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Experimental Validation of the Role of Trifluoroethanol as a Nanocrowder

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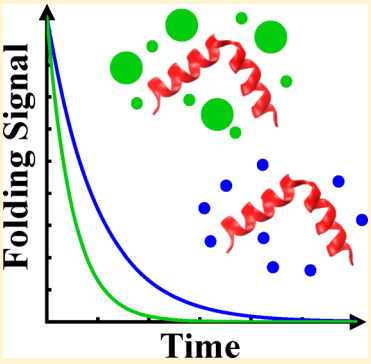
ABSTRACT: Trifluoroethanol (TFE) is commonly used to induce protein secondary structure, especially α-helix formation. Due to its amphiphilic nature, however, TFE can also self-associate to form micellelike, nanometer-sized clusters. Herein, we hypothesize that such clusters can act as nanocrowders to increase protein folding rates via the excluded volume effect. To test this hypothesis, we measure the conformational relaxation kinetics of an intrinsically disordered protein, the phosphorylated kinase inducible domain (pKID), which forms a helix−turn−helix in TFE solutions. We find that the conformational relaxation rate of pKID displays a rather complex dependence on TFE percentage (v/v): while it first decreases between 0 and 5%, between 5 and 15% the rate increases and then remains relatively unchanged between 15 and 30% and finally decreases again at higher percentages (i.e., 50%). This trend coincides with the fact that TFE clustering is maximized in the range of 15−30%, thus providing validation of our hypothesis. Another line of supporting evidence comes from the observation that the relaxation rate of a monomeric helical peptide, which due to its predominantly local interactions in the folded state is less affected by crowding, does not show a similar TFE dependence.



1. INTRODUCTION

While there are many ways to experimentally perturb a protein's stability, perhaps one of the most common is through the use of cosolvents. For example, guanidine hydrochloride (GdnHCl) and urea are frequently used to denature proteins, whereas several alcohols, such as hexafluoroisopropanol (HFIP) and trifluoroethanol (TFE), are known to induce secondary structure formation in polypeptides. Although there have been numerous efforts to understand how cosolvents act to change a protein's conformational preference, in each case, unanswered questions still remain. Herein, we study the conformational relaxation kinetics of two intrinsically disordered proteins (IDP) in different water/TFE mixtures, aiming to gain a better understanding of the mechanism with which this cosolvent influences the dynamics of protein folding.

The protein-stabilizing effect of TFE has been studied extensively both experimentally and computationally since its discovery by Goodman and Listowsky.[1−3] One view on TFE's mechanism of action is that it more favorably surrounds the protein than water, effectively leading to dehydration of the protein backbone, which, consequently, leads to backbone−backbone hydrogen bond formation and hence promotes secondary structure stabilization.[4−12] Conversely, other studies suggest that rather than stabilize the folded state, TFE acts to destabilize the unfolded state by structuring the solvent and, as a result, increasing the folded population.[13−15] Not surprisingly, some proposed mechanisms fall somewhere in-between.[16−20] In addition, it has been shown that the amphiphilic TFE molecule is capable, at large volume percentages, of exposing and interacting with hydrophobic side chains, thereby leading to disruption of hydrophobic tertiary interactions.[21] Due to the complexity of protein−TFE interactions, one expects that TFE will affect protein folding kinetics in a nonlinear manner. Indeed, Hamada et al.[22] found that the folding rates of a set of globular proteins follow a chevron-like trend with increasing TFE concentration. The interpretation for these results was that at low TFE percentages, folding rates are increased due to stabilization of native hydrogen-bonding groups, whereas at higher percentages, folding rates are decreased in a similar manner as is found with denaturants, due to TFE's interaction with buried residues, as determined by a high correlation between the *m*-values of TFE and GdnHCl.[22]

One factor that is potentially important to TFE's effect on protein folding, but not considered by previous studies, is the ability of TFE to self-associate. For example, dynamic light scattering (DLS) and nuclear magnetic resonance (NMR) measurements, as well as molecular dynamics (MD) simulations, found that TFE molecules can form clusters.[23−28] This clustering is thought to be the result of the cosolvent's hydrophobic CF_3 groups shielding themselves from water in micellelike structures that have Stokes' radii of 0.55 nm.[23] Furthermore, TFE clustering does not show a monotonic dependence on its percentage; it reaches a maximum at about 30% (v/v), above which the clusters disassemble and the solution becomes more homogeneous. Taken together, these findings suggest that TFE could act as a molecular crowder, thus increasing folding rates at certain percentages via the excluded volume effect.[29] In addition, the viscosity of TFE/water mixtures doubles from 0% to 60% TFE.[30] Such a drastic

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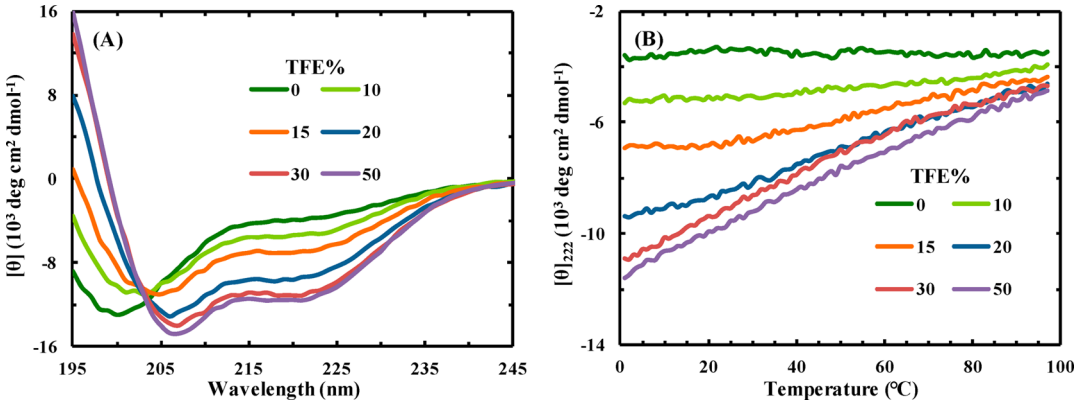

Figure 1. (A) CD spectra of pKID collected at 1 °C and in aqueous solutions of different TFE percentages, as indicated. (B) The corresponding CD *T*-melts of these samples at 222 nm.

increase in solvent viscosity could also have notable impacts on the folding rates of proteins in these solutions.

In order to gain insight into the effect of viscosity and cosolvent aggregation on protein folding kinetics, we have examined the conformational relaxation rates of two IDPs in different water/TFE solutions. IDPs are ideal candidates for this study, because they lack appreciable tertiary structure when isolated in buffer, simplifying our interpretations. Specifically, we studied the phosphorylated kinase inducible domain (pKID) peptide[31] and the late embryogenesis abundant (LEA) peptide.[32] We chose these two systems because both have folded states that are rich in α-helical content; however, pKID forms a helix−turn−helix (HTH) structure, whereas LEA folds into a monomeric α-helix. Previous experiments have shown that macromolecular crowding only has a small effect on the folding rate of monomeric α-helices, whereas proteins with appreciable nonlocal contacts experience more of a change.[33] Our hypothesis is that if TFE indeed behaves as a nanocrowder, it will affect the folding rate of pKID differently than that of LEA. Our results indeed reveal that the relaxation rate of pKID shows a complex dependence on the TFE percentage (in the range of 0−50%), with a maximum occurring between 15 and 30%, whereas that of LEA does not show such a dependence.

2. EXPERIMENTAL SECTION

Deuterated TFE was purchased from Cambridge Isotope Laboratories and stored in a drybox upon opening. Peptides were synthesized on a PS3 automated peptide synthesizer (Protein Technologies, MA) using Fmoc-protocols, purified by reverse-phase chromatography, and identified by matrix-assisted laser desorption ionization (MALDI) mass spectroscopy. Phosphorylated serine was incorporated into pKID (sequence DSVTDSQKRREILSRRPS*YRKILNDLSSDAPG−$CONH_2$, with S* representing phosphoserine) via the modified amino acid Fmoc-Ser(HPO_3Bzl)−OH. The sequence of the LEA peptide is AADGAKEKAGEAADGAKEKAGE−$CONH_2$. CD measurements were carried out on an Aviv 62A DS spectropolarimeter (Aviv Associates, NJ) with a 1 mm sample holder. The peptide concentration was in the range of 50−60 μM in H_2O and various concentrations of TFE (pH 7). Fourier transform infrared (FTIR) spectra were collected with 1 cm^{-1} resolution on a Magna-IR 860 spectrometer (Nicolet, WI) using a two-compartment CaF_2 sample cell of 56 μm path length. The details of the laser-induced temperature jump (*T*-jump) IR setup have been described elsewhere.[34] The amide hydrogen of peptides used in IR measurements has been exchanged to deuterium; the samples were prepared by directly dissolving lyophilized solids in D_2O solutions containing desired percentages of deuterated TFE (pH* 7). The final peptide concentration was between 1−2 mM.

The fractional helicity of the peptide, f_H, was estimated on the basis of its mean residue ellipticity at 222 nm, $[\theta]_{222}$, using the following relationship[35]

$$f_H = ([\theta]_{222} - [\theta]_C)/([\theta]_H - [\theta]_C) \quad (1)$$

where $[\theta]_H$ is defined as

$$[\theta]_H = (-44000 + 250T)[(n_H - a)/n_T] \quad (2)$$

and $[\theta]_C$ is defined as

$$[\theta]_C = 640 - 45T \quad (3)$$

where n_H is the number of helical residues in the peptide folded state (n_H was defined as 21 for pKID and 22 for LEA), n_T is the total number of residues in the peptide, a is the number of carbonyls in the helical structure not involved in intramolecular helical hydrogen bonding (a = 6 for pKID and 3 for LEA), and T is the temperature in Celsius.

3. RESULTS AND DISCUSSION

We chose pKID as our model system because a previous study has shown that TFE (10−40%) can significantly increase its helical content.[36] Consistent with this finding, our CD measurements indicate that the helicity of pKID increases with increasing TFE percentage from 0 to 30%, above which this increase levels off (Figure 1A). In addition, the thermal melt (*T*-melt) of this peptide, probed at 222 nm, indicates that TFE has an effect on the nature of the thermal unfolding transition (Figure 1B). Specifically, it appears that the unfolding transition becomes most cooperative when the percentage of TFE is approximately 15%, whereas at higher TFE concentrations (e.g., 50%) the *T*-melt is essentially linear. This type of transition has also been seen in other studies where TFE was used to induce helical structure formation;[35,37] however, a microscopic interpretation of this phenomenon is lacking. Due to the lack of baselines in these CD *T*-melts, as well as the changing nature of the *T*-melts themselves as a function of TFE percentage, no quantitative analysis was performed to extract additional information from this data. We did, however, use the mean residue ellipticity at 222 nm and the method developed by Baldwin and co-workers (Experimental Section) to estimate

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the fractional helicity (f_H) formed for each case. As shown (Table 1), the f_H values obtained for pKID in 0% and 30% TFE, 21% and 54%, respectively, are in good agreement with those obtained in previous studies.[36,38]

Table 1. Estimated Fractional Helicity (f_H) for pKID and LEA at 1 °C

pKID		LEA	
TFE (%)	f_H (%)	TFE (%)	f_H (%)
0	21	0	2
10	28	30	25
15	36	40	38
20	47	50	39
30	54		
50	57		

To determine the effect of TFE on the folding−unfolding kinetics of pKID, we measured its conformational relaxation rates in various concentrations of TFE using a laser-induced *T*-jump IR technique.[39] As shown (Figure 2), the *T*-jump-induced relaxation kinetics, probed at 1630 cm^{-1}, can be described by a single-exponential function. In addition, the relaxation rate does not show any measurable dependence on the initial temperature, suggesting that the folding−unfolding process of pKID involves a significant ($\geq 1.5k_BT$) free energy barrier.[40]

As indicated (Figure 3 and Table 2), in comparison to those measured in the absence of TFE, the relaxation rates obtained at low TFE percentages (up to 5% TFE) show a small but measurable decrease (~24%), which disappears completely upon increasing the TFE percentage to 15%. This nonmonotonic dependence is interesting, since such a trend has not been reported before. One possible explanation for the initial decrease in the relaxation rate is that it arises from a TFE-induced increase in the solution viscosity (η), since a previous study[30] has shown that a 5% TFE solution (in H_2O) has a viscosity of 1.00 cP, compared to 0.89 cP for pure water. Interestingly, this viscosity increase cannot completely account for the observed decrease in the relaxation rate (k_R). This is because, assuming $k_R \propto (\eta)^{-\alpha}$, where α ranges from 0.6 to 1.0,[41−43] an increase of η from 0.89 to 1.00 cP only leads to a decrease of k_R by ~12%, less than observed. This finding is entirely expected since, besides viscosity, the protein stability, which in this case is a function of TFE concentration, can also affect k_R. As discussed above, the helicity of pKID increases in the presence of TFE, suggesting that under these conditions the folded state is stabilized. As shown (Figure 4), an increased stability can result from either an increase in the folding rate (k_F), a decrease in the unfolding rate (k_U), or both. However, an increase in k_F would lead to an increase in k_R, as $k_R = k_F + k_U$. Thus, the decreased relaxation rate at 5% TFE is consistent with the notion that this alcohol, at relatively low percentages,

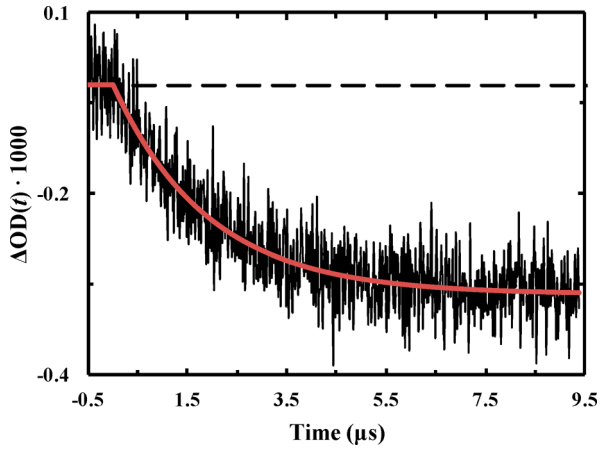

Figure 2. Representative trace of the relaxation kinetics of the pKID peptide in a 30% TFE solution in response to a *T*-jump from 5.7 to 11 °C, probed at 1630 cm^{-1}. The smooth line represents the best fit of this curve to a single-exponential function with a time constant of 1.8 ± 0.1 μs.

Table 2. Relaxation Time Constants (τ_R) of pKID and LEA at the Indicated Final Temperature (T_f)

pKID			LEA		
TFE (%)	T_f (°C)	τ_R (μs)	TFE (%)	T_f (°C)	τ_R (μs)
0	15.5	1.07 ± 0.09	30	24.2	0.21 ± 0.03
2.5	15.7	1.40 ± 0.10	40	24.7	0.36 ± 0.09
5	17	1.43 ± 0.08	50	23.1	0.18 ± 0.05
7.5	16.2	1.22 ± 0.09			
15	16.3	1.00 ± 0.30			
30	16.1	1.40 ± 0.10			
50	16.1	2.40 ± 0.50			

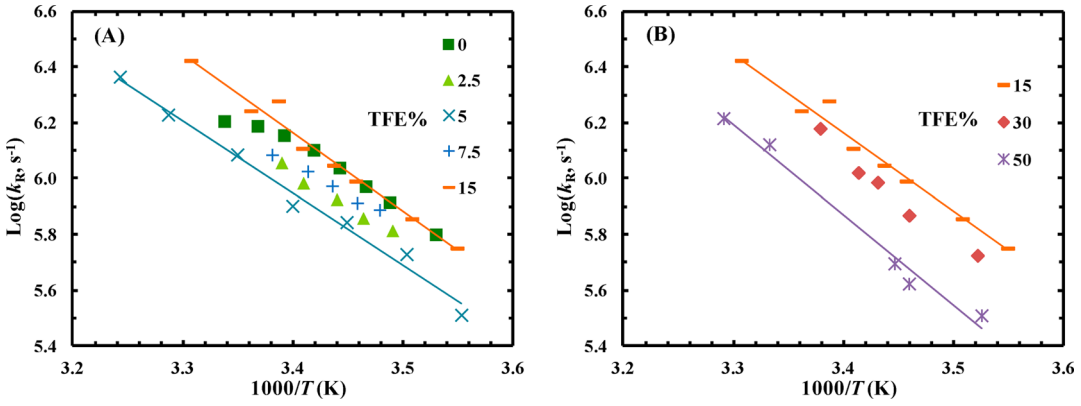

Figure 3. Temperature dependence of the relaxation rate constant of pKID measured for different TFE solutions, as indicated. For easy comparison, the results are presented in two panels: (A) 0−15% TFE and (B) 15−50% TFE. The solid lines shown are to guide the eye.

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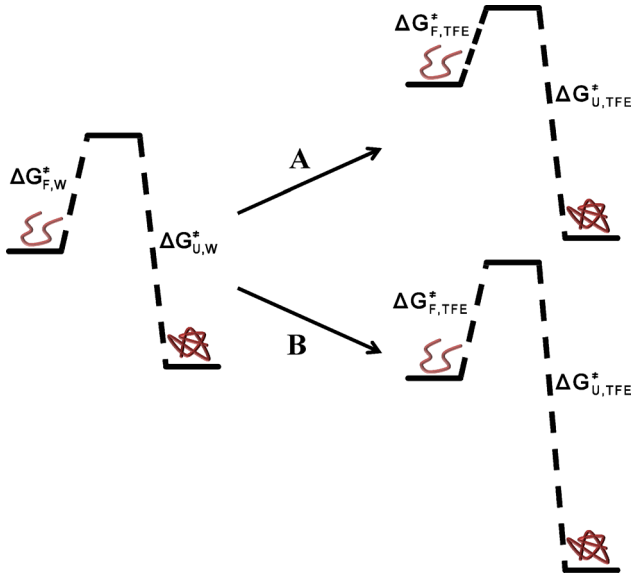

Figure 4. Cartoon illustration of the effect of TFE on the folding and unfolding free energy barriers of pKID. In scenario A, $\Delta G^{\ddagger}_{U,W} > \Delta G^{\ddagger}_{U,TFE}$ and $\Delta G^{\ddagger}_{F,W} = \Delta G^{\ddagger}_{F,TFE}$, whereas in scenario B $\Delta G^{\ddagger}_{U,W} = \Delta G^{\ddagger}_{U,TFE}$ and $\Delta G^{\ddagger}_{F,W} < \Delta G^{\ddagger}_{F,TFE}$.

can selectively stabilize the folded state, which kinetically manifests as an increase in the unfolding free energy barrier.

What is more surprising, however, is that upon further increasing the TFE percentage from 5 to 15%, the relaxation rate of pKID becomes larger (Figure 3). This faster relaxation rate remains unchanged, within experimental error, up to 30% TFE. Since both the helicity of pKID and the solution viscosity increase with increasing TFE concentration in this range of TFE percentages (i.e., 5−30%), both of which, as discussed above, would lead to a decrease in k_R, this kinetic trend is not anticipated and hence suggests that one needs to consider additional factors. One possible explanation, according to our hypothesis, is that this rate increase results from a crowding effect of TFE, which is known to form clusters in this concentration range. Such clusters, typically consisting of nine TFE molecules,[44] can occupy approximately 30% of the volume at 40% TFE, based on MD simulations.[28] Crowding, which preferentially destabilizes the more extended unfolded state through the excluded volume effect, increases protein folding rates.[29] However, unlike other commonly used macromolecular crowders, such as ficoll and dextran, which typically are assumed to be repulsive toward proteins, TFE interacts specifically with pKID. Thus, the observed concave upward dependence of the relaxation rate on TFE percentage, in the range of 0−30%, is a manifestation of the interplay of three factors, i.e., viscosity, stability and crowding.

While the results discussed above are consistent with the notion that TFE can act as a crowding agent at certain volume percentages, further validation of this claim is needed. Fortunately, TFE self-association or aggregation is not a monotonic function of its concentration, which peaks at around 30% and effectively vanishes at 70%.[24] This characteristic property of TFE clustering provides a simple means to test the validity of our hypothesis. Should the increased relaxation rate of pKID observed at 15−30% TFE solutions arise from crowding due to nearby TFE clusters, we would expect at higher concentrations of TFE, where these aggregates are less prevalent, the relaxation rates to, once again, decrease. Indeed, at 50% TFE the conformational relaxation rates of pKID become appreciably slower than those at 30% TFE, by a factor of approximately 1.7 (Figure 3 and Table 2). Thus, these results provide additional evidence in support of the notion that TFE clusters can act as nanocrowders. Furthermore, measurements of the conformational relaxation kinetics of another IDP, i.e. the LEA peptide, in water/TFE solutions also help support this claim. In nature, LEA proteins fold upon desiccation and are responsible for reducing aggregation of proteins in water-deficient conditions in both plants and animals.[45,46] Therefore, TFE, which causes the peptide backbone to be dehydrated, should be very effective in promoting LEA's folding to a monomeric α-helical structure, as observed (Figure 5A and Table 1). In addition, unlike that of pKID, the CD T-melt of LEA is more cooperative (Figure 5B), which may reflect its intrinsic ability to fold upon the removal of water. Perhaps more importantly, the folding of monomeric α-helices involves predominantly local interactions and thus diffusive motions over a relatively small length scale. As such, a previous study[32] has shown that their folding kinetics are much less affected by macromolecular crowding in comparison to folding processes that involve formation of substantial nonlocal interactions, such as the folding of β-sheet structures. In other words, we expect, unlike pKID, that LEA's relaxation rate will be less dependent on TFE clustering. Indeed, as shown (Figures 6 and 7 and Table 2), the T-jump-induced conformational relaxation rates of LEA are, within experimental uncertainties, practically the

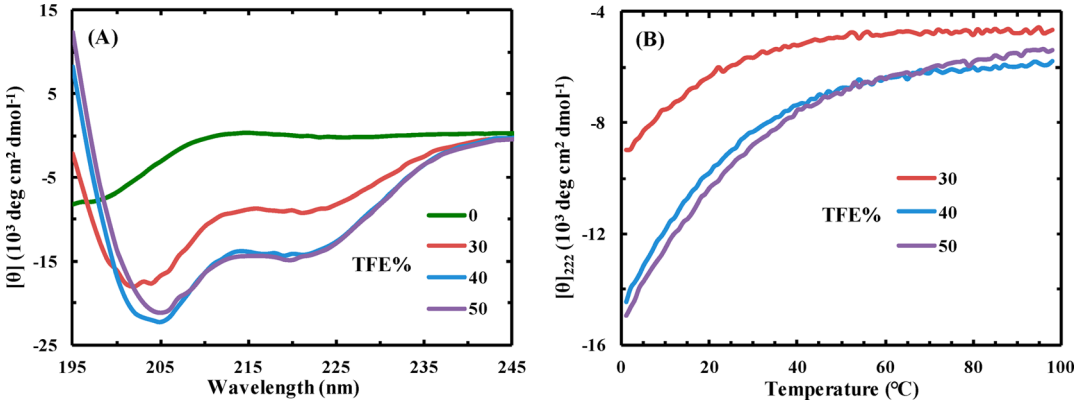

Figure 5. (A) CD spectra of LEA collected at 1 °C and in aqueous solutions of different TFE percentages, as indicated. (B) The corresponding CD T-melts of these samples at 222 nm.

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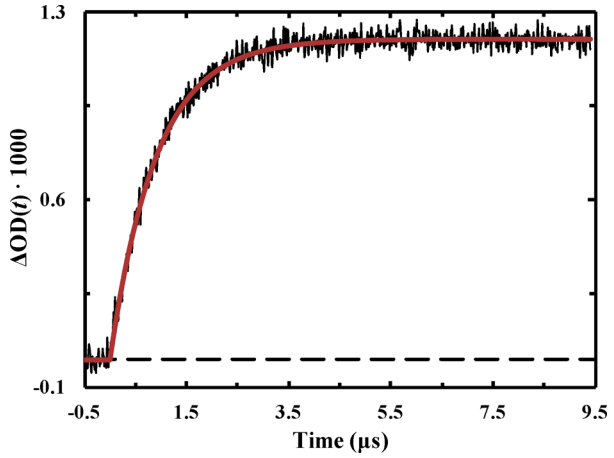

Figure 6. A representative trace of the relaxation kinetics of the LEA peptide in a 40% TFE solution in response to a *T*-jump from 3.8 to 8.4 °C, probed at 1664 cm^{-1}. The smooth line represents the best fit of this curve to a single-exponential function with a time constant of 0.9 ± 0.1 μs.

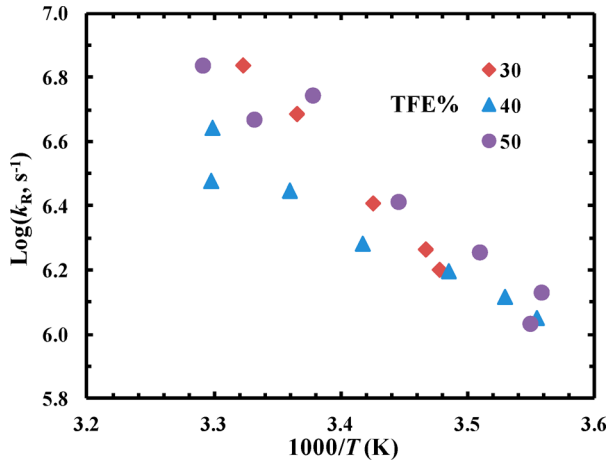

Figure 7. Relaxation rate constants of LEA versus temperature for different TFE solutions, as indicated.

same in the range of 30−50% TFE. Taken together, we believe that this difference in the relaxation kinetics of pKID and LEA supports the conclusion that TFE can act as a nanocrowder at certain concentrations. Additionally, it is worth noting that the relaxation rate of LEA is similar to that of a monomeric helical peptide derived from the ribosomal protein L9,[47] providing further evidence that the folding kinetics of naturally occurring helices are at, or near, the folding speed limit.

One alternative theory that has been proposed in the literature concerning TFE's interactions with proteins is that the clusters that are formed at certain volume percentages directly bind to hydrophobic residues in proteins;[48,49] however, both pKID and LEA are composed mainly of hydrophilic residues, with LEA having a slightly larger nonpolar residue composition. Although direct binding of these clusters to pKID could result in a change in the relaxation rates, such an event seems unlikely, since the kinetics of LEA are relatively unchanged throughout the TFE percentages examined.

In protein conformational studies it is common to use high concentrations of cosolvents, such as urea, alcohol, or TMAO, to experimentally control protein stability. Since many of these cosolvents have the tendency to self-associate, the crowding effect observed for TFE may also occur in other systems, an important aspect that has been largely overlooked. For example, in their MD simulations Cho et al.[50] found that a high concentration of TMAO leads to a reduction in the radius of gyration of several peptides, which led them to propose that TMAO can act as a molecular crowder. Using two-dimensional infrared (2D IR) spectroscopy, Ma et al.[51] also showed that TMAO can reduce the conformational entropy of proteins, thus further validating the crowding effect of TMAO aggregates. In this context, we expect that our observations in this study may be common for other cosolvents and thus should be taken into consideration in future studies when these molecules are used to tune the folding thermodynamics of proteins.

4. CONCLUSIONS

Alcohols are frequently used as cosolvents to enhance structure formation in peptides and proteins. In particular, TFE is remarkably effective in this regard and thus has found broad application. While previous studies have provided many insights into how TFE acts to achieve its structure-enhancing role, the potential effect of TFE clustering, which is maximized at approximately 30% TFE (v/v), has often been overlooked. To investigate whether TFE clusters affect the folding kinetics of proteins, herein we study the conformational relaxation kinetics of two intrinsically disorder proteins: one (i.e., pKID) forms a HTH conformation and the other (i.e., LEA) folds into an α-helix when TFE is present. Our results show that the relaxation rate of pKID has a complex dependence on TFE percentage in the range of 0−50%, whereas that of LEA is insensitive to TFE concentration. In particular, the maximum relaxation rate of pKID occurs at a TFE percentage (15−30%) where TFE clustering is also prevalent. Thus, based on these results, we propose that TFE can act as a nanocrowder and, through the excluded volume effect, increase the folding rate of proteins containing a substantial amount of nonlocal contacts.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Goodman, M.; Listowsky, I.; Masuda, Y.; Boardman, F. Conformational Aspects of Polypeptides. VIII. Helical Assignments via Far Ultrviolet Absorption Spectra and Optical Activity. *Biopolymers* **1963**, *1*, 33−42.

(2) Sonnichsen, F. D.; Van Eyk, J. E.; Hodges, R. S.; Sykes, B. D. Effect of Trifluoroethanol on Protein Secondary Structure: An NMR and CD Study Using a Synthetic Actin Peptide. *Biochemistry* **1992**, *31*, 8790−8798.

(3) Buck, M. Trifluoroethanol and Colleagues: Cosolvents Come of Age. Recent Studies with Peptides and Proteins. *Q. Rev. Biophys.* **1998**, *31*, 297−355.
(4) Rajan, R.; Balaram, P. A Model for the Interaction of Trifluoroethanol with Peptides and Proteins. *Int. J. Pept. Protein Res.* **1996**, *48*, 328−336.
(5) Konno, T.; Iwashita, J.; Nagayama, K. Fluorinated Alcohol, The Third Group of Cosolvents That Stabilize the Molten-Globule State Relative to a Highly Denatured State of Cytochrome *c*. *Protein Sci.* **2000**, *9*, 564−569.
(6) Diaz, M. D.; Berger, S. Preferential Solvation of a Tetrapeptide by Trifluoroethanol As Studied by Intermolecular NOE. *Magn. Reson. Chem.* **2001**, *39*, 369−373.
(7) Roccatano, D.; Colombo, G.; Fioroni, M.; Mark, A. E. Mechanism by Which 2,2,2-Trifluoroethanol/Water Mixtures Stabilize Secondary-Structure Formation in Peptides: A Molecular Dynamics Study. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99*, 12179−84.
(8) Diaz, M.; Fioroni, M.; Burger, K.; Berger, S. Evidence of Complete Hydrophobic Coating of Bombesin by Trifluoroethanol in Aqueous Solution: An NMR Spectroscopic and Molecular Dynamics Study. *Chem.—Eur. J.* **2002**, *8*, 1663−1669.
(9) Fioroni, M.; Diaz, M. D.; Burger, K.; Berger, S. Solvation Phenomena of a Tetrapeptide in Water/Trifluoroethanol and Water/Ethanol Mixtures: A Diffusion NMR, Intermolecular NOE, and Molecular Dynamics Study. *J. Am. Chem. Soc.* **2002**, *124*, 7737−7744.
(10) Starzyk, A.; Barber-Armstrong, W.; Sridharan, M.; Decatur, S. M. Spectroscopic Evidence for Backbone Desolvation of Helical Peptides by 2,2,2-Trifluoroethanol: An Isotope-Edited FTIR Study. *Biochemistry* **2005**, *44*, 369−376.
(11) Anderson, V. L.; Webb, W. W. A Desolvation Model for Trifluoroethanol-Induced Aggregation of Enhanced Green Fluorescent Protein. *Biophys. J.* **2012**, *102*, 897−906.
(12) Shao, Q.; Fan, Y.; Yang, L.; Gao, Y. Q. From Protein Denaturant to Protectant: Comparative Molecular Dynamics Study of Alcohol/Protein Interactions. *J. Chem. Phys.* **2012**, *136*, 115101.
(13) Cammers-Goodwin, A.; Allen, T. J.; Oslick, S. L.; McClure, K. F.; Lee, J. H.; Kemp, D. S. Mechanism of Stabilization of Helical Conformations of Polypeptides by Water Containing Trifluoroethanol. *J. Am. Chem. Soc.* **1996**, *118*, 3082−3090.
(14) Kentsis, A.; Sosnick, T. R. Trifluoroethanol Promotes Helix Formation by Destabilizing Backbone Exposure: Desolvation Rather than Native Hydrogen Bonding Defines the Kinetic Pathway of Dimeric Coiled Coil Folding. *Biochemistry* **1998**, *37*, 14613−22.
(15) Myers, J. K.; Pace, C. N.; Scholtz, J. M. Trifluoroethanol Effects on Helix Propensity and Electrostatic Interactions in the Helical Peptide from Ribonuclease T-1. *Protein Sci.* **1998**, *7*, 383−388.
(16) Thomas, P. D.; Dill, K. A. Local and Nonlocal Interactions in Globular-Proteins and Mechanisms of Alcohol Denaturation. *Protein Sci.* **1993**, *2*, 2050−2065.
(17) Walgers, R.; Lee, T. C.; Cammers-Goodwin, A. An Indirect Chaotropic Mechanism for the Stabilization of Helix Conformation of Peptides in Aqueous Trifluoroethanol and Hexafluoro-2-propanol. *J. Am. Chem. Soc.* **1998**, *120*, 5073−5079.
(18) Main, E. R. G.; Jackson, S. E. Does Trifluoroethanol Affect Folding Pathways and Can It Be Used as a Probe of Structure in Transition States? *Nat. Struct. Biol.* **1999**, *6*, 831−835.
(19) Yiu, C. P. B.; Mateu, M. G.; Fersht, A. R. Protein Folding Transition States: Elicitation of Hammond Effects by 2,2,2-Trifluoroethanol. *Chembiochem* **2000**, *1*, 49−55.
(20) Yamaguchi, K.; Naiki, H.; Goto, Y. Mechanism by Which the Amyloid Fibrils of a Beta(2)-Microglobulin Fragment Are Induced by Fluorine-Substituted Alcohols. *J. Mol. Biol.* **2006**, *363*, 279−288.
(21) Buck, M.; Schwalbe, H.; Dobson, C. M. Characterization of Conformational Preferences in a Partly Folded Protein by Heteronuclear NMR Spectroscopy: Assignment and Secondary Structure Analysis of Hen Egg-White Lysozyme in Trifluoroethanol. *Biochemistry* **1995**, *34*, 13219−13232.
(22) Hamada, D.; Chiti, F.; Guijarro, J.; Kataoka, M.; Taddei, N.; Dobson, C. Evidence Concerning Rate-Limiting Steps in Protein Folding from the Effects of Trifluoroethanol. *Nat. Struct. Biol.* **2000**, *7*, 58−61.
(23) Gast, K.; Zirwer, D.; Muller-Frohne, M.; Damaschun, G. Trifluoroethanol-Induced Conformational Transitions of Proteins: Insights Gained from the Differences between Alpha-Lactalbumin and Ribonuclease A. *Protein Sci.* **1999**, *8*, 625−634.
(24) Hong, D.-P.; Hoshino, M.; Kuboi, R.; Goto, Y. Clustering of Fluorine-Substituted Alcohols as a Factor Responsible for Their Marked Effects on Proteins and Peptides. *J. Am. Chem. Soc.* **1999**, *121*, 8427−8433.
(25) Gast, K.; Siemer, A.; Zirwer, D.; Damaschun, G. Fluoroalcohol-Induced Structural Changes of Proteins: Some Aspects of Cosolvent−Protein Interactions. *Eur. Biophys. J. Biophys.* **2001**, *30*, 273−283.
(26) Scharge, T.; Cezard, C.; Zielke, P.; Schutz, A.; Emmeluth, C.; Suhm, M. A. A Peptide Co-Solvent under Scrutiny: Self-Aggregation of 2,2,2-Trifluoroethanol. *Phys. Chem. Chem. Phys.* **2007**, *9*, 4472−4490.
(27) Jalili, S.; Akhavan, M. Molecular Dynamics Simulation Study of Association in Trifluoroethanol/Water Mixtures. *J. Comput. Chem.* **2010**, *31*, 286−294.
(28) Gerig, J. T. Toward a Molecular Dynamics Force Field for Simulations of 40% Trifluoroethanol−Water. *J. Phys. Chem. B* **2014**, *118*, 1471−80.
(29) Zhou, H. X.; Rivas, G. N.; Minton, A. P. Macromolecular Crowding and Confinement: Biochemical, Biophysical, and Potential Physiological Consequences. *Annu. Rev. Biophys.* **2008**, *37*, 375−397.
(30) Gente, G.; La Mesa, C. Water−Trifluoroethanol Mixtures: Some Physicochemical Properties. *J. Solution Chem.* **2000**, *29*, 1159−1172.
(31) Radhakrishnan, I.; Pérez-Alvarado, G. C.; Parker, D.; Dyson, H. J.; Montminy, M. R.; Wright, P. E. Solution Structure of the KIX Domain of CBP Bound to the Transactivation Domain of CREB: A Model for Activator:Coactivator Interactions. *Cell* **1997**, *91*, 741−752.
(32) Furuki, T.; Shimizu, T.; Chakrabortee, S.; Yamakawa, K.; Hatanaka, R.; Takahashi, T.; Kikawada, T.; Okuda, T.; Mihara, H.; Tunnacliffe, A.; Sakurai, M. Effects of Group 3 LEA Protein Model Peptides on Desiccation-Induced Protein Aggregation. *Biochim. Biophys. Acta* **2012**, *1824*, 891−7.
(33) Mukherjee, S.; Waegele, M. M.; Chowdhury, P.; Guo, L.; Gai, F. Effect of Macromolecular Crowding on Protein Folding Dynamics at the Secondary Structure Level. *J. Mol. Biol.* **2009**, *393*, 227−36.
(34) Serrano, A. L.; Waegele, M. M.; Gai, F. Spectroscopic Studies of Protein Folding: Linear and Nonlinear Methods. *Protein Sci.* **2012**, *21*, 157−70.
(35) Luo, P.; Baldwin, R. L. Mechanism of Helix Induction by Trifluoroethanol: A Framework for Extrapolating the Helix-Forming Properties of Peptides from Trifluoroethanol/Water Mixtures Back to Water. *Biochemistry* **1997**, *36*, 8413−21.
(36) Hua, Q. X.; Jia, W. H.; Bullock, B. P.; Habener, J. F.; Weiss, M. A. Transcriptional Activator−Coactivator Recognition: Nascent Folding of a Kinase-Inducible Transactivation Domain Predicts Its Structure on Coactivator Binding. *Biochemistry* **1998**, *37*, 5858−66.
(37) Jasanoff, A.; Fersht, A. R. Quantitative Determination of Helical Propensities from Trifluoroethanol Titration Curves. *Biochemistry* **1994**, *33*, 2129−2135.
(38) Zor, T.; Mayr, B. M.; Dyson, H. J.; Montminy, M. R.; Wright, P. E. Roles of Phosphorylation and Helix Propensity in the Binding of the KIX Domain of CREB-Binding Protein by Constitutive (c-Myb) and Inducible (CREB) Activators. *J. Biol. Chem.* **2002**, *277*, 42241−8.
(39) Huang, C. Y.; Getahun, Z.; Zhu, Y.; Klemke, J. W.; DeGrado, W. F.; Gai, F. Helix Formation via Conformation Diffusion Search. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *99*, 2788−2793.
(40) Lin, C. W.; Culik, R. M.; Gai, F. Using VIPT-Jump to Distinguish Between Different Folding Mechanism: Application to BBL and Trpzip. *J. Am. Chem. Soc.* **2013**, *135*, 7668−7673.
(41) Klimov, D.; Thirumalai, D. Viscosity Dependence of the Folding Rates of Proteins. *Phys. Rev. Lett.* **1997**, *79*, 317−320.
(42) Jas, G. S.; Eaton, W. A.; Hofrichter, J. Effect of Viscosity on the Kinetics of α-Helix and β-Hairpin Formation. *J. Phys. Chem. B* **2001**, *105*, 261−272.

(43) Zagrovic, B.; Pande, V. Solvent Viscosity Dependence of the Folding Rate of a Small Protein: Distributed Computing Study. *J. Comput. Chem.* **2003**, *24*, 1432−6.
(44) Fioroni, M.; Burger, K.; Mark, A. E.; Roccatano, D. A New 2,2,2-Trifluoroethanol Model for Molecular Dynamics Simulations. *J. Phys. Chem. B* **2000**, *104*, 12347−12354.
(45) Shimizu, T.; Kanamori, Y.; Furuki, T.; Kikawada, T.; Okuda, T.; Takahashi, T.; Mihara, H.; Sakurai, M. Dessication-Induced Structuralization and Glass Formation of Group 3 Late Embryogenesis Abundant Protein Model Peptides. *Biochemistry* **2010**, *49*, 1093−1104.
(46) Furuki, T.; Shimizu, T.; Kikawada, T.; Okuda, T.; Sakurai, M. Salt Effects on the Structural and Thermodynamic Properties of a Group 3 LEA Protein Model Peptide. *Biochemistry* **2011**, *50*, 7093−7103.
(47) Mukherjee, S.; Chowdhury, P.; Bunagan, M. R.; Gai, F. Folding Kinetics of a Naturally Occurring Helical Peptide: Implication of the Folding Speed Limit of Helical Proteins. *J. Phys. Chem. B* **2008**, *112*, 9146−9150.
(48) Reiersen, H.; Rees, A. R. Trifluroethanol May Form a Solvent Matrix for Assisted Hydrophobic Interactions between Peptide Side Chains. *Protein Eng.* **2000**, *13*, 739−743.
(49) Chatterjee, C.; Gerig, J. T. Interactions of Trifluoroethanol with the Trp-Cage Peptide. *Biopolymers* **2007**, *87*, 115−123.
(50) Cho, S. S.; Reddy, G.; Straub, J. E.; Thirumalai, D. Entropic stabilization of proteins by TMAO. *J. Phys. Chem. B* **2011**, *115*, 13401−13407.
(51) Ma, J.; Pazos, I. M.; Gai, F. New Microscopic Insights into the Protein-Stabilizing Effect of TMAO. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 8476−8481.