system the lower is the stabilising influence of 5 mol% urea.

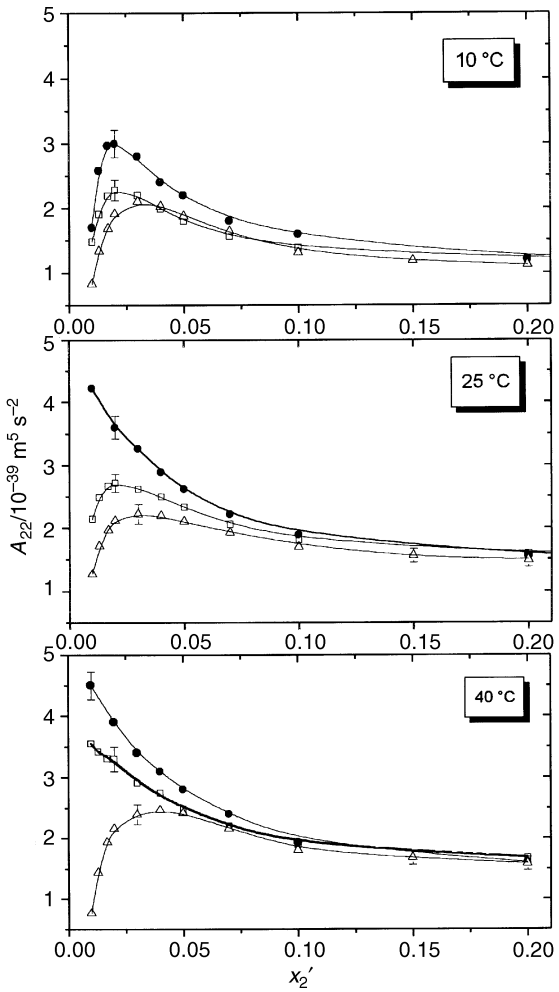

Fig. 4 Comparison of the A_{22} parameter with respect to the self-association of *t*-BuOD: for D_2O + *t*-BuOD + urea-d_4[$x_3 = 0.05$ (●) and 0.12 (△)] and D_2O + *t*-BuOD (□) [from ref. 1] mixtures at different temperatures as a function of $x_2' = x_2/(x_1 + x_2)$.

The addition of 12 mol% urea (open triangles in Fig. 4) causes in the water-rich region a clear deassociation of the *t*-BuOH molecules, since with decreasing x_2', the A_{22} parameter, after having passed through a weak maximum, strongly decreases. A further remarkable fact is that in the ternary system with the high urea content almost no temperature dependence is to be seen, which means that here the urea influence dominates the temperature influence.

After having determined the A_{22} parameter as a function of mixture composition and temperature, we can ask the question if one can establish a relation between the translational dynamic behaviour and the self-association behaviour of *t*-BuOH. We will restrict our following considerations to the range of low x_2' values, where the characteristic, different behaviour of the A_{22} parameter occurs.

By comparing the results in Fig. 1b and Fig. 4 at x_2' concentrations below $x_2' \approx 0.03$, we recognise that when strong deassociation occurs, the self-diffusion coefficient of *t*-BuOH remains nearly constant (at 10 and 25 °C) or decreases (at 40 °C) compared to the binary system. From a comparison of Fig. 1a and Fig. 4 in the same x_2' range we see that enhancement of self-association is connected with a small raise of *D* (at 10 and 25 °C). Summarising we can state that we find an indication, that the translational diffusivity of deassociated single *t*-BuOH molecules, presumably surrounded by a clathrate-like hydration shell,[33] is smaller than that of self-associated *t*-BuOH with less hydration water per alcohol molecule. This implies that the clusters of hydrophobically associated *t*-BuOH molecules can not be a rigid complex but rather a very flexible and fluctuating entity, a fact which is not surprising for the relatively weak hydrophobic interaction.

The results in Fig. 4 reveal that the urea effect on the "hydrophobic binding" depends strongly on the urea concentration. Relatively large amounts (*ca.* 7 M) of urea are needed for the destabilisation of the hydrophobic association of *t*-BuOH. Unfolding simulations on proteins in the presence of urea by Tirado-Rives *et al.*[34] and Thirumalai *et al.*,[35] as well as experimental examinations of the urea effect on peptides[14] led to the same conclusion. In these investigations 6 to 8 M of urea were required to achieve protein denaturation.

In order to examine further details with regard to the mixture composition and concentration dependence of the urea effect, we selected three fixed *t*-BuOD concentrations in the water-rich region, namely $x_2' = 0.02$, 0.04 and 0.2 and varied the urea concentration up to 15 mol% at 25 °C. The results are summarised in Fig. 5 and in Tables S8 and S9.‡ For the discussion, the binary *t*-BuOD + water system shall again be our reference system. It can be seen from Fig. 5 that the A_{22}-values and hence the association tendency, shows for the lowest *t*-BuOD concentration $x_2' = 0.02$ a pronounced maximum at $x_3 = 0.035$, but at the double *t*-BuOD concentration a weaker maximum appears at the double mole fraction of urea $x_3 = 0.07$. The maximum finally disappears at the highest alcohol concentration. Since we need for the double concentration of hydrophobic groups the double urea concentration at which a maximum stabilisation of the hydrophobic interaction occurs, there might be a direct relation between

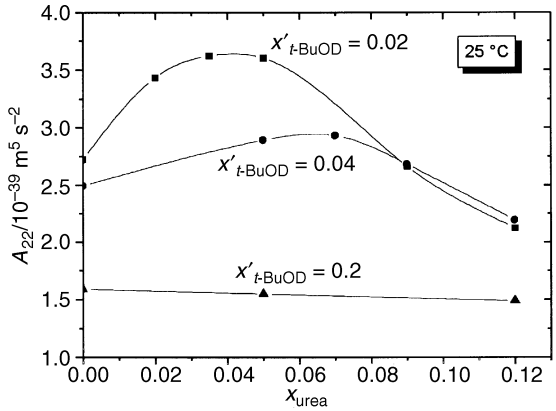

Fig. 5 A_{22} parameter with respect to the association of *t*-BuOD: for D_2O + *t*-BuOD [x'_2 = 0.02 (■), 0.04 (●) and 0.2 (▲)] + urea-d_4 mixtures at 25 °C as a function of the urea concentration.

the total number of water molecules in hydrophobic hydration spheres and the number of urea molecules at the maximum. With a hydration number $n_h = 25$ taken from ref. 29 we obtain with the above figures at the stabilisation maximum a ratio of water molecules in hydrophobic hydration spheres to urea molecules of about 12 : 1.

5 Conclusion

Measurements performed in this work aimed to clarify some aspects of the nature of the urea effect on the self-association of *t*-BuOH in aqueous solutions in analogy to the urea effect on complex aqueous protein solutions. Regarding the effect of urea on the translational dynamics of *t*-BuOH, it was shown that urea can, depending on the mixture composition, speed up or slow down the self-diffusion of the alcohol. But this is valid only below a certain transition temperature above which urea slows down the *t*-BuOH dynamics over the whole composition range. At low alcohol concentrations we find an indication that the *t*-BuOH self-association is connected with an increase in its translational mobility, implying that mainly the hydration water number determines the diffusivity of *t*-BuOH in the aqueous mixtures.

Regarding the urea effect on the translational dynamics of water, it was found that urea slows down the mean self-diffusion of water molecules. Therefore, a water structure-breaking mechanism of urea suggested in earlier literature,[31] which should be associated with a raise of the overall water mobility, could not be confirmed.

It was also shown that addition of relative small amounts of urea to diluted aqueous alcohol solutions stabilises the hydrophobic interaction, the stabilisation reaching a maximum at a characteristic total hydrophobic hydration water to urea mole ratio of 12 to 1. Addition of 12 mol% urea causes a clear destabilisation of the hydrophobic association in the water-rich region. Provided that a native protein structure is essentially determined by the hydrophobic interaction of side chains, the use of relatively large amounts of urea to achieve protein denaturation would be predicted on the basis of the results of the present work. It can be concluded that the same molecular mechanism is responsible for the destabilisation of hydrophobically associated small molecules by urea as it is for the denaturation of large biomolecules. Therefore, in the light of the present results, any proposed denaturation mechanism which suggests that the need of high concentrations of urea is connected with the extraordinary great size of biomolecules, should be reconsidered. Furthermore, the present paper demonstrates that small model molecules, such as low molecular alcohols, can be used, applying the NMR *A* parameter method, to monitor dynamic processes and weak association phenomena occurring in complex multi-component systems.

Acknowledgements

The authors thank the Deutsche Forschungsgemeinschaft (DFG) for financial support within the DFG-Schwerpunktprogramm (SPP 736): "Stoffeigenschaften komplexer fluider Gemische".

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