Counteracting Osmolyte Trimethylamine N-Oxide Destabilizes Proteins at pH below Its pK$_a$

MEASUREMENTS OF THERMODYNAMIC PARAMETERS OF PROTEINS IN THE PRESENCE AND ABSENCE OF TRIMETHYLAMINE N-OXIDE

Earlier studies have reported that trimethylamine N-oxide (TMAO), a naturally occurring osmolyte, is a universal stabilizer of proteins because it folds unstructured proteins and counteracts the deleterious effects of urea and salts on the structure and function of proteins. This conclusion has been reached from the studies of the effect of TMAO on proteins in the pH range 6.0–8.0. In this pH range TMAO is almost neutral (zwitterionic form), for it has a pK$_a$ of 4.66 ± 0.10. We have asked the question of whether the effect of TMAO on protein stability is pH-dependent. To answer this question we have carried out thermal denaturation studies of lysozyme, ribonuclease-A, and apo-$\alpha$-lactalbumin in the presence of various TMAO concentrations at different pH values above and below the pK$_a$ of TMAO. The main conclusion of this study is that near room temperature TMAO destabilizes proteins at pH values below its pK$_a$, whereas it stabilizes proteins at pH values above its pK$_a$. This conclusion was reached by determining the $T_m$ (midpoint of denaturation), $\Delta H_m$ (denaturational enthalpy change at $T_m$), $\Delta C_p$ (constant pressure heat capacity change), and $\Delta G_m$ (denaturational Gibbs energy change at 25 °C) of proteins in the presence of different TMAO concentrations. Other conclusions of this study are that $T_m$ and $\Delta G_m$ depend on TMAO concentration at each pH value and that $\Delta H_m$ and $\Delta C_p$ are not significantly changed in the presence of TMAO.

Many organisms are known to accumulate low molecular weight organic molecules (osmolytes) in their tissues in response to harsh environmental stresses. These osmolytes are generally categorized into three groups, namely amino acids and their derivatives, polyhydric alcohols, and methylimines (1). Molecules of the first two groups are “compatible osmolytes,” which means that cells accumulate these osmolytes to high concentrations without significantly perturbing protein functions under physiological conditions (1–4). Molecules of the third group, which reverse the perturbations caused by urea, are known as “counteracting osmolytes” (2, 5). One such counteracting osmolyte is trimethylamine N-oxide (TMAO), which is present in high concentrations in coelacanth (sharks) and marine elasmobranchs (rays) (6). The effect of TMAO on protein stability and enzyme activity has been widely studied. This osmolyte has been shown in vitro to do the following: (i) increase the melting temperature as well as the unfolding free energy of proteins (7–11); (ii) offset the destabilizing effects of urea (8, 10, 11); (iii) restore the enzyme activity that is lost upon urea treatment (12, 13); (iv) force the folding of unstructured proteins (4, 12–15); (v) favor the protein self-association and polymerization of microtubules (16–18); (vi) correct temperature-sensitive folding defects (19); and (vii) interfere with the formation of scrapie prion protein (20). TMAO has been shown in vivo to counteract the damaging effects of salts (21), hydrostatic pressure (22, 23), and urea (24, 25) on proteins.

TMAO is a compound that has a pK$_a$ in the pH range 4.56–4.75 (11, 26). Thus, this compound can exist in zwitterionic and positively charged forms, depending on the pH of the medium. Although TMAO-facilitated stabilization of proteins has been studied at length (8, 11–13, 27), these studies were carried out in the pH range 6.0–8.0 in which TMAO is almost neutral. To date, no studies have been carried out at pH values below the pK$_a$ of TMAO, where the osmolyte exists predominantly in the positively charged form. Thus, it is not known what the effect of the positively charged TMAO on protein stability is. To answer this question we have investigated the effects of both positively charged and neutral forms of TMAO on the thermal denaturation of three model proteins, namely, pancreatic RNase A, hen egg white lysozyme, and bovine apo-$\alpha$-lactalbumin ($\alpha$-LA) in the pH range 2.0–7.0. In this article we report for the first time that the positively charged form of TMAO destabilizes all proteins.

EXPERIMENTAL PROCEDURES

Commercial lyophilized preparations of RNase A (type III-A), hen egg white lysozyme, and bovine apo-$\alpha$-lactalbumin were purchased from Sigma. Guanidinium chloride (GdmCl) was the ultrapure sample from Schwarz/Mann. TMAO was obtained from Sigma. These and other chemicals, which were of analytical grade, were used without further purification.

RNase A, lysozyme, and holo-$\alpha$-lactalbumin solutions were dialyzed extensively against 0.1 M KCl, pH 7.0. $\alpha$-LA was prepared by adding 4 mM EGTA to the solution of holoprotein (with Ca$^{2+}$ bound). Protein stock solutions were filtered using 0.45-µm Millipore filter paper. All three proteins gave a single band during polyacrylamide gel electrophoresis. Concentration of the protein was determined experimentally using molar absorption coefficient (M$^{-1} \cdot$ cm$^{-1}$) values of 9800 at 277.5 nm for RNase A (28), 39,000 at 280 nm for lysozyme (29), and 29,210 at 280 nm for $\alpha$-LA (30). The concentration of GdmCl stock solution was determined by refractive index measurements (31). All solutions for optical measurements were prepared in the desired degassed buffer containing 0.1 M KCl. For various pH ranges, the buffers used were 0.1 M KCl, 5 mM EGTA, 20 mM Hepes (for pH 3.0–7.0), 50 mM Tris (for pH 7.0–10.0), and 100-mM HEPES (for pH 10.0–12.0).

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0.05 M glycine hydrochloride buffer (pH range 2.0–3.5), 0.05 M citrate buffer (pH 4.0–4.5) and 0.05 M cacodylic acid buffer (pH range 5.0–7.0). The pH of the protein solutions changes upon the addition of TMAO; hence, the required pH was adjusted by adding either HCl or NaOH. Because the change in pH may also occur upon heating or upon the addition of GdmCl, the pH of the solution was therefore measured after the denaturation experiment. It has been observed that the change in pH is not significant.

Thermal denaturation studies were carried out in a Jasco V-560 UV-visible spectrophotometer equipped with a Peltier-type temperature controller (ETC-500T) at a heating rate of 1 °C/min. This scan rate was found to provide adequate time for equilibration. Each sample was heated from 20 to 85 °C. The change in absorbance with increasing temperature was followed at 287 nm for RNase A, 300 nm for lysozyme, and 295 nm for α-LA. About 650 data points of each transition curve were collected. After denaturation, the sample was immediately cooled down to measure reversibility of the reaction. All solution blanks showed negligible change in absorbance with temperature and were, therefore, neglected during the data analysis. The raw absorbance data were converted into a molar absorption coefficient (m^−1 cm^−1) at a given wavelength λ. Each heat-induced transition curve was analyzed for Tm (midpoint of denaturation) and ΔHm (denaturation enthalpy change) at Tm using a non-linear least squares method according to the relation shown in Equation 1,

\[
y(T) = \frac{y_D(T) + y_N(T)\exp(-\Delta H_m/R(1/T_1 - 1/T_m))}{1 + \exp(-\Delta H_m/R(1/T_1 - 1/T_m))} \tag{Eq. 1}
\]

where y(T) is the optical property at temperature T (kelvin), y_D(T) and y_N(T) are the optical properties of the native and denatured protein molecules at T (kelvin), respectively, and R is the gas constant. In the analysis of the transition curve, it was assumed that a parabolic function describes the dependence of the optical properties of the native and denatured protein molecules (i.e., y_D(T) = a_D + b_D T + c_D T^2 and y_N(T) = a_N + b_N T + c_N T^2, where a_D, b_D, c_D, a_N, b_N, and c_N are temperature-independent coefficients) (32). A plot of ΔH_m versus Tm, at each concentration of TMAO gave the value of ΔC_p, the constant pressure heat capacity change. As shown in Equation 2,

\[
\Delta G_m(T) = \Delta H_m \left(\frac{T_m - T}{T_m}\right) - \Delta C_p \left(T_m - T\right) + T \ln \left(\frac{T}{T_m}\right) \tag{Eq. 2}
\]

using values of Tm, ΔH_m, and ΔC_p, the value of ΔG_m at any temperature T, ΔG_m(T), was estimated with the help of the Gibb-Helmholtz equation.

Heat-induced denaturations of lysozyme, RNase A, and α-LA were measured by near-UV difference spectral measurements. For each protein, thermal experiments were performed in triplicate at each pH and each [TMAO], the molar concentration of TMAO. This osmolyte has pK values in the range 4.56–4.75 (11, 26). Thus, the pH range chosen was to have TMAO in positively charged and neutral states. The selected [TMAO] range was such as to have minimal to maximal effects on the capacity change. As shown in Equation 2, ΔG_m values for the range 4.56–4.75 (11, 26). Thus, the pH range chosen was to have TMAO in positively charged and neutral states. The selected [TMAO] range was such as to have minimal to maximal effects on the capacity change.

Results

Heat-induced denaturation curves of proteins in the presence of different TMAO concentrations (0, 0.25, 0.50, 0.75, and 1 M) at five different pH values were measured by following the changes in Δε_280 of lysozyme, Δε_287 of RNase A, and Δε_285 of α-LA as a function of temperature. Denaturation of each protein was reversible at all pH values in the entire range of [TMAO]. It was observed that for each protein an increase in [TMAO] raised the Tm if pH was <5 and reduced Tm if pH was >5. Panels A and B of Figs. 1-3 show representative denaturation curves of lysozyme, RNase A, and α-LA, respectively. Each denaturation curve of a protein at a given [TMAO] and pH was analyzed for Tm and ΔH_m using a nonlinear least squares method that involves fitting the entire (Δε, T) data to the transition curve to Equation 1 with eight free parameters (\( \alpha_N, b_D, c_N, \alpha_D, b_N, c_N, T_m, \) and \( \Delta H_m \)). The only exception was that in the analysis of the transition curves of α-LA in the presence and absence of TMAO at pH 4.0, the least squares method involved fitting the entire (Δε, T) data according to Equation 1 with fixed values of \( \alpha_N, b_D, c_N, \alpha_D, b_N, c_N, T_m, \) and \( \Delta H_m \). The only exception was that in the analysis of the transition curves of α-LA in the presence and absence of TMAO at pH 4.0, the least squares method involved fitting the entire (Δε, T) data according to Equation 1 with fixed values of \( \alpha_N, b_D, c_N, \alpha_D, b_N, c_N, T_m, \) and \( \Delta H_m \). The only exception was that in the analysis of the transition curves of α-LA in the presence and absence of TMAO at pH 4.0, the least squares method involved fitting the entire (Δε, T) data according to Equation 1 with fixed values of \( \alpha_N, b_D, c_N, \alpha_D, b_N, c_N, T_m, \) and \( \Delta H_m \). The only exception was that in the analysis of the transition curves of α-LA in the presence and absence of TMAO at pH 4.0, the least squares method involved fitting the entire (Δε, T) data according to Equation 1 with fixed values of \( \alpha_N, b_D, c_N, \alpha_D, b_N, c_N, T_m, \) and \( \Delta H_m \).
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Fig. 2. Representative thermal denaturation profiles of RNase A in the presence of different concentrations of TMAO at pH 7.0 and pH 2.0. Denaturation curves measured by following changes in \( \Delta \varepsilon_{222} \), of RNase A in the absence and presence of various concentrations of TMAO at pH values of 7.0 (A) and 2.0 (B). Curve numbers have the same meanings as in Fig. 1. The insets in panels A and B show the thermal denaturation curves measured by \( \Delta \varepsilon_{222} \), and the curve numbers have the same meanings as those in Fig. 1. For the sake of clarity, the CD melting curve at 0.5 M TMAO is not shown.

DISCUSSION

Heat denaturation curves (plots of \( \Delta \varepsilon \) versus temperature) of lysozyme, RNase A, and \( \alpha \)-LA were measured in the presence and absence of TMAO at different pH values. Because these conformational transition curves were used for the estimation of different thermodynamic quantities, a few comments are therefore necessary. Analysis of thermal denaturation curves according to Equation 1 assumes that the transition between the native and denatured states is a two-state process. This is indeed true for these proteins in the absence of TMAO (34, 35). However, no calorimetric data are available for these proteins in the presence of TMAO. To check whether the two-state assumption is also valid in the presence of TMAO, thermal denaturation curves were measured by following changes in the \( \Delta \varepsilon_{222} \) of each protein in the presence of 0.5 and 1 M TMAO at different pH values (e.g. see insets in Figs. 1–3). These transition curves were analyzed for \( T_m \) and \( \Delta H_m \) according to Equation 1. At a given pH, the plots of \( \Delta H_m \) versus [GdmCl] and \( T_m \) versus [GdmCl], which were found to be linear, were used to get values of \( T_m \) and \( \Delta H_m \) in the absence of GdmCl (see Fig. 4). The \( T_m \) and \( \Delta H_m \) values of lysozyme evaluated in this manner at pH values 6.0 and 5.0 are given in Table I.

To estimate the \( \Delta C_p \) of a protein, \( \Delta H_m \) values are determined at a number of \( T_m \) values by varying the solvent condition, and the slope of the straight line of the \( \Delta H_m \) versus \( T_m \) plot is used to evaluate \( \Delta C_p \) (i.e. \( \Delta C_p = -\frac{\Delta H_m}{\Delta T_m} \)). However, it is seen in Table I that at a given pH the variation in \( \Delta H_m \) of proteins with a change in [TMAO] is not significant, for the mean error from the triplicate measurements of \( \Delta H_m \) at the given pH and [TMAO] is in the range 2–5%. The only exceptions were the results of \( \alpha \)-LA at pH values of 7.0 and 4.0. This is the reason for estimating \( \Delta C_p \) of the protein in the presence of a given [TMAO] using \( \Delta H_m, T_m \) data obtained at different pH values. For a protein at a given [TMAO], all 15 \( \Delta H_m, T_m \) values obtained from triplicate measurements at all five pH values were used to construct the \( \Delta H_m \) versus \( T_m \) plot. Representative \( \Delta H_m \) versus \( T_m \) plots of \( \alpha \)-LA in the absence and presence of 1 M TMAO are shown in Fig. 5. Such plots were analyzed for \( \Delta C_p \), and Table I shows values of the \( \Delta C_p \) of proteins at different TMAO concentrations. It should be noted that a “plus/minus” (±) with each \( \Delta C_p \) in Table I is the fit error (S.D.).

At a constant pH and osmolyte concentration, \( \Delta G_p \), the value of \( \Delta G_p \) at 25 °C, was estimated using Equation 2 with known values of \( \Delta H_m, T_m \), and \( \Delta C_p \). However, this estimation requires a large extrapolation. Hence, a large error may be associated with \( \Delta G_p \) determination due to errors in the estimations of \( \Delta H_m, T_m \), and \( \Delta C_p \). We have used Becktel and Schellman’s procedure (33) to determine the maximum and minimum errors associated with the \( \Delta G_p \) determination in a given solvent condition. Because there were three independent measurements of \( \Delta H_m \) and \( T_m \) of a protein at the given pH and [TMAO], we obtained six values of \( \Delta G_p \) (three maximum and three minimum values). All of these six values were used to determine the average \( \Delta G_p \) and the mean error. It was observed that the mean error associated with the \( \Delta G_p \) estimation was in the range 5–9% for all proteins. The average values of \( \Delta G_p \) are given in Table I.

Fig. 6 shows plots of ln\( K_D \) (\( = -\frac{\Delta G_p}{RT} \)) versus ln \( \alpha \) (\( \alpha \) is the activity coefficient of TMAO estimated using activity coefficient data reported by Lin and Timasheff (11). Fig. 7 shows the effect of TMAO on the far- and near-UV CD spectra of lysozyme, RNase A, and \( \alpha \)-LA under native and denatured conditions.
Our observation on denatured proteins is in agreement with the report that TMAO is nonperturbational in its effects on fully solvent-exposed amide protons (26). Hence, a comparison of a thermodynamic property of denaturation in the presence and absence of TMAO is valid.

Because the thermodynamic parameters of proteins reported here are obtained by an indirect method based on equilibrium denaturation, it is therefore necessary to validate them against those obtained directly by a thermodynamic method. A comparison of the $\Delta H_m$, $T_m$, and $\Delta C_p$ of proteins in the absence of TMAO (Table I) with those obtained from differential scanning calorimetry measurements (34, 35) gave excellent agreement between the values of each thermodynamic quantity obtained by these two different methods; for example, the calorimetric values of $\Delta C_p$ are 1.60, 1.22, and 1.55 kcal mol$^{-1}$ K$^{-1}$ for lysozyme, RNase A, and $\alpha$-LA, respectively. This agreement led us to believe that our measurements of transition curves and our analysis of these curves for thermodynamic parameters are authentic and accurate.

Equation 2 was used to estimate $\Delta G_D^\circ$, the value of $\Delta G_D$ at 25 °C. It is obvious that the accuracy of this estimate will depend on the accuracy with which $T_m$, $\Delta H_m$, and $\Delta C_p$ are determined. It has been observed that the error in the determination of $T_m$ which is 0.1–0.6% for all proteins, has no significant effect. On the other hand, if errors in the determinations of $\Delta H_m$ and $\Delta C_p$ are considered, the method of Becktel and Schellman (33) gave mean errors in the range 5–9%, with which the $\Delta G_D^\circ$ values of all proteins are estimated. This then means that the change in the stability parameter ($\Delta G_D^\circ$) on increasing [TMAO] is not large (see Table I). However, there is a definite trend in the variation of $\Delta G_D^\circ$ with [TMAO]. It is also seen in Table I that the effect of osmolytes on the protein stability in terms of $T_m$ is significantly large. One of the important conclusions reached from the results presented in Table I is that TMAO stabilizes proteins if pH is $\geq$5.0 and destabilizes proteins if pH is $<5.0$. It is noteworthy that our findings at pH 5.0 and above is consistent with earlier reports (7–11, 36). However, no report is available for the effect of TMAO on protein stability at pH values of $<5.0$.

A large body of data suggests the following. (i) The preferential exclusion of the osmolyte from the protein domain (i.e. the preferential hydration of the protein) stabilizes the native state by shifting the equilibrium, N state $\leftrightarrow$ D state, toward the N state. (ii) The preferential binding of the osmolyte to the denatured protein destabilizes the N state by shifting the equilibrium toward the D state. (iii) The observed effect of the osmolyte on the denaturation equilibrium is a balance between these two opposing effects (37). Thus, the stabilization effect of TMAO on all the three proteins at a pH of $\geq$5.0 could be due to the dominating preferential exclusion effect, and the destabilization of proteins at pH $<5.0$ could be due to the dominating preferential binding effect. In the absence of data on the preferential exclusion of TMAO from the domains of RNase A, lysozyme, and $\alpha$-LA and the preferential binding of TMAO with...
these proteins, we have used the Wyman linkage relation (38) to determine the preferential exclusion/binding parameter. Briefly, upon changing only the osmolyte concentration, the change in the equilibrium constant of the two-state denaturation is given by the difference in the number of osmolyte molecules bound by the denatured and native protein molecules, as shown in Equation 4,

\[
\Delta G = \Delta H - T \Delta S = K_d \Delta N
\]

(Eq. 4)

where \( K_d \) is the equilibrium constant of protein denaturation at 25°C, \( a \) is the thermodynamic activity of the osmolytes, and \( \Delta N \) is the difference between the number of molecules of the osmolyte bound per mole of protein between the denatured (\( \beta \)) and native (\( \alpha \)) states. Furthermore, if \( \Delta \alpha \) the value of the slope of the plot of \( \ln K_d \) versus \( \ln a \), is negative, the preferential exclusion effect dominates over the preferential binding effect; if \( \Delta \alpha \) is positive, the preferential binding effect dominates over the preferential exclusion effect. It is interesting to recall the study of Wang and Bolen (27), who determined the transfer-free energies of protein groups (side chains and peptide backbone unit) and showed that both binding and exclusion effects are observed when a protein is present in a TMAO solution.

We have determined \( \Delta \alpha \) values of all proteins from the plots of \( \ln K_d \) versus \( \ln a \) at different pH values. It is seen in Fig. 6 that for all proteins the slope \( \Delta \alpha \) is negative at pH values \( \leq 5.0 \), suggesting that TMAO is preferentially excluded from the domains of RNase A, lysozyme, and \( \alpha \)-LA. Although the preferential interaction parameters of these proteins have not been reported, there is experimental evidence for the preferential hydration of proteins in the presence of TMAO near neutral pH (11). Using the procedure of Wang and Bolen (27) and their data on the transfer-free energies of protein groups, we have determined transfer-free energies of lysozyme, RNase A, and \( \alpha \)-LA from water to 1 M TMAO solution. It has been observed that this transfer-free energy change of each protein is positive, suggesting that the effect of the exclusion of osmolytes from the peptide backbone domi-
inates over the preferential binding of the osmolytes with side chains. It is also seen in Fig. 6 that for all proteins the slope (Δν) is positive at pH values <5.0, suggesting that the effect of the binding of TMAO with side chains dominates over the effect of the exclusion of TMAO from the peptide backbone. However, no data on the preferential binding and transfer-free energies of protein groups from water to TMAO solution at pH values <5.0 are available to support our argument.

At present we do not have any explanation for the above-mentioned peculiar behavior of TMAO, i.e., that it stabilizes proteins at pH >5.0 and destabilizes proteins at pH <5.0. It may, however, be noted that the pKₐ of TMAO is 4.66 ± 0.10. Hence, the zwitterionic form of TMAO dominates over its positively charged form at all values of pH greater than the pKₐ, and the positively charged form dominates at all values of pH less than the pKₐ. We are therefore tempted to suggest that the zwitterionic form of TMAO stabilizes proteins, whereas the positively charged form of TMAO destabilizes proteins. To support this proposition, we have studied heat-induced denaturation of RNase A and lysozyme in the presence of 1 M glycine betaine at pH values of 6.0 and 2.0. It has been observed that 1 M glycine betaine (pKₐ = 2.17) stabilizes both proteins at pH 6.0, where it exists as a zwitterion, and loses its stabilizing effect at pH 2.0, where it is positively charged. That is why, as suggested by Yancey et al. (2), molecules existing in charged forms are not used by nature as osmolytes.

The ΔHₘ values of many proteins in the presence of various osmolytes near neutral pH have been reported (11, 39–42). One of the conclusions of these studies is that the ΔHₘ of proteins in the presence of osmolytes may increase, decrease, or remain unchanged. ΔHₘ of RNase-T1 has been measured in the presence of different concentrations of TMAO near neutral pH, and it has been reported that it increases with increasing [TMAO] (11). It is also seen in Table I that the ΔHₘ of lysozyme, RNase A, and α-LA in the pH range 5.0–7.0 increases with [TMAO], although this increase in ΔHₘ is not very significant. It is also seen in Table I that ΔHₘ of each protein decreases with the [TMAO] in the pH range of 5.0, where TMAO acts as a destabilizer. Furthermore, results shown in Fig. 6 show that the preferential binding of TMAO occurs at pH values of <5.0 for Δν > 0. Δν is noteworthy that additives that are destabilizers decrease the ΔHₘ of a protein because of preferential binding with proteins (34).

To understand the thermodynamic basis of the mechanism of stabilization of the proteins by TMAO under physiological conditions, we have determined the ΔHₘ° (enthalpy change at 25 °C) and ΔSₘ° (entropy change at 25 °C) of lysozyme, RNase A, and α-LA in the presence of different concentrations of TMAO at all pH values. It has been observed that at pH values ≥5.0, both ΔΔHₘ° (difference in ΔHₘ° of the protein in the presence and absence of TMAO) and ΔΔSₘ° (difference in ΔSₘ° of the protein in the presence and absence of TMAO) are > 0. The representative data (ΔΔHₘ° and ΔΔSₘ°) are given in Table II and suggest that protein stabilization by TMAO is enthalpically unfavorable and entropically favorable. However, the unfavorable enthalpy change outweighs the favorable entropy change to yield an unfavorable free energy change (ΔΔGₘ° of > 0). Thus, the stabilization of all the proteins by TMAO is enthalpically controlled at pH 5.0 and above. It is noteworthy that Zou et al. (43) measured ΔHₘ° (enthalpy change of transfer) and ΔSₘ° (entropy change of transfer) of protein groups upon transference from water to TMAO solution using isothermal titration calorimetry near neutral pH and at 25 °C. From these measurements they predicted that protein stabilization by TMAO is enthalpically controlled.

It has been observed that at pH values of <5.0 there is a decrease in ΔHₘ° of lysozyme and α-LA upon transferring them from water to each [TMAO], whereas the ΔΔSₘ° for this process increases for lysozyme and decreases for α-LA. The representative data (ΔΔHₘ° and ΔΔSₘ°) of these proteins are given in Table II. These results suggest that the enthalpic contribution to protein destabilization outweighs the entropic contribution leading to a ΔΔGₘ° of < 0; hence, destabilization is under enthalpic control. In the case of RNase A, both ΔΔHₘ° and ΔΔSₘ° increase upon transferring the protein from water to different TMAO concentrations. The representative (ΔΔHₘ°, ΔΔSₘ°) data are given in Table II, where it is seen that the destabilization of RNase A is under entropic control.

The ΔCₘ° of several proteins have been measured in the presence of various osmolytes, and it has been observed that depend-

\[ \text{Equation 4} \]

\[ \Delta H_m^\circ \]
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In the presence of TMAO, the apolar and polar contributions remain unchanged. The pH values during the studies are 7.0 for RNase-A and 4.0 for lysozyme. The highest pH values are 7.0 for RNase-A and 4.0 for lysozyme. The lowest pH values are 2.0 for lysozyme and RNase-A, and 4.0 for α-LA.

Table II
Change in stability parameters on transferring proteins from 0 M to 1 M TMAO at two extreme pH values and 25 °C

| Protein      | Highest pH | Lowest pH | ΔHf° | ΔSf° | ΔGf° | ΔHd° | ΔSd° | ΔGd° |
|--------------|------------|-----------|------|------|------|------|------|------|
| Lysozyme     | 2620       | 4.66      | 1230 |       |      |      |      |      |
| RNase-A      | 4060       | 9.56      | 1210 |       |      |      |      |      |
| α-LA         | 4480       | 12.07     | 880  | 800  | 3.7  |      |      |      |

where ΔASAapo and ΔASAap, respectively, are changes in the accessible surface area of apolar and polar groups on denaturation, and ΔCp and ΔCpol are the constant pressure heat capacity changes associated with the exposure of 1 mol A² of apolar and polar groups, respectively (44, 45).ΔCpol is >0 and ΔCpol is <0 in water (44). It has been reported that the structure of the native and denatured states of proteins is not affected in the presence of TMAO (7, 26). The results shown in Fig. 7 support these reports. Thus, the change in accessible surface area (ΔASA) values of polar and apolar groups in the presence of TMAO are expected to be unperturbed. If this is the case, it is obvious that the ΔCp of a protein will depend only on the dependencies of ΔCpol and ΔCpol on solvent composition. Therefore, no change in the ΔCp of proteins upon transferring them from water to TMAO solution means that the apolar and polar contributions remain either unchanged or changed in such a way that they lead to no change in the observed ΔCp of proteins.

This study clearly demonstrates that the zwitterionic form of TMAO stabilizes proteins, for both Tm and ΔGd° increase with [TMAO], whereas the positively charged form of the osmolyte destabilizes proteins in terms of Tm and ΔGd°. Furthermore, we have demonstrated that the zwitterionic form is excluded from the protein surface for a Δν of <0, whereas positively charged form binds to proteins for a Δν of >0.

Recently, Chilson and Chilson (46) have shown that TMAO failed to refold acid and that GdmCl denatured lactate dehydrogenase at pH 2.3. These observations are in agreement with our finding that TMAO at this pH is a protein destabilizer. Thus, one practical aspect of our study is that TMAO cannot be used to refold proteins at pH below its pKₐ. Interestingly, there are many marine animals that are bottom dwellers and use TMAO as an osmolyte to counteract the deleterious effects of pressure on proteins (47). These animals are also known to have a H⁺ pump at their cell membrane to maintain (near) pH homeostasis (48). As suggested by our study, one obvious reason for having this H⁺ pump is that TMAO can protect the cellular proteins and enzymes against pressure denaturation, which is possible only when pH is above the pKₐ of the osmolyte. This is one possible biological implication of our study of TMAO-protein interaction at pH <5.0.

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