The Osmolyte TMAO Modulates Protein Folding Cooperativity by Altering Global Protein Stability

Prashant N. Jethva‡ and Jayant B. Udgaonkar*†,‡,∥

*National Centre for Biological Sciences, Tata Institute of Fundamental Research, Bengaluru 560065, India
∥Indian Institute of Science Education and Research, Pune 411008, India

Supporting Information

ABSTRACT: The folding of many globular proteins from the unfolded (U) to the native (N) state appears to be describable by a two-state N ↔ U model, which has led to the general belief that protein folding occurs in a highly cooperative manner. One reason for the widespread belief in “two-state folding” is that protein folding reactions are invariably studied by ensemble averaging probes and not by probes that can distinguish as well as quantify the multiple conformations that may be present. Consequently, how cooperativity is affected by protein stability, protein sequence, and solvent conditions is poorly understood. In this study, hydrogen exchange coupled to mass spectrometry (HX-MS) of the PI3K SH3 domain was carried out in the presence of a stabilizing osmolyte, trimethylamine N-oxide (TMAO). By showing that HX occurs under the EX1 regime even in the presence of 2 M TMAO, we were able to examine the temporal evolution of the populations of the different conformations present together. A strong link between protein folding cooperativity and protein stability is revealed. Increasing stability is accompanied by an increase in the ruggedness of the free energy landscape as well as diminished cooperativity; the number of amide sites simultaneously opening up their structure decreases with an increase in TMAO concentration. A comparison of the effect of TMAO to that of urea on the intrinsic dynamics of the PI3K SH3 domain indicates that TMAO counteracts the effect of urea not only on protein stability but also on protein folding cooperativity.

Proteins sample many partially to completely unfolded conformational states of high free energy under native conditions, according to their Boltzmann distribution.1–6 It is still not understood whether the transition to each of the partially unfolded intermediate states is cooperative or noncooperative in nature. Moreover, the factors that determine the extent of cooperativity are not well understood.7 It seems that a dynamic coupling between long-range (hydrophobic and electrostatic) and short-range (main chain hydrogen bonding) interactions defines the overall cooperativity (smoothness) of the folding free energy landscape. Strong coupling would lead to highly cooperative folding (two-state folding), with one large thermodynamic barrier separating the two ground (N and U) states on the folding free energy landscape. On the other hand, partial coupling would lead to reduced cooperativity (multistate folding), with more than one thermodynamic barrier separating the two ground states, while the absence of coupling would lead to an absence of cooperativity and a continuum of partially folded intermediate states, with many small thermodynamic barriers separating two ground states.8,9 Protein stability appears to be a major factor in determining cooperativity,10–13 and factors that affect protein stability, such as changes in solvent conditions, pressure, and temperature, are expected to modulate cooperativity.11 A common class of cosolvents that alter protein stability are the osmolytes, but little about how osmolytes modulate the cooperative nature of the protein folding reaction is known.

Stabilizing osmolytes are small chemical molecules, including methyl amines, amino acids, and polyols, which have been selected evolutionarily to help organisms to cope with the protein denaturing effects of environmental stress.10,14,15 Trimethylamine N-oxide (TMAO) belongs to the methyl amine class of osmolytes and stabilizes proteins against urea-induced denaturation under osmotic stress conditions.14,16 How TMAO stabilizes proteins is poorly understood. Stabilization could occur because of the dominant unfavorable interaction between TMAO and the polypeptide backbone.17–20 Alternatively, TMAO could effect a change in water structure or act through a nanocrowding mechanism.21,22 TMAO stabilizes folding intermediates23 and accelerates the folding of proteins.18,24–26 Because water–water hydrogen bonding appears to become stronger than protein–water hydrogen bonding in the presence of TMAO,17 the osmolyte is expected to modulate both the stability and dynamics of the native state and, thereby, the cooperativity of folding.11–13,27
Biochemistry

Under most experimental conditions, the native (N) state or the unfolded (U) state is predominantly present, obscuring the presence of sparsely populated, high-free energy, intermediate states whose presence or absence defines the cooperativity of the folding reaction. Delineating the cooperativity of the formation of such intermediate states requires a probe that can detect these intermediates in transient coexistence with the N and/or U states. While experimental probes such as hydrogen exchange, thiorelabeling, and nuclear magnetic resonance (NMR) spectroscopy have proven to be invaluable for characterizing such transient intermediate states, it is hydrogen exchange coupled to mass spectrometry (HXSMS) that is the method of choice for providing information about the dynamics of conformational conversion between coexisting populations of different states and, hence, the cooperativity of protein folding reactions even under native conditions.

SH3 domains are small globular proteins and are considered to be archetypal of two-state folding proteins. They play important roles in signal transduction, where they provide the interaction surface for multimeric protein complexes. The SH3 domain of the PI3 kinase was initially characterized as a cooperatively folding protein (two-state folding), but subsequent equilibrium and kinetic folding studies showed that it (un)folds via multiple intermediate states. Previous unfolding studies using HXSMS as a probe have shown that the unfolding of the PI3K SH3 domain is a highly heterogeneous process. The initial structure opening events occur through a continuum of intermediate states. Only at a later stage does the remaining structure unfold in a cooperative manner. Furthermore, structural characterization of the unfolding events has shown that the cooperatively unfolding/folding core of the protein comprises β strands 1, 2, and 3 in the absence of denaturant and β strands 1, 2, 3, and 4 in the presence of 5 M urea. In this manner, the extent of folding cooperativity is greater under strongly destabilizing conditions.

In the study presented here, the native state dynamics of PI3K SH3 domain was studied in the absence and presence of increasing concentrations of TMAO. In the absence of TMAO, the PI3K SH3 domain samples two partially unfolded intermediate states (I state 1 and I state 2) noncooperatively. The cooperative opening of I state 1 leads to the global unfolding of the PI3K SH3 domain. The cooperative nature of the opening of I state 2 suggests that under native conditions, the unfolding of the PI3K SH3 domain is a barrier-limited transition. Upon stabilization by TMAO, the cooperativity of folding was found to decrease. Under very strongly stabilizing conditions, the PI3K SH3 domain no longer unfolds cooperatively. In 2 M TMAO, there does not appear to be any large kinetic barrier for unfolding but instead many small distributed barriers. Furthermore, an additional partially unfolded intermediate state (I state 3) is sampled, suggesting that the change in thermodynamic stability also modulates the ruggedness of the free energy landscape.

MATERIALS AND METHODS

Buffers, Chemical Reagents, and Experimental Conditions. Buffers and chemical reagents, including TMAO, were of the highest purity grade and procured from Sigma-Aldrich (St. Louis, MO). Guanidine hydrochloride (GdnHCl) of the highest purity grade was procured from United States Biochemicals (USB) (Cleveland, OH). Sodium phosphate (50–100 mM) and 3-(N-morpholino)propanesulfonic acid (MOPS) (50 mM) were used as buffers at pH 7.2 and 6.2, respectively. The concentrations of the stock solutions of GdnHCl and TMAO were determined by measuring the refractive index on an Abbe refractometer (Thermo Scientific, Waltham, MA). All the pH values mentioned were not corrected for the isotope effect. All the experiments were carried out at 25 °C.

Protein Purification. Purification of the PI3K SH3 domain was carried out as described previously. The concentration of the protein was determined by measurement of the absorbance at 280 nm, using an ε280 of 17900 cm⁻¹ M⁻¹. The purity of the purified protein was checked by electrospray ionization mass spectrometry (ESI-MS) (Figure S1).

Fluorescence- and Circular Dichroism (CD)-Monitored Equilibrium Unfolding Studies. GdnHCl-induced equilibrium unfolding studies in the absence and presence of different concentrations of TMAO were carried out by monitoring the change in the intrinsic tyrosine (Tyr) fluorescence on the stopped-flow module (SMF-4, Biologic, Science Instruments, Seyssinet-Pariset, France). Tyrosine residues were excited at 268 ± 1 nm (1 cm path length), and emission was collected using a 300 ± 10 nm band-pass filter (Asahi Spectra, Torrance, CA). The protein concentration was 15–25 μM. The equilibrium unfolding curves were fitted to a two-state (N ↔ U) model (see the data analysis in the Supporting Information). Far-ultraviolet (far-UV) circular dichroism spectra were acquired using a Jasco J-815 spectropolarimeter (0.2 cm path length). The protein concentration used for far-UV CD measurements was 20–25 μM.

Deuteration of Protein. The protein was deuterated in the manner described previously. In brief, the protein was dissolved in D2O-containing buffer at pH 7.2, followed by a temperature jump to 70 °C for 10 min. The protein was then immediately placed in ice for 25 min. Finally, the deuterated protein was equilibrated at 25 °C before the start of the experiment. Protein integrity after deuteration was checked by far-UV CD spectroscopy (see Figure S2).

Hydrogen Exchange Kinetics. The hydrogen exchange (HX) reaction was initiated by diluting deuterated protein (500 μM, pH 7.2) 20-fold with protonated exchange buffer (with and without different concentrations of TMAO at pH 7.2 and 25 °C). For the back-exchange (BKEX) control, deuterated buffer at the same pH was used instead of protonated buffer. At the specified time of HX, the reaction mixture was injected into a G-2S, Hi-trap (GE Healthcare, Chicago, IL) desalting column coupled to an ÄKTA basic high-performance liquid chromatograph (GE healthcare). The protein was eluted from the column using Milli-Q water acidified to pH 2.6; 50 μL of desalted and quenched protein was injected via a 50 μL loop, into the HDX module attached to a nanoAquity ultraperformance liquid chromatograph (Waters, Milford, MA), coupled with a Synapt G2 HD mass spectrometer (Waters). The protein was first collected in a C-18 reverse phase trap column at a flow rate of 75 μL/min followed by elution with a gradient from 3 to 40% acetonitrile in 2 min, at a flow rate of 40 μL/min inside the HDX module. The eluted protein was infused directly into the Synapt G2 mass spectrometer. The entire HDX module was maintained at 4–8 °C, to minimize back-exchange of deuteriums during chromatography.
Instrument Parameters. The capillary voltage, desolvation temperature, and source temperature of the Synapt G2 mass spectrometer were set at 3 kV, 100 °C, and 35 °C, respectively, during the acquisition of HX mass spectra. Blank runs between two HX reactions were carried out to ensure that there was no carryover from the previous run.

Data Analysis. Intact Protein Analysis. Twenty scans (of 1 s each) from the TIC (total ion count) chromatogram were combined to obtain the protein mass spectrum. Each mass spectrum was further analyzed with MassLynx (version 4.1, Waters) using the background subtraction, smoothing, and centroid functions. The highest-intensity charge state (+11 charge state) signal was normalized with respect to the total area under the curve using Origin (version 8, Origin Lab Corp., Northampton, MA). Unimodal and bimodal mass distributions were fitted to a single Gaussian distribution equation (eq 1) and the sum of two Gaussian (eq 2) distribution equations, respectively. Parameters (centroid mass and area under the curve) for both mass distributions were analyzed separately. The mass of the protonated protein (9276 Da) was subtracted from the observed mass of the protein at a particular time of HX to obtain the number of protected deuteriums. The number of protected deuteriums (in the high-mass distribution) as a function of time of HX was fitted to a double- or triple-exponential decay equation to obtain apparent rate constants and amplitudes of each kinetic phase of exchange. The change in the area under the low-mass distribution (fraction unfolded) as a function of time was fitted to a single-exponential equation. The peak width at 20% of the mass distribution height was obtained using HXExpress2, an Excel-based macro program. All the plots and fitting of data were carried out using SigmaPlot (version 12, Systat Software, San Jose, CA).

Mass distributions were fit to the following equations.

\[ M(t) = \frac{A_1}{\sqrt{\pi/2 \times w_1}} \times e^{-2(C-C_1)^2/w_1^2} \]  

where \( M(t) \) is the mass distribution at time \( t \), \( A_1 \) is the area under the distribution, \( C_1 \) is the centroid of the mass distribution at time \( t \), \( w_1 \) is the full width at half-maximum of the Gaussian distribution, and \( C \) is the linearly variable \( m/z \) axis.

\[ M(t) = \left[ \frac{A_1}{\sqrt{\pi/2 \times w_1}} \times e^{-2(C-C_1)^2/w_1^2} \right] + \left[ \frac{A_2}{\sqrt{\pi/2 \times w_2}} \times e^{-2(C-C_2)^2/w_2^2} \right] \]  

where \( M(t) \) is the mass distribution at time \( t \), \( A_2 \), \( C_2 \), and \( w_2 \) are the area under the distribution, the centroid, and the peak width at half-maximum for each Gaussian distribution \( i \), respectively, and \( C \) is the linearly variable \( m/z \) axis.

Peptide Analysis. For peptide analysis, the protein was digested by being passed through an immobilized pepsin column (Applied Biosystems, Foster City, CA) kept inside the HDX module. The protein was passed through the pepsin column at a flow rate of 50 μL/min for 4 min with Milli-Q water containing 0.05% formic acid. Protein fragments thus generated were initially trapped inside a C-18 reverse phase trap column, followed by separation using a C-18 reverse phase analytical column (gradient from 10 to 40% acetonitrile containing 0.1% formic acid) over 10 min. The MS/tandem MS method was used to obtain the MS/MS spectra of different protein fragments. The ProteinLynx Global Server (PLGS, version 2.4, from Waters) was used to identify each peptide and its retention time from the MS/MS mass spectrum.

To determine the centroid mass, as well as the width at 20% of the peak height of the isotopic mass distribution, intensity values for the isotopic mass distributions were exported to HXExpress2. The isotopic mass distributions were fitted to a single binomial function. The fractional deuterium content in each sequence segment was calculated according to eq 3.

\[ \text{fraction } D = \frac{m(t) - m(5\% D)}{m(100\% D) - m(5\% D)} \]  

where \( m(t) \) is the observed centroid mass at time \( t \) of HX. The centroid mass of each peptide in the BKEX control sample was considered as the 100% deuterated control, and the centroid mass of each peptide at 180 d (days) of exchange was considered as the 5% deuterated control. The plot of the fraction of deuterium retained as a function of time was fitted to a single-, double-, or triple-exponential decay equation to determine the apparent rate constants and amplitudes of the HX reaction.

RESULTS

TMAO Stabilizes the PI3K SH3 Domain. The PI3K SH3 domain has seven Tyr residues dispersed throughout the protein sequence. Tyr fluorescence is quenched by the single tryptophan (Trp) residue in the folded state of the protein due to fluorescence resonance energy transfer (FRET). Hence, the Tyr fluorescence spectrum is a good tool for monitoring the tertiary structure of the protein. Figure 1 shows Tyr fluorescence-monitored GdnHCl-induced equilibrium unfolding curves in the presence of increasing concentrations of TMAO. These were normalized by the fluorescence signal at 4 M GdnHCl. The solid lines represent fits to a two-state model for unfolding (see Figure S3 for the parameters obtained from the fits).

![Figure 1. Equilibrium unfolding studies of the PI3K SH3 domain in the absence and presence of different concentrations of TMAO at pH 7.2 and 25 °C. Shown are tyrosine fluorescence-monitored, GdnHCl-induced equilibrium unfolding curves in the presence of increasing concentrations of TMAO. These were normalized by the fluorescence signal at 4 M GdnHCl. The solid lines represent fits to a two-state model for unfolding (see Figure S3 for the parameters obtained from the fits).](image-url)
concentration of TMAO (Figure S3). The value of $m_{opt}$, which is a measure of the change in the solvent accessible surface area upon unfolding, was found to decrease in the presence of TMAO. This suggested that the U state becomes compact upon addition of TMAO.

It should be noted that upon addition of TMAO, the fluorescence emission spectra of the native (N) and unfolded (U) proteins showed no change in their emission maxima (Figure S4). However, a gradual decrease in the Tyr fluorescence intensity and a concomitant increase in the Trp fluorescence intensity were observed for the U state in the presence of increasing concentrations of TMAO. This increase in the level of fluorescence resonance energy transfer from Tyr to Trp suggests that the U state becomes more compact in the presence of TMAO. Tyr fluorescence also decreased for the native protein in the presence of TMAO; however, a concomitant increase in Trp fluorescence was not observed. Figure S4 also shows that the secondary structure of the native and unfolded proteins remained unperturbed upon addition of TMAO. It is also seen that the protein remains fully soluble, in its native and unfolded states, even in 2 M TMAO (Figure S4B,D).

**Effect of TMAO-Induced Stabilization on Mass Distributions.** The HX reaction in the absence and presence of TMAO was carried out by diluting the deuterated protein into the protonated exchange buffer at the same pH. At a specific time of HX, the reaction was quenched by desalting followed by the acquisition of the mass spectrum. Figure 2 and Figure S5 show the mass distributions [+]1 charge state, the most intense charge state (Figure S1)] at different times of HX in the absence and presence of different concentrations of TMAO. The unimodal mass distributions at the earliest time point of HX (5 s) indicated the presence of only one population of the native protein in the absence and presence of TMAO. As was shown previously, in the absence of TMAO, the mass distribution remained unimodal during the initial 300 s of HX and shifted gradually to a lower mass (Figure 2, 0 M TMAO). After 300 s, the mass distribution became bimodal with the appearance of a lower mass distribution corresponding to the completely exchanged out protein (unfolded state). The observation of the bimodal mass distribution suggested that the protein undergoes cooperative structural opening in the absence of TMAO. To further validate the observations described above, the charge state distributions at all time points of HX in the absence of TMAO were deconvoluted and analyzed. Figure S6 shows that the deconvoluted mass distributions also behaved in a manner similar to that of the mass distributions of the +11 charge state.

The presence of TMAO resulted in a significant change in the observed mass distributions (Figure 2). As in the absence of TMAO, the mass distribution was observed to shift gradually up to 300 s of HX, and at later time points, the mass distribution became bimodal. Bimodality in the mass distributions was observed at TMAO concentrations of ≤1.5 M, suggesting that unfolding occurs in a barrier-limited cooperative manner. However, in the presence of 2 M TMAO, the mass distribution remained unimodal at all times of HX, suggesting that under the most stabilizing solvent conditions, the protein exchanged out all its protected deuteriums by noncooperative openings only.

Figure 2 shows that the two components of the bimodal mass distribution were very well separated on the m/z scale in the absence of TMAO but that in the presence of increasing concentrations of TMAO, the two components begin to merge with each other. To quantify this behavior, the change in the peak width of the bimodal mass distribution was analyzed. An analysis of the kinetics of this change can provide valuable information about the cooperative nature of a protein folding reaction.52 It is seen from the kinetics (Figure S7) that at the initial time points of HX, the peak width was constant but increased at later time points due to the appearance of the low mass distribution. The magnitude of the increase in the peak width gave the number of cooperatively opening residues. This difference was $15 \pm 1$, $13 \pm 1$, $8 \pm 1$, and $6 \pm 1$ Da for 0, 0.5, 1.0, and 1.5 M TMAO, respectively, suggesting that the cooperativity of folding decreased in a TMAO concentration-dependent manner. The absence of a peak width maximum for HX in the presence of 2 M TMAO indicated a complete loss of protein folding cooperativity. It should be noted that the protein remained soluble and stable during prolonged incubation in the presence of 2 M TMAO (Figure S8).

**Effect of TMAO-Induced Stabilization on the Kinetics of Hydrogen Exchange.** Figure 3 shows that in the absence of TMAO, the high mass distributions shifted gradually to low mass in two kinetic phases, the fast and slow phases of noncooperative HX. At later time points, HX appeared to occur cooperatively. The area under the low mass distribution increased in an exponential manner (Figure 3A,F). The fast and slow phases of noncooperative HX into the species giving
rise to the high mass distribution indicated that the N state transiently sampled partially unfolded intermediate states I_2 (fast phase) and I_3 (slow phase), as had been shown previously. The slow phase of cooperative HX suggested that global opening of I_3 led to the sampling of the U state. It was seen that the apparent rate constants for the slow noncooperative formation of I_2 and cooperative formation of U were similar (Table S1). Eighteen ± 1 deuteriums were found to exchange cooperatively in the absence of TMAO, as also seen previously. Many SH3 domains are known to undergo partial cooperative unfolding. The half-life (apparent rate) of global unfolding of the PI3K SH3 domain is in good agreement with the previously reported half-lives of other SH3 domains.

In the presence of 0.5 (Figure 3B,G) and 1.0 M TMAO (Figure 3C,H) also, the high mass distribution shifted into two kinetic phases and the cooperative opening of I_2 led to global unfolding. When HX was carried out in the presence of TMAO, the kinetic phases corresponding to the formation of I_2 and U were slower than in the absence of TMAO. The important difference between HX in the absence and HX in the presence of TMAO was the decrease in the number of cooperatively opening residues, which was 15 ± 1 and 11 ± 1 in 0.5 and 1 M TMAO, respectively, in good agreement with the numbers obtained from the peak width analysis (Figure S7).

In 1.5 M TMAO also, bimodal mass distributions were observed at later times of HX. Unlike the case of HX at lower TMAO concentrations, however, the gradual shift in the high mass distribution was found to occur in three kinetic phases (Figure 3D,I). This result suggested that apart from sampling I_2 and I_3, the protein populated an additional partially unfolded intermediate state (I_1) in 1.5 M TMAO. The number of residues opening cooperatively was reduced even further to 7 ± 1 in the presence of 1.5 M TMAO.

When HX was carried out in the presence of 2 M TMAO, the mass distribution was found to remain unimodal throughout the exchange process. The kinetics of the change in the number of protected deuteriums fit to a triple-exponential decay equation (Figure 3E). Thus, the protein lost all its protected deuteriums in a noncooperative (diffusive) manner and sampled even the U state by noncooperative diffusive opening of the polypeptide backbone. The apparent rate constants for the formation of I_2 and U were very similar. The triple-exponential decay suggested that in the presence of 2 M TMAO, the protein exchanges during the noncooperative formation of I_N, I_2, and U.

**Mechanism of HX in the Presence of TMAO.** To determine whether structural openings are noncooperative or cooperative, it is necessary to know whether HX in a particular solvent condition occurs in the EX1 or EX2 regime (see the Supporting Information). When HX occurs in the EX1 regime, it becomes possible to distinguish cooperative from noncooperative structural unfolding. The most definitive way to identify the HX regime is to measure the pH dependence of the observed apparent rate constant of HX (see the text of the Supporting Information). In the case of TMAO concentrations of ≤1.5 M, the mass distribution showed bimodality at later time points of HX. This suggested that for these lower TMAO concentrations, HX occurs in the EX1 regime. To determine which HX regime was operative in the presence of 2 M TMAO, where only unimodal mass distributions were observed, HX in the presence of 2 M TMAO was also carried out at pH 6.2 and 25 °C. In the EX2 regime, the observed apparent rate constant of HX is expected to decrease 10-fold with a decrease in the pH of the HX reaction by 1 unit, while in the EX1 regime, it is expected to remain constant. Figure 4 and Table S1show that the apparent rate constants and the numbers of deuteriums exchanged during all three kinetic phases of HX in the presence of 2 M TMAO were similar at pH 6.2 and 7.2, suggesting that HX occurs in the EX1 regime in the presence of 2 M TMAO. It should be noted that the protein stability is
However, a mere comparison of the apparent rate constants of HX at two different pH values for only the intact protein may not provide a correct picture of the HX regime. Therefore, the pH dependencies of the apparent rate constants of HX into different sequence segments of the protein were determined. To study the kinetics of HX into different sequence segments in the presence of 2 M TMAO, pepsin proteolysis of the protein was carried out at pH 2.6 after the HX reaction was quenched. Figure S10 shows the different sequence segments, which were separated by reverse phase chromatography (RP-LC) followed by identification through mass spectrometry (MS). To obtain quantitative information from isotopic mass distributions arising from the different sequence segments, each isotopic mass distribution was fitted to a single binomial equation to obtain the width and centroid. From the centroid mass, the fraction of deuterium retained, averaged over all the amide sites within the sequence segment, was obtained (see Materials and Methods).

Figures S11 and S12 show that as in the case of the whole intact protein, all the sequence segments showed a gradual shift in unimodal mass distributions (noncooperative opening) at all time points of HX at pH 7.2 and 6.2. The kinetics of the change in the fraction of deuterium retained were identical at pH 7.2 and 6.2 for most of the sequence segments, and hence, global fits of the HX data were carried out, except for sequence segment 71–81 (β strand 5) (Figure S13). The apparent rate constants obtained from the global fits were very similar to the apparent rate constants obtained at pH 7.2 (Figure 5 and Table S2). Hence, as in the case of HX into the whole intact protein, HX into individual sequence segments was also independent of pH. HX into sequence segment 71–81 could be described by a two-exponential equation, and the apparent rate constants of the fast and slow phases of noncooperative HX at pH 6.2 were more than 10- and 3-fold slower, respectively, than the apparent rate constant at pH 7.2. This suggested that for this sequence segment only, HX occurred in the EX2 regime during the fast phase and in the EXX regime during the slow phase of noncooperative HX. Furthermore, to identify any subtle changes in the mass distributions due to the change in pH during HX, the kinetics of the change in peak width during HX into different sequence segments were determined. Figure S14 shows that all the sequence segments showed very similar peak widths at both pH values at all times.
of exchange. These observations suggested that in the presence of 2 M TMAO, HX did indeed occur in the EX1 regime.

**TMAO Dependence of Opening/Unfolding Rate Constants.** The dependence of the kinetics of HX on TMAO concentration is a measure of the amount of TMAO-disfavored surface area in each partially unfolded intermediate state. The U state, which exposes a large amount of polypeptide backbone surface area, would be destabilized greatly by the unfavorable interaction of TMAO with the polypeptide backbone. Likewise, any intermediate state that exposes a large polypeptide backbone surface area would form with an apparent rate constant that would be greatly affected by TMAO concentration. Figure 6A shows that the logarithm of the apparent rate constant for the formation of IN, I₃, and I₂ was found to be slightly faster than those for the formation of U, at higher concentrations of TMAO (Figure 6). As previously observed for urea (Figure 6B), the logarithm of the apparent rate constants of formation of IN, I₃, and I₂ were found to have strong and similar dependencies on TMAO concentration. This weak dependence suggests that similar to the case of urea (Figure 6B), TMAO did not affect the TS state for the formation of I₃, hence, the apparent rate constant remained unaffected by the presence of TMAO. As previously observed for urea (Figure 6B), the logarithms of the apparent rate constants for the formation of I₂ and U were also found to have strong and similar dependencies on TMAO concentration. However, contrary to the observation made in the case of urea, where the apparent rate constants for the formation of I₂ and U became very similar at high urea concentrations, the apparent rate constants for the formation of I₂ were found to be slightly faster than those for the formation of U, at higher concentrations of TMAO (Figure 6). The dependence of the apparent rate constant of formation of I₃ on TMAO concentration could not be measured because there were insufficient data points. The observation that the global unfolding rate constant decreases with an increase in TMAO concentration suggests that the TS for global unfolding is destabilized in the presence of TMAO. While this result is consistent with TMAO interacting unfavorably with the polypeptide backbone, it is possible that TMAO also acts via other mechanisms.²¹,²²

**Structural Characterization of Partially Unfolded States in the Presence of 2 M TMAO.** To understand how the intrinsic dynamics of different regions of the PI3K SH3 domain were modulated by the presence of 2 M TMAO, pepsin proteolysis of the protein was carried out as mentioned above. Figure S11 shows the isotopic mass distributions of different sequence segments at different times of HX in the presence of 2 M TMAO at pH 7.2 and 25 °C. The isotopic mass distributions corresponding to all the sequence segments showed the gradual shift along the m/z axis and fit well to a single binomial distribution at all time points of HX.⁵¹ The most striking differences between HX in the absence and presence of 2 M TMAO were observed for sequence segments 1–11 (β strand 1), 23–35 (β strand 2), and 51–71 (β strands 3 and 4). All three sequence segments were known to exchange cooperatively in the absence of TMAO,⁶³ but they exchanged noncooperatively in the presence of 2 M TMAO. To characterize how protected deuteriums exchanged out in each sequence segment, the kinetics of the change in the fraction of deuterium retained were fit to a single-, double-, or triple-exponential decay equation. Table S2 shows the apparent rate constants and amplitudes of HX for each sequence segment.

Figure 7 shows the kinetics of HX into each sequence segment. Sequence segment 36–50 (nSrc loop) exchanged its protected deuteriums within 200 s of HX in a single-exponential phase. Sequence segments 1–11 (β strand 1), 12–22 (RT loop), 23–35 (β strand 2), 59–71 (β strand 4), and 71–81 (β strand 5) exchanged protected deuteriums in two exponential phases. The observation of two apparent rate constants of exchange for sequence segment 1–11 (β strand 1) suggests that it takes part in the formation of I₃ as well as the gradual noncooperative formation of the U state. The apparent rate constants of HX into sequence segments 12–22, 23–35, and 59–71 indicate that they exchanged during the formation of I₁ and the noncooperative formation of U. The apparent rate constants for sequence segment 71–81 suggest that it exchanged during the formation of I₃ only. Sequence segment 51–71 (a β hairpin comprises β strands 3 and 4) exchanged protected deuteriums in three exponential phases (Figure 7). This suggests that it exchanged during the noncooperative formation of I₃, I₂, and U. Figure 8 maps the HX kinetics and the fraction of deuterium protected in each partially unfolded intermediate state onto the structure of the N state.

**DISCUSSION**

It has been suggested recently⁵¹ that the cooperativity of unfolding can be modulated by modulating the thermodynamic stability of a protein. Under destabilizing solvent conditions, in which either GdnHCl or urea is present, it was seen that the cooperativity of unfolding of monellin⁵¹ and of the PI3K SH3 domain increased. Under stabilizing solvent conditions, cooperativity is expected to decrease. In the study presented here, it is shown that TMAO, an osmolyte known to counteract the destabilizing effect of urea, reduces the cooperativity of the unfolding of the PI3K SH3 domain.
Furthermore, it is also shown that upon stabilization, the energy landscape becomes more rugged.

Multiple Structural Openings of the PI3K SH3 Domain in the Absence of TMAO. As reported previously, and as demonstrated in this study (Figure 4), HX occurs in the EX1 regime in the absence of TMAO. Hence, the gradual shift in the high mass distribution can be attributed to noncooperative structural openings of the N state.

Figure 7. Kinetics of HX into different sequence segments of the PI3K SH3 domain in the presence of 2 M TMAO at pH 7.2 and 25 °C. Dashed lines are the fractions of deuterium retained (1.0) in each sequence segment in the BKEX control sample. The solid lines are fits to single-exponential (sequence segment 36−50), double-exponential (sequence segments 1−11, 12−22, 23−35, 59−71, and 71−81), and triple-exponential (sequence segment 51−71) equations. The inset for sequence segment 51−71 shows early times of HX. The bar graph shows the fraction of deuterium back-exchanged in the first 5 s of HX. The apparent rate constants and amplitudes obtained from the fits are listed in Table S2. The error bars represent the spread in the data from two separate experiments.

Figure 8. Comparative analysis of the structures of different partially unfolded intermediate states in the absence (0 M) of any cosolvent, under a strongly destabilizing condition (5 M urea) and under a strongly stabilizing condition (2 M TMAO) at pH 7.2 and 25 °C. The lengths of the equilibrium arrow for cooperative opening indicate the size of the cooperative unit.
(Figure 2 and Figure S5). Such gradual, noncooperative opening of the structure could be due to the independent loss of structure at each amide residue, one at a time. The opening and closing events at the individual amide sites could be either parallel or sequential to each other. The apparent rate constant of HX obtained from the gradual shift in mass distribution would be the mean apparent rate constant of HX for all the residues that are exchanging during that particular exponential phase.

As shown previously, the shift in the high mass distribution, which occurs in two kinetic phases, arises from the transient sampling of two partially unfolded high-free energy intermediates (I<sub>h</sub> and I<sub>I</sub>) by the N state (Figure 3). The apparent rate constant for the two phases are very different from each other, the transition states (TSs) preceding the formation of I<sub>h</sub> and I<sub>I</sub> must be structurally very different. I<sub>h</sub> forms the fastest, suggesting that it is nativelike it forms on breathing openings/motions are believed to be important for core and exchange fully buried amide sites. Such native state protein occurs, and solvent molecules penetrate the protein important tertiary interactions are broken, global unfolding of buried residues exchange out during the formation of I<sub>2</sub>. Once energy intermediates (IN and I<sub>2</sub>) by the N state (Figure 3). The apparent rate constant of formation of I<sub>2</sub> is very similar to the apparent rate constant of structure at each amide residue, one at a time. The opening and closing events at the individual amide sites could be either parallel or sequential to each other. The apparent rate constant of HX obtained from the gradual shift in mass distribution would be the mean apparent rate constant of HX for all the residues that are exchanging during that particular exponential phase.

Hence, it cannot directly delineate noncooperative structural openings from a cooperative structural opening. 11

To understand how thermodynamic stability modulates the conformational space sampled by the P13K SH3 domain, the stability of the protein was increased by the addition of TMAO (Figure 1), and the native state dynamics was studied by HX at increasing TMAO concentrations. It is seen that up to the increase in stability provided by the addition of 1.5 M TMAO, the mass distribution behaved qualitatively as it does in the absence of TMAO (Figure 2 and Figure S5): the protein appears to sample the same conformational space as it does in the absence of TMAO. Quantitative analysis (Figure 3 and Table S1) shows, however, that at 1.5 M TMAO the high mass distribution shifts in three kinetic phases. This suggests that apart from I<sub>h</sub> and I<sub>2</sub> the protein samples an additional partially unfolded intermediate state I<sub>1</sub>. A sampling of an additional intermediate state indicates an increase in the ruggedness of the energy landscape of the protein upon stabilization. Furthermore, the observation that the total number of noncooperatively exchanging deuteriums increases at the expense of the number of cooperatively exchanging deuteriums indicates that local unfolding dominates over global unfolding in the presence of increasing TMAO concentrations (Table S1). Upon further stabilization by the addition of 2 M TMAO, there appears to be a complete loss of cooperativity, as indicated by the observation that only unimodal mass distributions were observed at all time points of HX (Figure 2). Hence, it appears that TMAO-induced thermodynamic stabilization decreases the cooperativity of unfolding and converts an inherently cooperative unfolding transition into a completely noncooperative transition. Like TMAO, other osmolytes such as sarcosine are also expected to stabilize the P13K SH3 domain, in a manner similar to how they stabilize other proteins. Other osmolytes may also allow the visualization of previously unseen intermediates such as I<sub>3</sub>, but this remains to be demonstrated. It is also possible that TMAO and other osmolytes may alter the structure of conformations populated on a folding pathway, as has been shown for barstar, 19 but not in the case of the Fyn SH3 domain.

Previously, it had been shown that protein folding cooperativity increases upon addition of chemical denaturants. 14,15 It appears that TMAO counteracts not only the effect of a chemical denaturant such as urea 20 on stability but also the effect of urea on cooperativity.

**Structure of Partially Unfolded Intermediate States under Stabilizing Conditions.** It has been shown that the nSrc loop and RT loop behave as a single noncooperatively opening unit and that both exchange during the formation of I<sub>h</sub> and I<sub>2</sub> in the absence of any TMAO. The nSrc loop still exchanges out in a single kinetic phase (during the formation of I<sub>1</sub>) in the presence of 2 M TMAO, too, while the RT loop exchanges out in two kinetic phases (during the formation of I<sub>1</sub> and U) (Figure 7). This suggests that the RT loop has at least two groups of amide sites whose protection factors differ in the presence of 2 M TMAO but not in its absence. The RT loop has a partial β sheet character: a few of its residues are involved in intraloop hydrogen bonding. 20 It is possible that these hydrogen-bonded amide sites exchange slowly under strongly stabilizing solvent conditions and do so only during the formation of U. Amide sites that have less protection because they are not hydrogen-bonded appear to exchange out during the formation of I<sub>1</sub>. Both loop regions are known to be very dynamic in solution 20 and to expose a large surface area of the polypeptide backbone to solvent. It appears that compaction of both loops by TMAO 25,26 slows the dynamics. Non-hydrogen-bonded amide sites might be very dynamic in the absence of

5859

DOI: 10.1021/acs.biochem.8b00698
Biochemistry 2018, 57, 5851–5863
TMAO, and hence, the RT loop exchanged out in a single kinetic phase.\textsuperscript{13}

Sequence segments $1\text{–}11$ ($\beta$ strand 1), $23\text{–}35$ ($\beta$ strand 2), and $51\text{–}71$ ($\beta$ strands 3 and 4) unfold cooperatively in the absence of TMAO,\textsuperscript{13} but they show only gradual noncooperative openings in the presence of 2 M TMAO (Figure S11). Previously, sequence segment $1\text{–}11$ was found to exchange in three kinetic phases, and the apparent rate constants of formation of $I_1$ and $U$ were similar in the absence of TMAO. Thus, one fewer kinetic phase is seen for $\beta$ strand 1 (Figure 7 and Table S2). $\beta$ strand 2 was found to exchange out cooperatively in the absence of any TMAO. However, in the presence of 2 M TMAO, it exchanges not only noncooperatively but also in two kinetic phases leading to the formation of $I_1$ and $U$. This is likely because $\beta$ strand 2 is hydrogen bonded with $\beta$ strands 1 and 3. TMAO differentially affects the intrinsic dynamics of $\beta$ strands 1 and 3. $\beta$ strand 1 behaves approximately the same in the absence and presence of TMAO, while the dynamics of $\beta$ strand 3 is greatly affected by the presence of TMAO (see below). This asymmetric stabilization around $\beta$ strand 2 appears to lead to the partitioning of amide sites within $\beta$ strand 2, which then exchange out with rate constants that differ by up to an order of magnitude. In the presence of 2 M TMAO, $\beta$ strand 3 exchanges out during all the three kinetic phases of HX, leading to the formation of $I_N$, $I_1$, and $U$, while $\beta$ strand 1 exchanges out only during the formation of $I_N$ and $U$. Hence, amide sites in $\beta$ strand 2 that exchange out during the formation of $I_1$ must be coupled structurally to $\beta$ strand 3, and amide sites that exchange out during the formation of $U$ could be coupled structurally to both $\beta$ strands 1 and 3.

The observation that sequence segment $51\text{–}71$ exchanges out in three kinetic phases suggests that $\beta$ strands 3 and 4 ($\beta$ hairpin) take part in the noncooperative formation of all three states, $I_N$, $I_1$, and $U$. Sequence segment $59\text{–}71$ ($\beta$ strand 4), which is a smaller (second half) segment of sequence segment $51\text{–}71$, exchanges out in two kinetic phases, whose apparent rate constants suggest that it exchanges out during the formation of $I_1$ and $U$. This directly suggests that sequence segment $51\text{–}58$ ($\beta$ strand 3) of sequence segment $51\text{–}71$ must exchange out during the formation of $I_N$. Furthermore, subtraction of the amplitude of HX for sequence segment $59\text{–}71$ ($\beta$ strand 4) from the amplitude of HX for sequence segment $51\text{–}71$ ($\beta$ strands 3 and 4) indicates that sequence segment $51\text{–}58$ ($\beta$ strand 3) exchanges out in all three kinetic phases, too (Table S2). This is in sharp contrast to the cooperative exchange of sequence segment $51\text{–}58$ in the absence of TMAO. This change in the nature of the exchange of sequence segment $51\text{–}58$ in the presence of 2 M TMAO could be due to sequence specific stabilization by TMAO.

Sequence segment $71\text{–}81$ ($\beta$ strand 5) exchanges out in two kinetic phases. Quantitative analysis shows that during the fast and slow phases of noncooperative exchange it exchanges out in the EX2 regime and EXX regime of HX, respectively (Figure S and Table S2). This could possibly be due to the nature of hydrogen bonding of $\beta$ strand 5. $\beta$ strand 5 has two groups of amide sites, one that is hydrogen bonded to $\beta$ strand 1 and another that is solvent exposed and not involved in hydrogen bonding. This could lead to an order of magnitude difference in the observed apparent rate constants for the two kinetic phases of HX.

**Native State Dynamics in the Presence of TMAO and Urea.** In natural environments, many organisms may face challenging conditions such as a high concentration of urea, a high temperature, or a high salt concentration.\textsuperscript{14} Furthermore, the cellular milieu is crowded because of the presence of a variety of macromolecules.\textsuperscript{15} To deal with protein denaturing environmental conditions and to maintain functionally relevant macromolecular states, organisms synthesize many small molecules. An osmolyte such as urea destabilizes proteins by being preferentially enriched at the protein surface, presumably because it binds to the polypeptide backbone,\textsuperscript{70,67–70} while TMAO stabilizes proteins by being preferentially excluded from the polypeptide backbone.\textsuperscript{17,71} Hence, urea and TMAO are a special pair of cosolvent osmolytes that are found in nature and that counteract each other in their effects on the dynamics of the polypeptide backbone.

The PI3K SH3 domain has been found to sample the same conformational space in the absence and presence of urea.\textsuperscript{15} In the presence of increasing concentrations of urea, the cooperativity of unfolding increases. Until now, it was not known whether TMAO affects the cooperativity of protein unfolding. This study shows that by reducing the cooperativity of unfolding, TMAO counters the effect of urea not only on stability but also on cooperativity. Furthermore, it also shows that in the presence of TMAO local fluctuations dominate over global unfolding events. The ability of TMAO to counteract the effect of urea on polypeptide backbone dynamics may be important in restoring the functionally relevant backbone fluctuations that are important for protein function.\textsuperscript{72}

**Testing the Model for Thermodynamic Stability and Cooperativity.** Proteins are marginally stable molecules. Because of this marginal stability, the native state of a protein samples many partially unfolded to completely unfolded conformations. It is not understood whether a protein switches from one conformation to another by cooperative structural opening or by a gradual noncooperative structural opening. However, a recent study has shown that the cooperativity of structural opening can be modulated by changing the stabilities of the unfolded state by adding a denaturant.\textsuperscript{79} Subsequent studies have shown that protein destabilizing conditions such as a high pH\textsuperscript{17} or a mutation\textsuperscript{12} also increase the cooperativity of unfolding.

These observations suggest that an increase in cooperativity of unfolding is due to the decrease in the stability of the protein. To confirm the connection established between protein stability and cooperativity, it had become important to establish that stabilization of the protein would lead to a decrease in the cooperativity of unfolding. This study shows that this is indeed the case: stabilization of the PI3K SH3 domain upon addition of the osmolyte TMAO is accompanied by switching of the inherently cooperative unfolding reaction of the PI3K SH3 domain to a completely noncooperative unfolding reaction.

**CONCLUSION**

This study provides important new insight into the dynamic relationship between thermodynamic stability and the cooperativity of protein folding. The extent of cooperativity of the unfolding of the PI3K SH3 domain decreases upon addition of the stabilizing osmolyte TMAO: fewer amide sites exchange out cooperatively as the concentration of TMAO present during HX is increased. In the presence of 2 M TMAO, cooperativity is completely lost as the protein is very strongly stabilized: all amide sites undergo HX in a noncorrelated (noncooperative) manner. The protein then

DOI: 10.1021/acs.biochem.8b00698

Biochemistry 2018, 57, 5851–5863
**Biochemistry**

unfolds in a completely noncooperative, uphill manner, suggesting that refolding under such strongly stabilizing native conditions would also be completely noncooperative, but downhill. It is important to note that the concentrations of TMAO at which its effect on cooperativity is seen are similar to concentrations of TMAO found in the cells of marine organisms.14

**REFERENCES**


Direct evidence for a two-state protein unfolding transition from hydrogen-deuterium exchange, mass spectrometry, and NMR. Protein Sci. 5, 1060–1066.


