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*

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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-α-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), ΔH_m (denaturational enthalpy change at T_m), ΔC_p (constant pressure heat capacity change), and $\Delta G_D°$ (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 $\Delta G_D°$ depend on TMAO concentration at each pH value and that ΔH_m and ΔC_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 methylamines (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),[1] 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 scrape 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-α-lactalbumin (α-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 α-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-α-lactalbumin solutions were dialyzed extensively against 0.1 M KCl, pH 7.0. α-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} 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 α-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

[1] The abbreviations used are: TMAO, trimethylamine *N*-oxide; α-LA, apo-α-lactalbumin; GdmCl, guanidinium chloride; CD, circular dichroism; ΔC_p, constant-pressure heat capacity change; T_m, midpoint of

denaturation; ΔH_m, enthalpy change of denaturation; ΔG_D, Gibbs energy change; $\Delta G_D°$, Gibbs energy change at 25 °C.

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-505T) 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 co-efficient (M^{-1} cm^{-1}) at a given wavelength λ. Each heat-induced transition curve was analyzed for T_m (midpoint of denaturation) and ΔH_m (denaturational enthalpy change at T_m) using a non-linear least squares method according to the relation shown in Equation 1,

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

where $y(T)$ is the optical property at temperature T (kelvin), $y_N(T)$ and $y_N(D)$ 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. $y_N(T) = a_N + b_N T + c_N T^2$ and $y_D(T) = a_D + b_D T + c_D T^2$, where a_N, b_N, c_N, a_D, b_D, and c_D are temperature-independent coefficients) (32). A plot of ΔH_m versus T_m at each concentration of TMAO gave the value of ΔC_p, the constant pressure heat capacity change. As shown in Equation 2,

$$\Delta G_D(T) = \Delta H_m\left(\frac{T_m - T}{T_m}\right) - \Delta C_p\left[(T_m - T) + T\ln\left(\frac{T}{T_m}\right)\right] \quad \text{(Eq. 2)}$$

using values of T_m, ΔH_m, and ΔC_p the value of ΔG_D at any temperature T, $\Delta G_D(T)$, was estimated with the help of the Gibbs-Helmholtz equation.

Heat-induced denaturations of lysozyme, RNase A, and α-LA were measured by near-UV difference spectral measurements. For each protein, measurements were performed in triplicate at each pH and each [TMAO], the molar concentration of TMAO. This osmolyte has pK_a 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 thermal stability of proteins, keeping in view its solubility in solution as well as its tendency to precipitate proteins at higher temperatures. To see whether the stabilizing/destabilizing effect of TMAO depends on the physicochemical properties of proteins, we had chosen three proteins of different pI and hydrophobicity values, namely lysozyme, RNase A, and α-LA; pI and hydrophobicity (kcal mol^{-1}) values, respectively, are 10.7 and 890 for lysozyme, 9.5 and 780 for RNase A, and 5 and 1050 for α-LA.

Isothermal (25 °C) far- and near-UV spectra of lysozyme, RNase A, and α-LA were measured at least three times in a Jasco J-715 spectropolarimeter equipped with a Peltier-type temperature controller (PTC-348-WI). All solution blanks showed negligible change in ellipticity in the entire wavelength range and were, therefore, neglected during the data analysis. The raw CD data were converted into mean residue ellipticity (deg cm^2 $dmol^{-1}$) at the entire wavelength using the relation shown in Equation 3,

$$[\theta]_\lambda = \theta_\lambda M_o/10lc \quad \text{(Eq. 3)}$$

where θ_λ is the observed ellipticity (millidegrees) at the wavelength λ, M_o is the mean residue weight of the protein, c is the protein concentration (mg/cm^3), and l is the pathlength (centimeters).

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
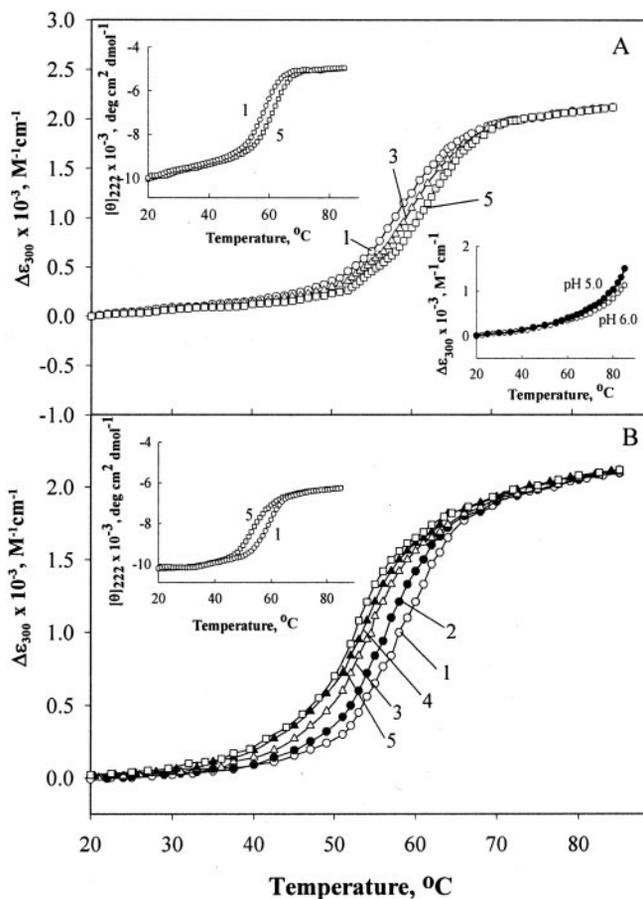

FIG. 1. **Representative thermal denaturation profiles of lysozyme in the presence of different concentrations of TMAO at pH 6.0 and pH 2.0.** *Curves 1, 2, 3, 4,* and *5* represent the denaturation profiles of lysozyme in 0, 0.25, 0.5, 0.75 and 1 M TMAO, respectively, at pH 6.0 (*A*) and pH 2.0 (*B*). For the sake of clarity, curves 2 and 4 are not shown in *panel A*. *Insets* in the *left corners* of *panels A* and *B* show thermal denaturation monitored by change in the mean residue ellipticity at 222 nm; the *curve numbers* have the same meaning as those in the *panels A* and *B* and, for the sake of clarity, the denaturation curve at 0.5 M TMAO is not shown. The *inset* in the *right corner* of *panel A* represents the incomplete transition curves of lysozyme in the absence of GdmCl at the indicated pH values.

changes in $\Delta\epsilon_{300}$ of lysozyme, $\Delta\epsilon_{287}$ of RNase A, and $\Delta\epsilon_{295}$ of α-LA as a function of temperature. Denaturation of each protein was reversible at the all pH values in the entire range of [TMAO]. It was observed that for each protein an increase in [TMAO] raised the T_m if pH was ≥5 and reduced T_m 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 T_m and ΔH_m using a nonlinear least squares method that involves fitting the entire ($\Delta\epsilon$, T) data of the transition curve to Equation 1 with all eight free parameters (a_N, b_N, c_N, a_D, b_D, c_N, T_m, and ΔH_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 ($\Delta\epsilon_{295}$, T) data according to Equation 1 with fixed values of a_N, b_N, and c_N, for it has been observed that the temperature-dependence of y_N is independent of pH and [TMAO]. Table I shows values of T_m and ΔH_m of all three proteins in the presence and absence of TMAO at different pH values. It should be noted that in the case of lysozyme we could not obtain complete denaturation curves in the measurable temperature range at the pH values 6.0 and 5.0 (see $\Delta\epsilon_{300}$ versus temperature plot in the *inset* of Fig. 1A). To
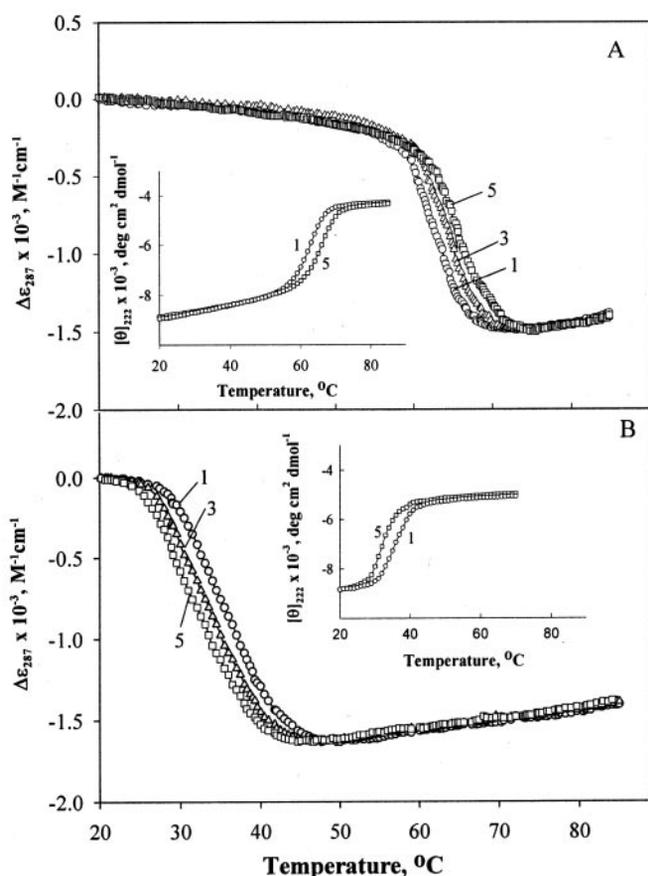
FIG. 2. **Representative thermal denaturation profiles of RNase A in the presence of different concentrations of TMAO at pH 7.0 and pH 2.0.** Denaturation curves measured by following the changes in $\Delta\epsilon_{287}$ of RNase A in the absence and presence of various concentrations of TMAO at pH 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 $[\theta]_{222}$, 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.

bring down thermal denaturation in the measurable temperature range, measurements were carried out in the presence of 2 M GdmCl at these pH values. To correct for the effect of GdmCl on T_m and ΔH_m, we also measured thermal denaturation curves of lysozyme at three more concentrations, namely 1.5, 3, and 3.5 M GdmCl at pH values of 6.0 and 5.0 (transition curves not shown). These transition curves were analyzed for T_m and ΔH_m according to Equation 1. At a given pH, the plots of ΔH_m *versus* [GdmCl] and T_m *versus* [GdmCl], which were found to be linear, were used to get values of T_m and ΔH_m in the absence of GdmCl (see Fig. 4). The T_m and ΔH_m values of lysozyme evaluated in this manner at pH values 6.0 and 5.0 are given in Table I.

To estimate the ΔC_p of a protein, ΔH_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 ΔH_m *versus* T_m plot is used to evaluate ΔC_p (*i.e.* $\Delta C_p = (\delta \Delta H_m/\delta T_m)_p$) (33). However, it is seen in Table I that at a given pH the variation in ΔH_m of proteins with a change in [TMAO] is not significant, for the mean error from the triplicate measurements of ΔH_m at the given pH and [TMAO] is in the range 2–5%. The only exceptions were the results of α-LA at pH values of 7.0 and 4.0. This is the reason for estimating ΔC_p of the protein in the presence of a given [TMAO] using (ΔH_m, T_m) data obtained at different pH values. For a protein at a given [TMAO], all 15 (ΔH_m, T_m) values obtained from triplicate measurements at all five pH values were used to construct the ΔH_m *versus* T_m plot. Repre-

sentative ΔH_m *versus* T_m plots of α-LA in the absence and presence of 1 M TMAO are shown in Fig. 5. Such plots were analyzed for ΔC_p, and Table I shows values of the ΔC_p of proteins at different TMAO concentrations. It should be noted that a "plus/minus" ($\pm$) with each ΔC_p in Table I is the fit error (S.D.).

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

Fig. 6 shows plots of $\ln K_D^\circ$ ($= -\Delta G_D^\circ/RT$) *versus* $\ln a$ (a 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 α-LA under native and denatured conditions.

DISCUSSION

Heat denaturation curves (plots of $\Delta\epsilon$ *versus* temperature) of lysozyme, RNase A, and α-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 $[\theta]_{222}$ 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 ΔH_m and T_m according to Equation 1, and these values of thermodynamic quantities were compared with those obtained from difference absorption measurements given in Table I. It has been observed that both measurements gave, within the experimental error range, identical values of T_m and ΔH_m. This comparison suggests that heat-induced denaturation of proteins in the presence of TMAO is a two-state process.

To compare a thermodynamic quantity of a protein in the presence of TMAO with that in its absence, it is necessary to show that the structural characteristics of the two end states, *i.e.* the N and D states of heat denaturations of lysozyme, RNase A, and α-LA, are not affected on the addition of TMAO. It is seen in Fig. 7 that the far- and near-UV CD spectra of both native and denatured proteins in the absence and presence of TMAO are, within the experimental error range, identical. Furthermore, it has been observed that the near-UV absorption spectra of the two end states of proteins were also unperturbed in the presence of TMAO (*e.g.* see Figs. 1–3). These observations on the native proteins are consistent with the x-ray results showing that the native structure of proteins is unperturbed by TMAO (7) and with the size exclusion chromatography results suggesting that TMAO has no effect on the dimensions of the native proteins

FIG. 3. **Representative thermal denaturation profiles of α-LA in the presence of different concentrations of TMAO at pH 7.0 and pH 4.0.** Denaturation curves of α-LA in the absence and presence of various concentrations of TMAO are shown at pH values of 7.0 (*A*) and 4.0 (*B*). Curve numbers have the same meanings as in Fig. 1. The *insets* shown in *panels A* and *B* are the melting curves measured by $[\theta]_{222}$, and the *curve numbers* have the same meaning as those in Fig. 1. For the sake of clarity, the denaturation profile of the protein in the presence of 0.5 M TMAO is not shown.
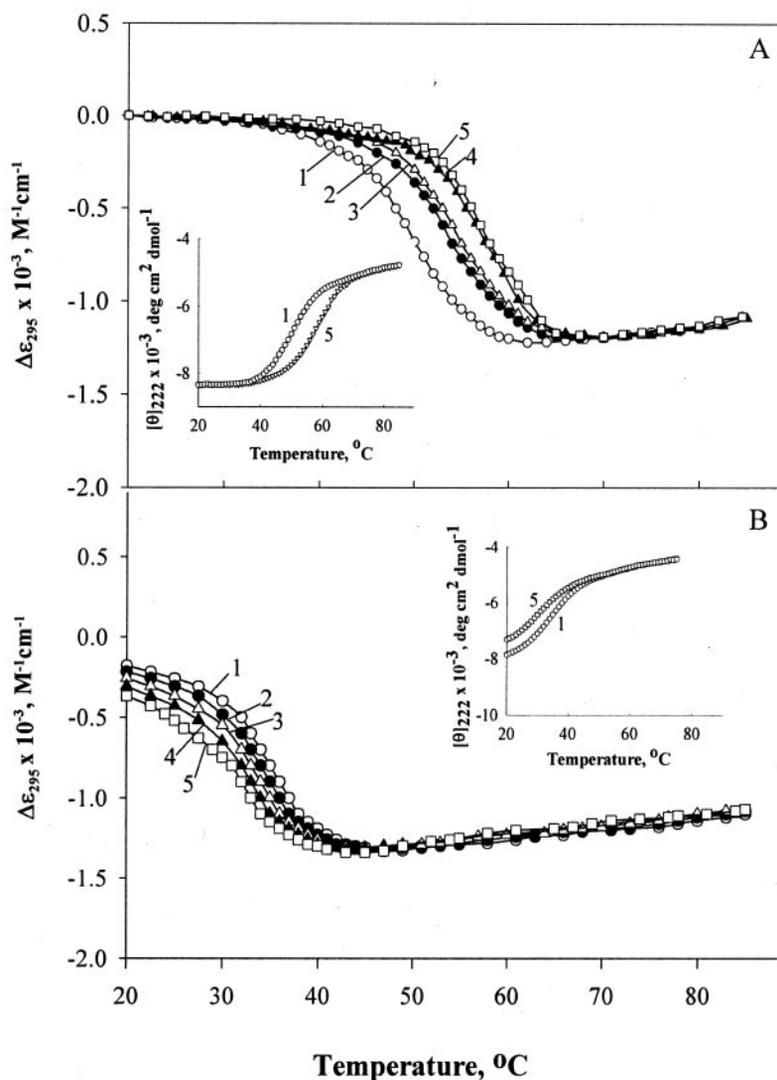
(4, 12, 13). Our observation on denatured proteins is in agreement with the report that TMAO is nonperturbational in its effects on fully solvent-exposed amide protons (26). Hence, a comparison of a thermodynamic property of denaturation in the presence and absence of TMAO is valid.

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 ΔH_m, T_m, and ΔC_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 ΔC_p are 1.60, 1.22, and 1.55 kcal mol^{-1} K^{-1} for lysozyme, RNase A, and α-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 $\Delta G_D°$, the value of ΔG_D at 25 °C. It is obvious that the accuracy of this estimate will depend on the accuracy with which T_m, ΔH_m, and ΔC_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 ΔH_m and ΔC_p are considered, the method of Becktel and Schellman (33) gave mean errors in the range 5–9%, with

which the $\Delta G_D°$ values of all proteins are estimated. This then means that the change in the stability parameter ($\Delta G_D°$) on increasing [TMAO] is not large (see Table I). However, there is a definite trend in the variation of $\Delta G_D°$ 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 ≥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 ↔ 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 ≥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 α-LA and the preferential binding of TMAO with

TABLE I

Thermodynamic parameters of lysozyme, RNase A, and apo-α-lactalbumin in the presence of TMAO

	Lysozyme[a,b]															
[TMAO]	pH 6.0			pH 5.0			pH 3.5			pH 2.5			pH 2.0			ΔC_p
	T_m	ΔH_m	$\Delta G_D°$	T_m	ΔH_m	$\Delta G_D°$	T_m	ΔH_m	$\Delta G_D°$	T_m	ΔH_m	$\Delta G_D°$	T_m	ΔH_m	$\Delta G_D°$	
0.00	84.0	128	12.77	80.0	118	11.05	77.0	110	9.74	63.7	92	6.82	58.0	84	5.63	1.62 ± 0.08
0.25	84.9	129	13.30	81.0	120	11.70	75.0	106	9.33	61.8	90	6.61	55.8	82	5.35	1.60 ± 0.93
0.50	85.3	131	13.56	82.1	121	11.77	73.5	103	8.78	60.6	87	6.16	54.5	81	5.12	1.59 ± 0.07
0.75	85.5	133	13.60	82.8	125	12.20	72.5	100	8.20	59.3	85	5.79	53.3	80	4.90	1.62 ± 0.19
1.00	86.8	135	14.00	83.5	126	12.40	72.0	96	7.63	58.3	84	5.63	52.9	79	4.76	1.63 ± 0.10

	RNase-A[a]															
[TMAO]	pH 7.0			pH 6.0			pH 4.4			pH 4.0			pH 2.0			ΔC_p
	T_m	ΔH_m	$\Delta G_D°$	T_m	ΔH_m	$\Delta G_D°$	T_m	ΔH_m	$\Delta G_D°$	T_m	ΔH_m	$\Delta G_D°$	T_m	ΔH_m	$\Delta G_D°$	
0.00	63.3	117	10.50	62.4	113	9.91	57.6	101	7.90	53.2	95	6.65	36.0	82	2.63	1.23 ± 0.05
0.25	63.8	119	10.80	62.9	114	10.10	56.5	99	7.51	52.0	93	6.28	35.1	81	2.44	1.24 ± 0.07
0.50	64.5	120	11.00	63.5	115	10.30	55.2	97	7.10	51.0	92	6.00	34.0	80	2.20	1.24 ± 0.10
0.75	65.2	121	11.20	64.6	116	10.50	55.0	96	7.00	50.5	90	5.80	33.6	79	2.05	1.23 ± 0.12
1.00	66.0	124	11.65	66.3	117	10.85	54.4	94	6.70	50.1	88	5.60	32.0	78	1.70	1.24 ± 0.09

	α-LA[a]															
[TMAO]	pH 7.0			PH 6.5			PH 6.0			pH 5.5			pH 4.0			ΔC_p
	T_m	ΔH_m	$\Delta G_D°$	T_m	ΔH_m	$\Delta G_D°$	T_m	ΔH_m	$\Delta G_D°$	T_m	ΔH_m	$\Delta G_D°$	T_m	ΔH_m	$\Delta G_D°$	
0.00	42.5	50	2.00	50.5	67	3.70	55.0	72	4.40	59.8	76	5.02	34.0	41	1.02	1.55 ± 0.07
0.25	43.5	53	2.26	53.8	68	4.00	56.4	73	4.60	61.8	81	5.70	33.5	38	0.87	1.52 ± 0.04
0.50	44.2	55	2.41	55.0	69	4.11	56.8	74	4.77	62.3	83	5.89	32.0	35	0.70	1.50 ± 0.08
0.75	46.4	58	2.80	57.8	73	4.74	58.8	75	4.99	62.8	84	6.18	30.2	34	0.52	1.48 ± 0.06
1.00	46.3	60	2.91	58.0	75	4.92	59.8	78	5.32	63.5	85	6.49	29.0	32	0.40	1.54 ± 0.12

[a] T_m, ΔH_m and ΔC_p are in °C, kcal mol^{-1}, and kcal mol^{-1} K^{-1}, respectively. From triplicate measurements the values of maximum errors from the mean are 0.1–0.6% and 2–5% in T_m, and ΔH_m, respectively. The error in $\Delta G_D°$ is in the range 5–9%.

[b] T_m and ΔH_m of lysozyme are corrected for the effect of 2 M GdmCl at pH values of 5.0 and 6.0 (see "Experimental Procedures" and "Results").
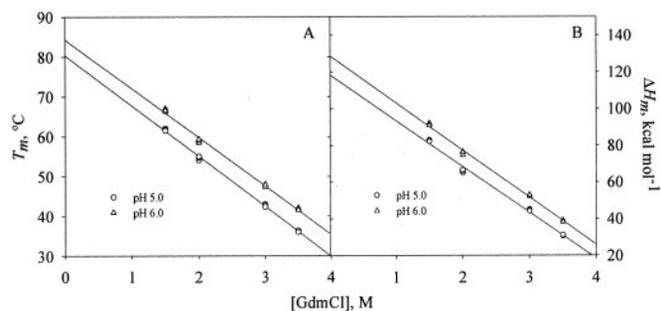

FIG. 4. **Effect of GdmCl on T_m and ΔH_m of lysozyme.** Plots of T_m *versus* [GdmCl] (*A*) and ΔH_m *versus* [GdmCl] (*B*) of lysozyme are shown at pH values of 6.0 and 5.0. Three independent measurements of T_m and ΔH_m were made at each [GdmCl] value. The *solid line* at each pH value shows the best fit of the total 12 points.

FIG. 5. **Representative ΔH_m *versus* T_m plots of the protein in the absence and presence of TMAO.** Plots of ΔH_m *versus* T_m are shown for α-LA in the absence (○) and presence of 1 M TMAO (△). Three independent measurements of ΔH_m and T_m were made at each pH. The *solid line* at a given osmolyte concentration shows the best fit of the total 15 points.

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,

$$\delta \ln K_D°/\delta \ln a = \Delta \nu = \nu^D - \nu^N \qquad \text{(Eq. 4)}$$

where $K_D°$ is the equilibrium constant of protein denaturation at 25 °C, a is the thermodynamic activity of the osmolytes, and $\Delta \nu$ is the difference between the number of molecules of the osmolyte bound per mole of protein between the denatured (ν^D) and native (ν^N) states. Furthermore, if $\Delta \nu$, 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 $\Delta \nu$ 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 pep-
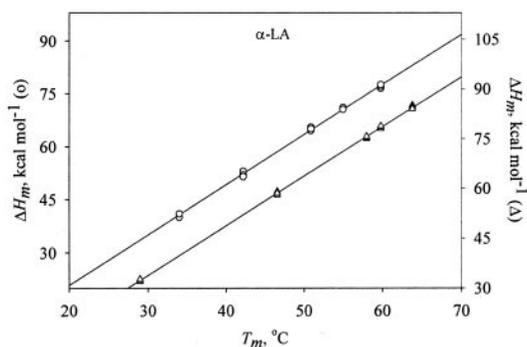
tide backbone unit) and showed that both binding and exclusion effects are observed when a protein is present in a TMAO solution.

We have determined $\Delta \nu$ 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 ($\Delta \nu$) is negative at pH values of ≥5.0, suggesting that TMAO is preferentially excluded from the domains of RNase A, lysozyme, and α-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 α-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 dom-
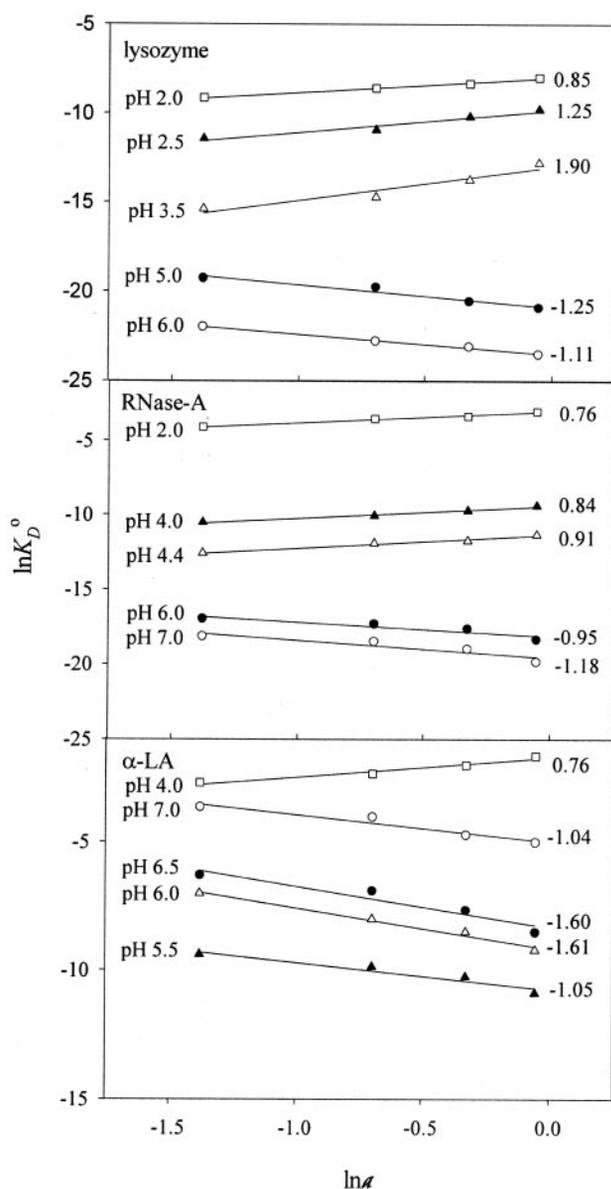
FIG. 6. **Wyman plots of the effect of TMAO on the denaturation of proteins at various pH values and 25 °C.** Plots of $\ln K_D°$ *versus* $\ln a$ for lysozyme, RNase A, and α-LA are shown at the indicated pH values. The number at the *right end* of each *line* indicates the slope, $\Delta\nu$ (see Equation 4), determined by the linear least squares analysis. The symbols on each line relate to the pH value given at the *left end* of the *line*.

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.[2] It has been observed that 1 M glycine betaine ($pK_a = 2.17$) 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 ΔH_m 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 ΔH_m of proteins in the presence of osmolytes may increase, decrease, or remain unchanged. ΔH_m 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 ΔH_m of lysozyme, RNase A, and α-LA in the pH range 5.0–7.0 increases with [TMAO], although this increase in ΔH_m is not very significant. It is also seen in Table I that ΔH_m 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 $\Delta\nu$ of >0. It is noteworthy that additives that are destabilizers decrease the ΔH_m 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 $\Delta H_D°$ (enthalpy change at 25 °C) and $\Delta S_D°$ (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 $\Delta\Delta H_D°$ (difference in $\Delta H_D°$ of the protein in the presence and absence of TMAO) and $\Delta\Delta S_D°$ (difference in $\Delta S_D°$ of the protein in the presence and absence of TMAO) are >0. The representative data ($\Delta\Delta H_D°$ and $\Delta\Delta S_D°$) 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 ($\Delta\Delta G_D°$ 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 $\Delta H_{tr}°$ (enthalpy change of transfer) and $\Delta S_{tr}°$ (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 $\Delta H_D°$ of lysozyme and α-LA upon transferring them from water to each [TMAO], whereas the $\Delta\Delta S_D°$ for this process increases for lysozyme and decreases for α-LA. The representative data ($\Delta\Delta H_D°$ and $\Delta\Delta S_D°$) 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 $\Delta\Delta G_D°$ of <0; hence, destabilization is under enthalpic control. In the case of RNase A, both $\Delta H_D°$ and $\Delta S_D°$ increase upon transferring the protein from water to different TMAO concentrations. The representative ($\Delta\Delta H_D°$, $\Delta\Delta S_D°$) data are given in Table II, where it is seen that the destabilization of RNase A is under entropic control.

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

inates over the preferential binding of the osmolytes with side chains. It is also seen in Fig. 6 that for all proteins the slope ($\Delta\nu$) 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 pK_a of TMAO is 4.66 ± 0.10. Hence, the zwiterionic form of TMAO dominates over its positively charged form at all values of pH greater than the pK_a, and the positively charged form dominates at all values of pH less than the pK_a. We are therefore tempted to suggest that the

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[2] R. Singh, I. Haque, and F. Ahmad, unpublished results.

FIG. 7. **Effect of TMAO on the secondary and tertiary structures of the native and denatured states of lysozyme, RNase A, and α-LA.** *A* and *D*, lysozyme. *Curve 1* depicts the far- (*A*) and near-UV (*D*) CD spectra of the native state in 0 M TMAO at pH values of 6.0 (——) and 2.0 (·–·–·) and in 1 M TMAO at pH values of 6.0 and 2.0 (····). *Curve 2* depicts the far- (*A*) and near-UV (*D*) CD spectra of the denatured state in 0 M TMAO at pH values of 6.0 (——) and 2.0 (·–·–·) and in 1 M TMAO at pH values of 6.0 and 2.0 (····). *B* and *E*, RNase A. *Curves 1* and *2* have the same meanings as in *panels A* and *D*, except that the highest pH is 7.0 instead of 6.0. *C* and *F*, α-LA. *Curve 1* represents the far- (*C*) and near-UV (*F*) CD spectra of the native state in the absence (——) and presence (····) of 1 M TMAO at pH 7.0. *Curve 2* depicts the far- (*C*) and near-UV (*F*) CD spectra of the denatured state in 0 M TMAO at pH values of 7.0 (——) and 4.0 (·–·–·) and in 1.0 M TMAO at pH values of 7.0 and 4.0 (····). *Curves 1* and *2* represent measurements at 25 and 85 °C, respectively.
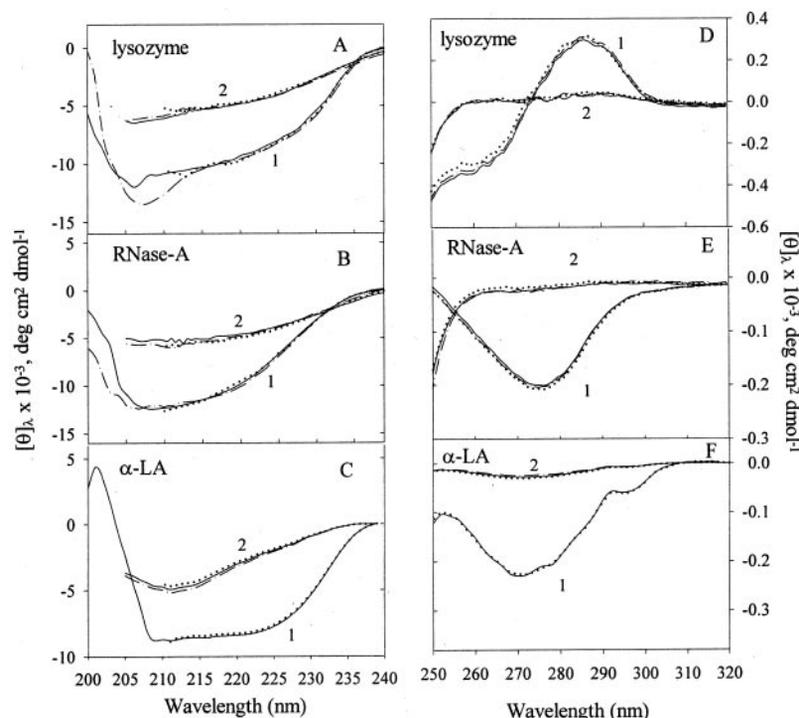
TABLE II

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[a,b]			Lowest pH[a,c]		
	$\Delta\Delta H_D°$	$\Delta\Delta S_D°$	$\Delta\Delta G_D°$	$\Delta\Delta H_D°$	$\Delta\Delta S_D°$	$\Delta\Delta G_D°$
Lysozyme	2620	4.66	1230	−640	1.10	−970
RNase-A	4060	9.56	1210	1410	7.51	−830
α-LA	4480	12.07	880	−900	−0.37	−800

[a] $\Delta\Delta H_D°$ and $\Delta\Delta G_D°$ are in kcal mol^{-1} and, $\Delta\Delta S_D°$ is in kcal mol^{-1} K^{-1}.
[b] The highest pH values during the studies are 7.0 for RNase-A and α-LA and 6.0 for lysozyme.
[c] The lowest pH values are 2.0 for lysozyme and RNase-A and 4.0 for α-LA.

ing on the protein and the osmolyte used, the ΔC_p may increase, decrease, or remain unchanged in the presence of osmolytes (11, 39–42). It is seen in Table I that the ΔC_p values of lysozyme, RNase A, and α-LA are unchanged upon the addition of TMAO. The current viewpoint regarding the ΔC_p of a protein is that it originates from the change in polar and nonpolar accessible surface areas on protein denaturation (44). This viewpoint is described by the relation shown in Equation 5,

$$\Delta C_p = \Delta C_p^{ap}\Delta ASA^{ap} + \Delta C_p^{pol}\Delta ASA^{pol} \qquad (Eq. 5)$$

where ΔASA^{ap} and ΔASA^{pol}, respectively, are changes in the accessible surface area of apolar and polar groups on denaturation, and ΔC_p^{ap} and ΔC_p^{pol} are the constant pressure heat capacity changes associated with the exposure of 1 mol·Å^2 of apolar and polar groups, respectively (44, 45); ΔC_p^{ap} is >0 and ΔC_p^{pol} is <0 in water (44). It has been reported that the structure of the native and denatured states of proteins are 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 ΔC_p of a protein will depend only on the dependencies of ΔC_p^{pol} and ΔC_p^{ap} on solvent composition. Therefore, no change in the ΔC_p 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 ΔC_p of proteins.

This study clearly demonstrates that the zwitterionic form of TMAO stabilizes proteins, for both T_m and $\Delta G_D°$ increase with [TMAO], whereas the positively charged form of the osmolyte destabilizes proteins in terms of T_m and $\Delta G_D°$. Furthermore, we have demonstrated that the zwitterionic form is excluded from the protein surface for a $\Delta\nu$ of <0, whereas positively charged form binds to proteins for a $\Delta\nu$ 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 pK_a. 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 neutral) 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 pK_a of the osmolyte. This is one possible biological implication of our study of TMAO-protein interaction at pH of <5.0.

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