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# Effects of the osmolyte TMAO (Trimethylamine-N-oxide) on aqueous hydrophobic contact-pair interactions

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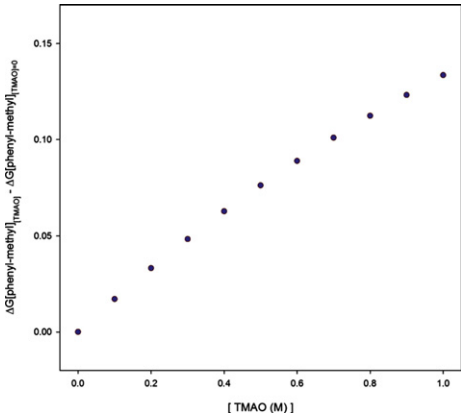
## HIGHLIGHTS

- We determine how the osmolyte TMAO influences hydrophobic contact-pair formation.
- TMAO disrupts interactions between hydrophobic moieties.
- This disruption is not dependent on the size of hydrophobes.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Osmolytes are small, soluble organic molecules produced by living organisms for maintaining cell volume. These molecules have also been shown to have significant effects on the stability of proteins. Perhaps one of the most studied osmolytes is Trimethylamine-N-oxide (TMAO). Thermodynamic studies of the effects of TMAO on proteins have shown that this molecule is a strong stabilizer of the protein folded state, thus being able to counteract the effects of protein denaturants such as urea and guanidine hydrochloride. Most studies of TMAO effects on bio-molecular stability have until now been focused on how the osmolyte reduces the solubility of polypeptide backbones, while the effects of TMAO on hydrophobic interactions are still not well understood. In fact, there are few experimental data measuring the effect of TMAO on hydrophobic interactions. This work studies phenyl and alkyl contact pairs as model hydrophobic contact pairs. The formation of these contact pairs is monitored using fluorescence, i.e., through the quenching of phenol fluorescence by carboxylate ions; and a methodology is developed to isolate hydrophobic contributions from other interactions. The data demonstrate that the addition of TMAO to the aqueous solvent destabilizes hydrophobic contact pairs formed between alkyl and phenyl moieties. In other words, TMAO acts as a "denaturant" for hydrophobic interactions.


## 1. Introduction

Environmentally stressed organisms often use small, soluble, organic molecules for maintaining cell volume [1]. The cellular cytoplasm of these organisms accumulates these molecules at moderate to high concentrations in order to compensate for stress factors such as osmotic

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pressure, dehydration, high temperature and pressure [1]. Hence, these molecules are often referred to as "osmolytes." Compensating for stress factors is not the only way osmolytes influence organisms. Almost three decades ago it was discovered that many osmolytes stabilize the folded state of proteins indiscriminately [2] and the study of osmolyte effects on proteins has led to a much greater understanding of the molecular thermodynamics of the protein folding process [3–10].

Of all osmolytes, the effects of TMAO (trimethylamine N-oxide) on protein stability are perhaps most well studied experimentally [6,10–29]. The addition of TMAO to the protein solvent medium has been shown to protect various proteins against urea and guanidine hydrochloride denaturation and to also induce structure in proteins that are normally disordered under dilute in-vitro conditions. The thermodynamic analysis of Bolen and co-workers has demonstrated that the addition of TMAO (and other stabilizing osmolytes) to water converts the aqueous medium into a "poorer" solvent of the polypeptide backbone [3–6,9–14,21,30–39]. This causes the unfolded state to become more unstable relative to the folded state of the protein and protects the protein against denaturants that convert water into a "better" solvent of the amide bond. However, the effects of TMAO on interactions between hydrophobic moieties of proteins are not so well understood. Different theoretical calculations make contradictory predictions of the effect of TMAO on pair-wise hydrophobic interactions. Garde et al. predict that TMAO addition has minimal effect on the thermodynamics of hydration of methane-like solutes, [40,41] while the results of Paul and Patey indicate that TMAO can disrupt interactions between hydrophobic solutes [42]. In contrast, the calculations of Graziano [43] and Daggett [44] argue that TMAO addition enhances the magnitude of hydrophobic interactions. The only analysis based on experimental data is Bolen's calculated transfer free energies of hydrophobic amino acid side chains of the Nank4-7 protein [45]. These free energies are shown to take negative values when the side chains are transferred from pure water to 1 M TMAO solutions, indicating favorable TMAO interactions with the side-chains.

These conflicting predictions point to the necessity of having experimental data that can unambiguously quantify the effects of TMAO addition on interactions existing between hydrophobic solutes dissolved in water, thereby resolving the differences between these predictions. This work examines how TMAO affects interactions between non-polar phenyl and alkyl moieties dissolved in aqueous solution by examining the quenching of phenol fluorescence by a variety of aliphatic carboxylates. These studies were done in a series of TMAO concentrations in the 0–1.0 M range. In previous studies we have developed methodologies for isolating the contribution of alkyl-phenyl interactions to the fluorescence quenching data [46,47]. The same methodology will be used to determine how interactions between phenyl and alkyl groups are influenced by the addition of TMAO to the aqueous media. Our results indicate that hydrophobic contact pairs are not indifferent towards TMAO addition to the solvent and in fact, the presence of TMAO destabilizes hydrophobic contact pairs in all of our studied systems. Therefore, we suggest that TMAO does not protect proteins through altering hydrophobic interactions but rather acts as a "denaturant" towards interactions that are stabilized through the hydrophobic effect.

## 2. Experimental

### 2.1. Materials

TMAO, sodium formate, sodium acetate, sodium propionate and sodium hexanoate were purchased from Sigma (St. Louis, MO). The phenol was purchased from J.T. Baker Chemical Co. (Phillispburg, NJ). All experiments were performed at an adjusted pH of 8.5 with an ionic strength of 0.3 M adjusted with NaCl. The concentration of phenol in all samples was 200 μM. All samples were measured at room temperature (20 °C).

### 2.2. Methods

Steady-state fluorescence spectra were measured on a Fluorolog-3 Horiba Jobin Yvon spectrofluorometer (Edison, NJ). The sample was held in a $10 \times 3$ mm$^2$ quartz cuvette. All samples were prepared in triplicate. Fluorescence spectra were collected using an excitation wavelength set to 270 nm; excitation and emission slits were set to 5 nm band pass resolution. Quenching studies were performed by monitoring changes in the fluorescence intensity at the maximum emission of 297 nm as a function of quencher concentration. All statistical data analyses including linear and nonlinear regressions were performed using Sigma Plot 10 software (Point Richmond, CA).

## 3. Results and discussion

### 3.1. Results

In this work we have used fluorescence quenching methods for studying how TMAO affects the hydrophobic contribution to interactions between phenol and carboxylate ions. Carboxylate ions quench the fluorescence of phenol dynamically [46–49]. As an example, the quenching of phenol by sodium acetate is demonstrated in Fig. 1, this quenching follows linear Stern–Volmer behavior: [50]

$$\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + k_q \tau [Q] \quad (1)$$

Where $F_0$ is the fluorescence of phenol in the absence of quencher, $F$ is the fluorescence of phenol in the presence of $Q$ molar of quencher and $K_{sv}$ is the Stern–Volmer constant composed of two components: $k_q$ the quenching rate constant and $\tau$ the fluorescence lifetime of phenol in the absence of quencher. In this work we have studied the quenching of phenol by formate, acetate, propionate and hexanoate ions. In addition, we have determined the effects of TMAO on the quenching properties of these anions. The quenching in all cases are well represented by linear Stern–Volmer behavior (coefficient of determination greater than 0.99) and are given in Table 1. In addition, the effects of TMAO on phenol fluorescence have also been measured: the addition of TMAO significantly quenches the fluorescence of phenol via a linear Stern–Volmer profile (Fig. 2). When quencher concentrations are greater than 0.1 M, the rate of collisions between phenol and quencher molecules calculated by classical diffusion theory is comparable to the decay rate associated with the excited phenol fluorophor and collisional quenching must be present [50]. Consequently, the minimal curvature observed in all the Stern–Volmer plots does indicate that there is an absence of any additional ground state fluorophor association that can contribute to the quenching process [50]. This lack of pre-association makes the presence of both TMAO and carboxylate in the near vicinity of the dilute excited fluorophor statistically unlikely, making the contribution of a putative additional simultaneous quenching mechanism infeasible. The quenching of phenol fluorescence in a solution containing carboxylate and TMAO molecules can thus be assumed to be the result of two uncoupled kinetic pathways. Therefore, the $K_{SV}$ value measured for a carboxylate ion dissolved in an aqueous solvent that has a given TMAO concentration (C) will be:

$$K_{SV} = k_q(\text{carboxylate}) * \tau \quad (2)$$

Where $k_q$ is the rate constant for carboxylate quenching and $\tau$ is the fluorescence lifetime of phenol measured in the same aqueous solution (containing TMAO) when no carboxylate is present. If $\tau$ is constant, we would expect to see a near-linear dependence of $K_{SV}$ on TMAO concentration. However, because TMAO also quenches phenol fluorescence

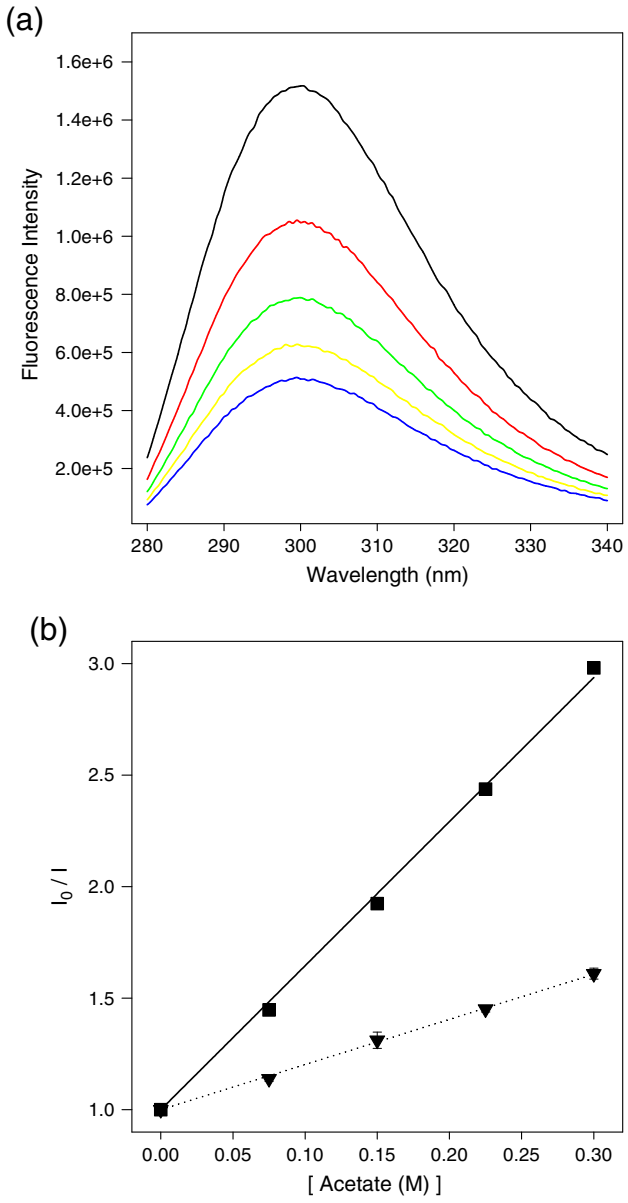


**Fig. 1.** (a) The quenching of phenol fluorescence by acetate ions (from top to bottom: 0 M, 0.075 M, 0.150 M, 0.225 M, 0.300 M). Excitation and emission slits are set to 2 nm bandpass; (b) Stern–Volmer plots for the quenching of phenol fluorescence by acetate in water (closed squares) and 0.7605 M TMAO (closed triangles).

via Stern–Volmer behavior, in Fig. 3, we observe a hyperbolic concentration dependence of $K_{SV}$ that can be expressed as:

$$K_{SV} = k_q(\text{carboxylate}) \times \frac{\tau_0}{1 + \tau_0 k_q(\text{TMAO}) \times C} \tag{2a}$$

where $\tau_0$ is the fluorescence lifetime of phenol measured in the absence of TMAO and carboxylate ion and $k_q$(TMAO) is the rate constant for TMAO quenching. Because TMAO quenching follows Stern–Volmer kinetics we can rewrite Eq. (2a) as:

$$K_{SV} = k_q(\text{carboxylate}) \times \tau_0 \frac{I(C)}{I_0} \tag{2b}$$

Where $I_o$ is the fluorescence intensity of phenol measured in the absence of any quencher and $I(C)$ is the fluorescence intensity of the same amount of phenol measured at [TMAO] = $C$; we can now define

**Table 1**
Compiled apparent Stern–Volmer constant ($K_{SV}$) values of various carboxylate ions, obtained from quenching phenol fluorescence. These values are plotted in Fig. 3.

| Quencher | [TMAO] (M) | Apparent $K_{SV}(M^{-1})$ |
|---|---|---|
| Formate | 0.000 | 4.4 ± 0.1 |
| | 0.152 | 2.99 ± 0.07 |
| | 0.304 | 2.40 ± 0.07 |
| | 0.456 | 2.15 ± 0.04 |
| | 0.608 | 1.67 ± 0.04 |
| | 0.761 | 1.47 ± 0.02 |
| Acetate | 0.000 | 5.8 ± 0.2 |
| | 0.152 | 4.4 ± 0.1 |
| | 0.304 | 3.4 ± 0.1 |
| | 0.456 | 2.7 ± 0.1 |
| | 0.608 | 2.31 ± 0.03 |
| | 0.761 | 2.02 ± 0.04 |
| Propionate | 0.000 | 5.9 ± 0.3 |
| | 0.152 | 4.2 ± 0.1 |
| | 0.304 | 3.31 ± 0.09 |
| | 0.456 | 2.70 ± 0.08 |
| | 0.608 | 2.3 ± 0.1 |
| | 0.761 | 1.87 ± 0.07 |
| Hexanoate | 0.000 | 6.1 ± 0.4 |
| | 0.152 | 4.3 ± 0.1 |
| | 0.304 | 3.5 ± 0.1 |
| | 0.456 | 2.8 ± 0.1 |
| | 0.608 | 2.3 ± 0.1 |
| | 0.761 | 2.02 ± 0.07 |

a "corrected" Stern–Volmer constant by multiplying $K_{SV}$ by the ratio $\frac{I_0}{I(C)}$, this parameter $K'_{SV}(C)$ now represents quenching by the carboxylate ion at any given concentration of added TMAO:

$$K'_{SV}(C) = K_{SV} \times \frac{I_0}{I(C)} = k_q \times \tau_0 \tag{2c}$$

The data shown in Fig. 4 and the fitting parameters listed in Table 2 demonstrate that the values of $K'_{SV}(C)$ are linearly well correlated with TMAO concentration. This observed linearity is consistent with the assumption that the quenching of phenol by TMAO and carboxylate co-solutes proceeds through uncoupled processes. In the subsequent analysis we will use the fitting parameters of Table 2 to calculate values of $K'_{SV}(C)$ at any given osmolyte concentration. The dependence of $K'_{SV}(C)$ on TMAO concentration will be used to quantify the effect of this osmolyte on hydrophobic interactions between the non-polar alkyl and phenyl moieties.

### *3.2. Identifying the contribution of contact pair formation to the quenching data*

The quenching of phenol fluorescence by carboxylate ions can be represented by Scheme 1:

$$F^* + Q \underset{\mathbf{k_{off}}}{\overset{\mathbf{k_{on}}}{\rightleftarrows}} \left[F^* \bullet Q\right] \xrightarrow{k_{pt}} F + Q$$

**Scheme 1.** Suggested mechanism for the quenching of excited state phenol ($F^*$) fluorescence by quencher molecules $Q$.

In this scheme $F^*$ is the excited fluorophor, $Q$ is the quencher, $[F^* \bullet Q]$ is the encounter complex formed between the quencher and fluorophor, $k_{pt}$ is the rate of energy transfer between the quencher and excited fluorophor within the encounter complex and $F$ is the fluorophor in the ground state. The quenching of phenol by acetate and formate has been shown to be a "reaction controlled" proton transfer process from the exited phenol hydroxide to the acetate ion [48,49]. In this case, because the quenching process is dynamic and not static, the Stern–Volmer quenching constant based on the parameters of Scheme 1 can be formulated as:

$$K_{sv} = k_q \tau = K_{ec} \times k_{pt} \times \tau \tag{3}$$

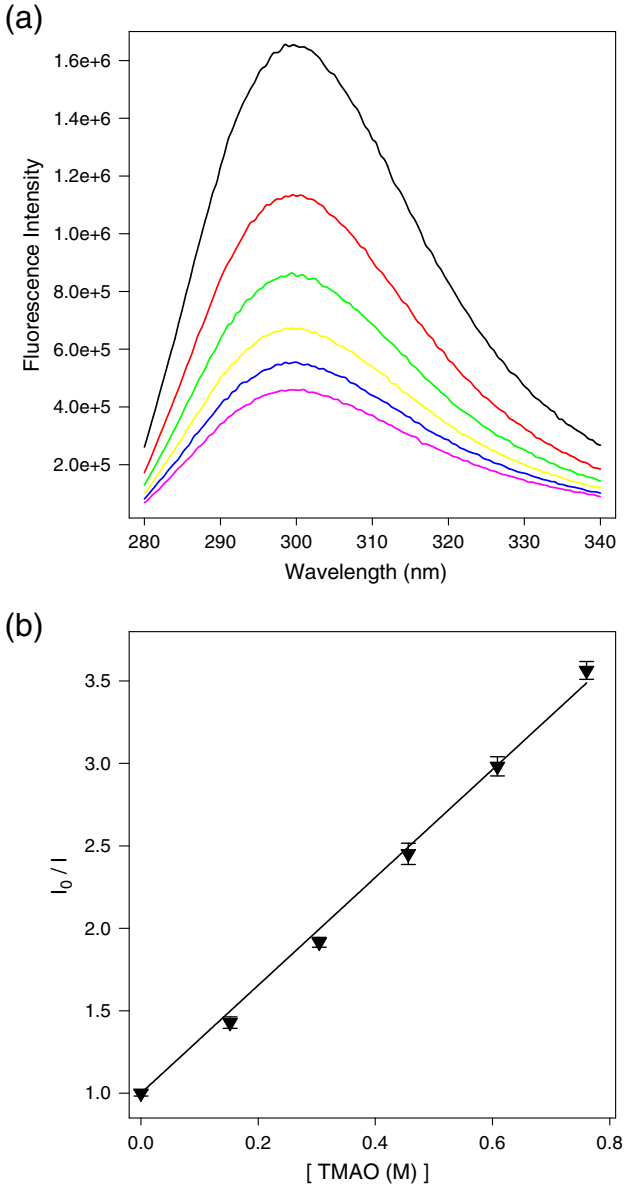


**Fig. 2.** (a) The quenching of phenol fluorescence by TMAO (from top to bottom: 0 M, 0.1521 M, 0.3042, 0.4563 M, 0.6084 M, 0.7605 M). Excitation and emission slits are set to 2 nm bandpass; (b) Stern–Volmer plot for the quenching phenol fluorescence by TMAO in the absence of carboxylate ions.

Where $k_q$ is the rate constant associated with dynamic quenching, $K_{ec}$ is the equilibrium constant associated with the formation of the encounter complex, $k_{pt}$ is the intrinsic rate of proton transfer from excited phenol to the carboxylate and $\tau$ is the fluorescence lifetime of phenol in the absence of quencher. Studies on the proton transfer process show that proton transfer occurs significantly, when the donor and acceptor moieties are separated by 0–5 water molecules [51]. Therefore, in order for the quenching process to compete with fluorescence the hydroxyl and carboxylate groups must be within 9 Å of one another. This value is smaller than the sum of the Van der Waals diameters of the quencher and fluorophor molecules. Therefore, we can assume that there is high probability that the encounter complex involves reacting molecules coming into van der Waals contact with one another. Because the parameter $\tau$ depends on TMAO concentration, the parameter $K'_{SV}(C)$ will be used to characterize the contribution of contact pair formation to the quenching process.

### 3.3. Isolating the contribution of the hydrophobic effect to contact pair formation

The hydrophobic contribution to $K_{sv}$ is isolated via the methodology we have previously developed [46,47]. Free energy of contact-pair formation between any alkyl-carboxylate and phenol $\Delta G_{ec}$, is assumed to be comprised of the following two interactions: a) the interaction between the carboxylate head group and the phenol moiety, b) the mostly hydrophobic interaction that exists between phenol and the alkyl tail. In other words:

$$\Delta G_{ec} \approx \Delta G_{head} + \Delta G_{alkyl} \tag{4}$$

If the free energy of contact formation of phenol and formate is subtracted from that of any given carboxylate:

$$\Psi = \{\Delta G_{ec}\}_{carboxylate} - \{\Delta G_{ec}\}_{formate} \approx \Delta G_{head} + \Delta G_{alkyl} - \Delta G_{formate} \tag{5}$$

The effect of any co-solute on contact pair formation can be calculated by subtracting the value of $\Psi$ at any given TMAO concentration $S$, from that of $\Psi$ determined when there is no co-solute present:

$$\Delta\Psi = \Psi_{[S]} - \Psi_{[S]=0} = \left(\Delta G_{head} + \Delta G_{alkyl} - \Delta G_{formate}\right)_{[S]} - \left(\Delta G_{head} + \Delta G_{alkyl} - \Delta G_{formate}\right)_{[S]=0} \tag{6}$$

Rearranging the terms gives us the following:

$$\Delta\Psi = \left\{(\Delta G_{head})_{[S]} - (\Delta G_{head})_{[S]=0}\right\} - \left\{(\Delta G_{formate})_{[S]} - (\Delta G_{formate})_{[S]=0}\right\} + \left\{\left(\Delta G_{alkyl}\right)_{[S]} - \left(\Delta G_{alkyl}\right)_{[S]=0}\right\} \tag{6a}$$

If the effect of TMAO on $\Delta G_{head}$ and $\Delta G_{formate}$ is assumed to be linear:

$$(\Delta G_{head})_{[S]} = (\Delta G_{head})_{[S]=0} + m^*[S] \tag{7a}$$

$$(\Delta G_{formate})_{[S]} = (\Delta G_{formate})_{[S]=0} + n^*[S] \tag{7b}$$

Where $n$ and $m$ are constants, inserting Eqs. (7a) and (7b) in Eq. (6) results in:

$$\Delta\Psi = (m-n)^*[S] + \left\{\left(\Delta G_{alkyl}\right)_{[S]} - \left(\Delta G_{alkyl}\right)_{[S]=0}\right\} \tag{8}$$

Formate is essentially a free carboxylate with a p$K_a$ of 3.8, the p$K_a$ of all other carboxylic acids varies from 4.75 (acetic acid) to 4.89 (octanoic acid) [52]. At pH 8.5 all carboxylate groups are essentially deprotonated.

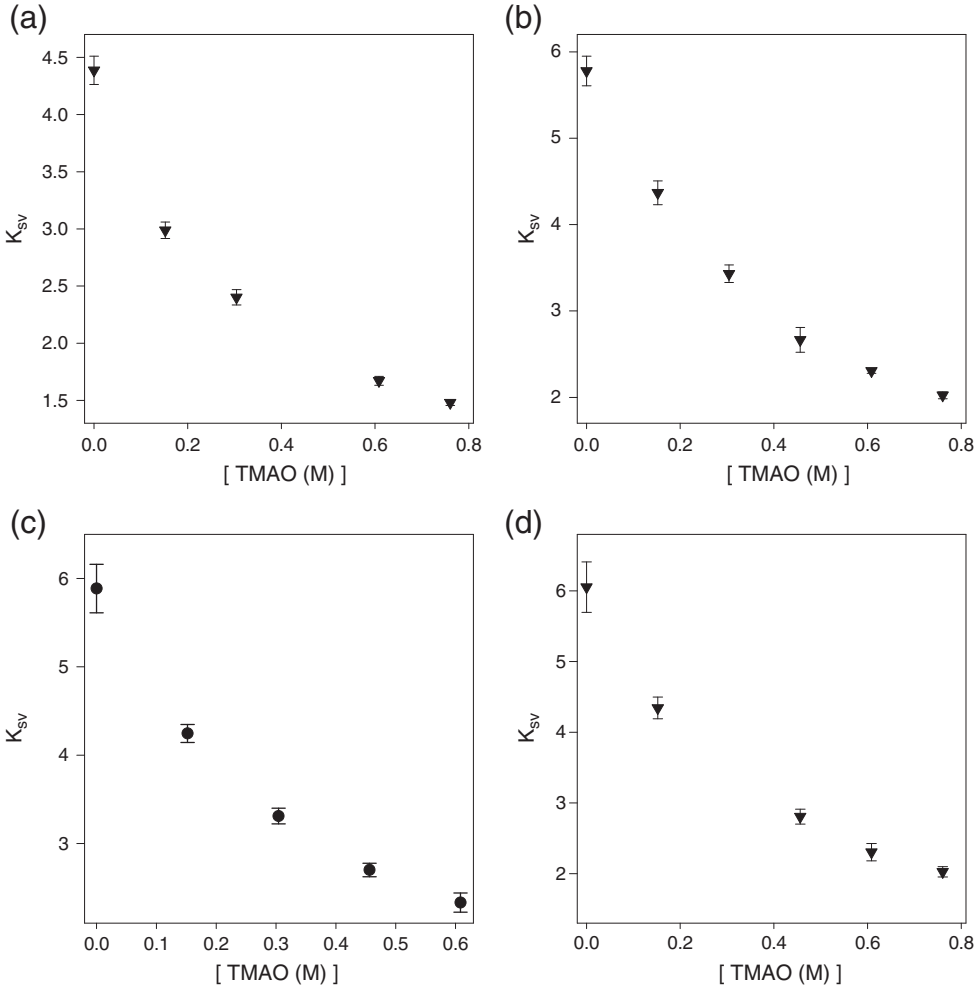


**Fig. 3.** The effects of TMAO on the apparent $K_{SV}$ quenching constants of carboxylate ions measured for phenol fluorescence: (a) formate, (b) acetate, (c) propionate, (d) hexanoate.

The electrostatic interaction of the various carboxylate head groups with phenol should thus be similarly affected by TMAO, therefore, the factor $(m\text{-}n)$ is likely to be a small constant term for all quenchers. Therefore $\Delta\Psi$ mostly reflects the difference between the alkyl–phenyl interaction in the presence and absence of TMAO. If the parameter $\Phi$ is defined for any given alkyl–carboxylate quencher:

$$\Phi = -RT \ln\left(\frac{(K_{SV})_{\text{alkyl-carboxylate}}}{(K_{SV})_{\text{formate}}}\right) \tag{9}$$

The values of $K'_{SV}(C)$ are obtained from the linear fits of Table 2. We may now define $\Delta\Phi$:

$$\Delta\Phi = \Phi_{[S]} - \Phi_{[S]=0} = -RT \ln\left(\frac{(K_{SV})_{\text{alkyl-carboxylate}}}{(K_{SV})_{\text{formate}}}\right)_{[S]} + RT \ln\left(\frac{(K_{SV})_{\text{alkyl-carboxylate}}}{(K_{SV})_{\text{formate}}}\right)_{[S]=0} \tag{10}$$

Substituting Eq. (3) in Eq. (10) we obtain:

$$\Delta\Phi = \Delta\Psi - RT \ln\left(\frac{\left(k_{pt}\right)_{\text{alkyl-carboxylate}}}{\left(k_{pt}\right)_{\text{formate}}}\right)_{[S]} + RT \ln\left(\frac{\left(k_{pt}\right)_{\text{alkyl-carboxylate}}}{\left(k_{pt}\right)_{\text{formate}}}\right)_{[S]=0} \tag{10a}$$

Because changes in activation free energies are related to changes in the thermodynamic free energies via linear free energy relationships [48,49], the TMAO dependence of the activation energy can be assumed to be linearly dependent on TMAO concentration:

$$\Delta\Phi = \Delta\Psi + \left(\Delta G^{\ddagger} + \alpha[S]\right)_{\text{alkyl-carboxylate}} - \left(\Delta G^{\ddagger} + \beta[S]\right)_{\text{formate}} - \left(\Delta G^{\ddagger}\right)_{\text{alkyl-carboxylate}} + \left(\Delta G^{\ddagger}\right)_{\text{formate}} = \Delta\Psi + (\alpha - \beta)[S] \tag{11}$$

Using the same arguments used for Eq. (7) the factor $(\alpha - \beta)$ is also likely to be a rather small constant term for all quenchers. Re-writing Eq. (11) yields:

$$\Delta\Phi = \left\{\left(\Delta G_{alkyl}\right)_{[S]} - \left(\Delta G_{alkyl}\right)_{[S]=0}\right\} + (m - n + \alpha - \beta)[S] \approx \left\{\left(\Delta G_{\text{alkyl}}\right)_{[S]} - \left(\Delta G_{alkyl}\right)_{[S]=0}\right\} \tag{11a}$$

In this equation, the first term isolates the contribution that the interaction between the hydrophobic alkyl and phenyl moieties makes to $K'_{SV}(C)$ values. Fig. 5 plots $\Delta\Phi$ calculated from the $K'_{SV}(C)$ values obtained from the correlation lines of Table 2 as a function of TMAO concentration. The values of $\Delta\Phi$ become more positive as the amount of TMAO is increased, indicating that the hydrophobic contact pair becomes more destabilized thermodynamically. From this figure we can conclude that TMAO disrupts the hydrophobic interaction between phenyl and alkyl chains.

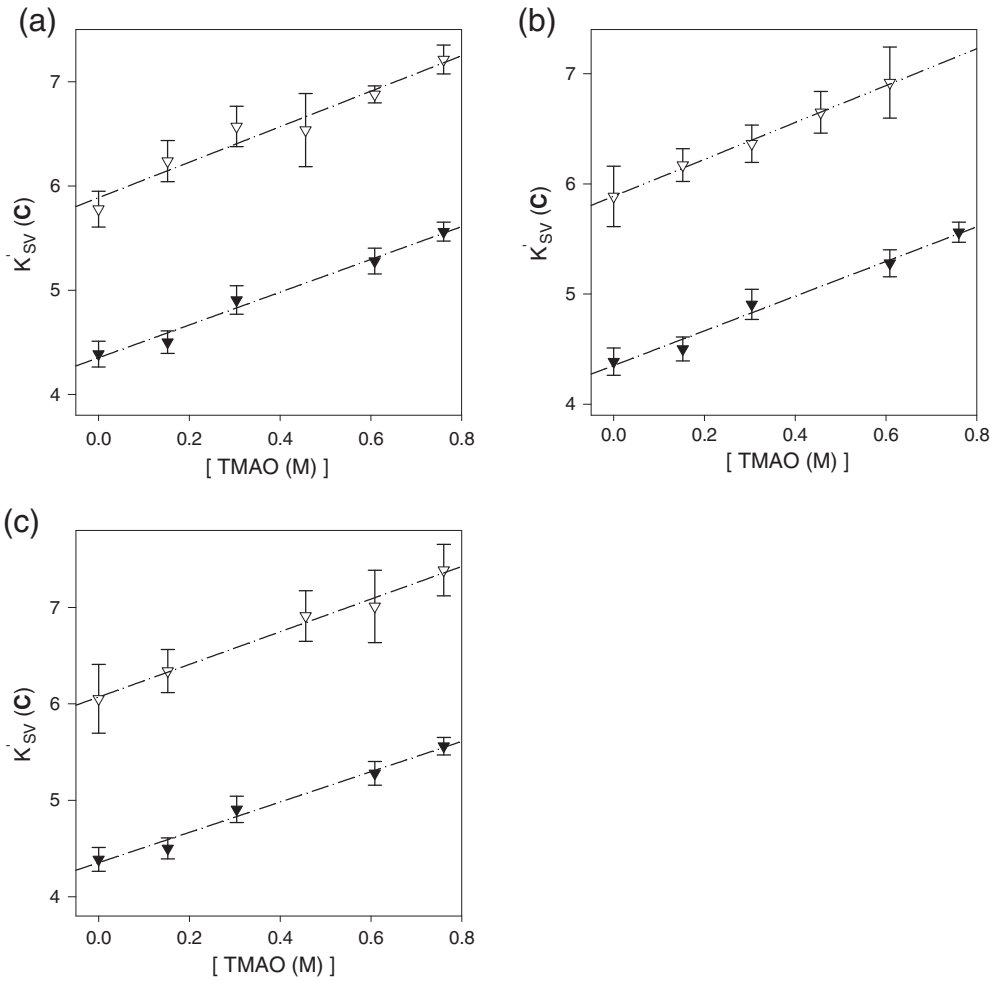


**Fig. 4.** The effects of TMAO on the corrected Stern–Volmer constants $K'_{SV}(C)$ of carboxylate ions measured for phenol fluorescence: (a) formate (closed triangles) and acetate (open triangles), (b) formate (closed triangles) and propionate (open triangles), (c) formate (closed triangles) and hexanoate (open triangles).

## 4. Conclusions

The unique folded structure of any protein results from the total sum contribution of a variety of interactions such hydrogen bonding, electrostatic interactions and the hydrophobic effect. Protein denaturation (or renaturation) experiments provide useful methodologies for assigning each separate contribution to the overall protein folding process. The correct determination of the contribution of each interaction however, requires us to understand how the denaturing (or renaturing) agent influences it. The hydrophobic effect plays a major role in stabilizing protein molecules but a clear picture of the effects of TMAO on hydrophobic interactions has eluded assignation for a long time. In this work we have re-visited this problem by studying the effects of TMAO on simple hydrophobic contact pairs formed between alkyl groups and a benzene ring. The system is simple enough to allow us to isolate the effect of TMAO on the alkyl–phenyl hydrophobic interaction. The data show that TMAO addition reduces interactions between hydrophobic contact pairs in manner consistent with the predictions of Paul and Patey and is consistent with the formal thermodynamic analyses of Bolen indicating that adding TMAO to aqueous solvent increases the solubility of hydrophobic moieties. In other words, TMAO can function as a "denaturing surfactant" for

**Table 2**
Linear regression fitting parameters obtained from correlating the data in Fig. 4.

| Quencher | $a$ ($M^{-2}$) | $y0$ ($M^{-1}$) | $r^2$ |
|---|---|---|---|
| Formate | 1.57 ± 0.12 | 4.3 ± 0.05 | 0.9840 |
| Acetate | 1.7 ± 0.2 | 5.89 ± 0.09 | 0.9456 |
| Propionate | 1.67 ± 0.05 | 5.89 ± 0.02 | 0.9969 |
| Hexanoate | 1.7 ± 0.1 | 6.07 ± 0.05 | 0.9880 |

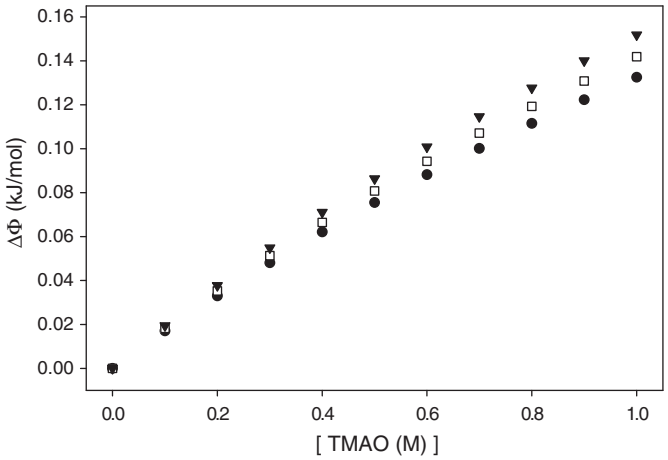


**Fig. 5.** TMAO concentration dependence of $\Delta\Phi$ as defined by Eq. (9); the quenchers are: acetate (closed circles), propionate (open squares), and hexanoate (closed triangles).

hydrophobic interactions. This result is of particular importance to theoreticians using protein unfolding/refolding data for developing algorithms that predict protein structure: the effects of TMAO (and perhaps other osmolytes) on the stabilizing hydrophobic interactions must now also be considered. In addition, understanding how TMAO affects hydrophobic interactions may also have potential practical application from a biotechnological standpoint. Recent work has demonstrated that the presence of TMAO promotes the association between chymotrypsin and trypsin inhibitor proteins [53]. One potential explanation of this enhanced binding may be that the presence of TMAO loosens the S1 pocket of chymotrypsin by weakening the hydrophobic interactions, thus making the enzyme adopt a more open "binding competent" form towards the trypsin inhibitor. Osmolytes like TMAO may function as important stabilizers for proteins that need to be stored and delivered in their biologically active oligomeric forms.

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## References

[1] P.H. Yancey, Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses, J. Exp. Biol. 208 (2005) 2819–2830.
[2] T. Arakawa, S.N. Timasheff, The stabilization of proteins by osmolytes, Biophys. J. 47 (1985) 411–414.
[3] M. Auton, D.W. Bolen, Predicting the energetics of osmolyte-induced protein folding/unfolding, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 15065–15068.
[4] M. Auton, D.W. Bolen, J. Rosgen, Structural thermodynamics of protein preferential solvation: osmolyte solvation of proteins, aminoacids, and peptides, Proteins 73 (2008) 802–813.
[5] M. Auton, A.C. Ferreon, D.W. Bolen, Metrics that differentiate the origins of osmolyte effects on protein stability: a test of the surface tension proposal, J. Mol. Biol. 361 (2006) 983–992.
[6] D.W. Bolen, G.D. Rose, Structure and energetics of the hydrogen-bonded backbone in protein folding, Annu. Rev. Biochem. 77 (2008) 339–362.
[7] D.K. Eggers, J.S. Valentine, Crowding and hydration effects on protein conformation: a study with sol–gel encapsulated proteins, J. Mol. Biol. 314 (2001) 911–922.
[8] F. Guo, J.M. Friedman, Osmolyte-induced perturbations of hydrogen bonding between hydration layer waters: correlation with protein conformational changes, J. Phys. Chem. B 113 (2009) 16632–16642.
[9] L.M. Holthauzen, D.W. Bolen, Mixed osmolytes: the degree to which one osmolyte affects the protein stabilizing ability of another, Protein Sci. 16 (2007) 293–298.
[10] A. Wang, D.W. Bolen, A naturally occurring protective system in urea-rich cells: mechanism of osmolyte protection of proteins against urea denaturation, Biochemistry 36 (1997) 9101–9108.
[11] R. Kumar, J.C. Lee, D.W. Bolen, E.B. Thompson, The conformation of the glucocorticoid receptor af1/tau1 domain induced by osmolyte binds co-regulatory proteins, J. Biol. Chem. 276 (2001) 18146–18152.
[12] L. Rajagopalan, J. Rosgen, D.W. Bolen, K. Rajarathnam, Novel use of an osmolyte to dissect multiple thermodynamic linkages in a chemokine ligand-receptor system, Biochemistry 44 (2005) 12932–12939.
[13] A.T. Russo, J. Rosgen, D.W. Bolen, Osmolyte effects on kinetics of FKBP12 C22A folding coupled with prolyl isomerization, J. Mol. Biol. 330 (2003) 851–866.
[14] M. Auton, D.W. Bolen, Additive transfer free energies of the peptide backbone unit that are independent of the model compound and the choice of concentration scale, Biochemistry 43 (2004) 1329–1342.
[15] D.D. Banks, L.M. Gloss, Equilibrium folding of the core histones: the H3–H4 tetramer is less stable than the H2A–H2B dimer, Biochemistry 42 (2003) 6827–6839.
[16] S.A. Celinski, J.M. Scholtz, Osmolyte effects on helix formation in peptides and the stability of coiled-coils, Protein Sci. 11 (2002) 2048–2051.
[17] Y. Feng, D. Liu, J. Wang, Native-like partially folded conformations and folding process revealed in the N-terminal large fragments of staphylococcal nuclease: a study by NMR spectroscopy, J. Mol. Biol. 330 (2003) 821–837.
[18] R.F. Gahl, M. Narayan, G. Xu, H.A. Scheraga, Trimethylamine-N-oxide modulates the reductive unfolding of onconase, Biochem. Biophys. Res. Commun. 325 (2004) 707–710.
[19] T.C. Gluick, S. Yadav, Trimethylamine N-oxide stabilizes RNA tertiary structure and attenuates the denaturating effects of urea, J. Am. Chem. Soc. 125 (2003) 4418–4419.
[20] M. Gonnelli, G.B. Strambini, No effect of trimethylamine N-oxide on the internal dynamics of the protein native fold, Biophys. Chem. 89 (2001) 77–85.
[21] M. Gulotta, L. Qiu, R. Desamero, J. Rosgen, D.W. Bolen, R. Callender, Effects of cell volume regulating osmolytes on glycerol 3-phosphate binding to triosephosphate isomerase, Biochemistry 46 (2007) 10055–10062.
[22] O. Gursky, Probing the conformation of a human apolipoprotein C-1 by amino acid substitutions and trimethylamine-N-oxide, Protein Sci. 8 (1999) 2055–2064.
[23] C.H. Henkels, J.C. Kurz, C.A. Fierke, T.G. Oas, Linked folding and anion binding of the *Bacillus subtilis* ribonuclease P protein, Biochemistry 40 (2001) 2777–2789.
[24] R. Kumar, J.M. Serrette, E.B. Thompson, Osmolyte-induced folding enhances tryptic enzyme activity, Arch. Biochem. Biophys. 436 (2005) 78–82.
[25] C. Lendel, V. Dincbas-Renqvist, A. Flores, E. Wahlberg, J. Dogan, P.A. Nygren, T. Hard, Biophysical characterization of Z(SPA-1)—a phage-display selected binder to protein A, Protein Sci. 13 (2004) 2078–2088.
[26] S.L. Lin, A. Zarrine-Afsar, A.R. Davidson, The osmolyte trimethylamine-N-oxide stabilizes the Fyn SH3 domain without altering the structure of its folding transition state, Protein Sci. 18 (2009) 526–536.
[27] S. Tranier, C. Iobbi-Nivol, C. Birck, M. Ilbert, I. Mortier-Barriere, V. Mejean, J.P. Samama, A novel protein fold and extreme domain swapping in the dimeric TorD chaperone from *Shewanella massilia*, Structure 11 (2003) 165–174.
[28] J. Tulla-Puche, I.V. Getun, C. Woodward, G. Barany, Native-like conformations are sampled by partially folded and disordered variants of bovine pancreatic trypsin inhibitor, Biochemistry 43 (2004) 1591–1598.
[29] V.N. Uversky, J. Li, A.L. Fink, Trimethylamine-N-oxide-induced folding of alpha-synuclein, FEBS Lett. 509 (2001) 31–35.
[30] M. Auton, D.W. Bolen, Application of the transfer model to understand how naturally occurring osmolytes affect protein stability, Methods Enzymol. 428 (2007) 397–418.
[31] I. Baskakov, D.W. Bolen, Forcing thermodynamically unfolded proteins to fold, J. Biol. Chem. 273 (1998) 4831–4834.
[32] I.V. Baskakov, R. Kumar, G. Srinivasan, Y.S. Ji, D.W. Bolen, E.B. Thompson, Trimethylamine N-oxide-induced cooperative folding of an intrinsically unfolded transcription-activating fragment of human glucocorticoid receptor, J. Biol. Chem. 274 (1999) 10693–10696.
[33] C.Y. Hu, H. Kokubo, G.C. Lynch, D.W. Bolen, B.M. Pettitt, Backbone additivity in the transfer model of protein solvation, Protein Sci. (5) (2010) 1011–1022, http://dx.doi.org/10.1002/pro.378.
[34] J. Rosgen, B.M. Pettitt, D.W. Bolen, Protein folding, stability, and solvation structure in osmolyte solutions, Biophys. J. 89 (2005) 2988–2997.
[35] J. Rosgen, B.M. Pettitt, D.W. Bolen, An analysis of the molecular origin of osmolyte-dependent protein stability, Protein Sci. 16 (2007) 733–743.
[36] T.O. Street, D.W. Bolen, G.D. Rose, A molecular mechanism for osmolyte-induced protein stability, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 13997–14002.
[37] S.N. Timasheff, G. Xie, Preferential interactions of urea with lysozyme and their linkage to protein denaturation, Biophys. Chem. 105 (2003) 421–448.
[38] A. Wang, A.D. Robertson, D.W. Bolen, Effects of a naturally occurring compatible osmolyte on the internal dynamics of ribonuclease A, Biochemistry 34 (1995) 15096–15104.
[39] P. Wu, D.W. Bolen, Osmolyte-induced protein folding free energy changes, Proteins 63 (2006) 290–296.
[40] M.V. Athawale, S. Sarupria, S. Garde, Enthalpy-entropy contributions to salt and osmolyte effects on molecular-scale hydrophobic hydration and interactions, J. Phys. Chem. B 112 (2008) 5661–5670.
[41] M.V. Athawale, J.S. Dordick, S. Garde, Osmolyte trimethylamine-N-oxide does not affect the strength of hydrophobic interactions: origin of osmolyte compatibility, Biophys. J. 89 (2005) 858–866.
[42] S. Paul, G.N. Patey, The influence of urea and trimethylamine-N-oxide on hydrophobic interactions, J. Phys. Chem. B 111 (2007) 7932–7933.
[43] G. Graziano, How does trimethylamine N-oxide counteract the denaturing activity of urea? Phys. Chem. Chem. Phys. 13 (2011) 17689–17695.
[44] B.J. Bennion, V. Daggett, The molecular basis for the chemical denaturation of proteins by urea, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 5142–5147.
[45] L.M. Holthauzen, J. Rosgen, D.W. Bolen, Hydrogen bonding progressively strengthens upon transfer of the protein urea-denatured state to water and protecting osmolytes, Biochemistry 49 (2010) 1310–1318.
[46] T.A. Shpiruk, M. Khajehpour, The effect of urea on aqueous hydrophobic contact-pair interactions, Phys. Chem. Chem. Phys. 15 (2013) 213–222.
[47] D.L. Beauchamp, M. Khajehpour, The effect of lithium ions on the hydrophobic effect: does lithium affect hydrophobicity differently than other ions? Biophys. Chem. 163–164 (2012) 35–43.
[48] D.K. Kunimitsu, A.Y. Woody, E.R. Stimson, H.A. Scheraga, Thermodynamic data from fluorescence spectra. II. Hydrophobic bond formation in binary complexes, J. Phys. Chem. 72 (1968) 856–866.
[49] A.Y. Moon, D.C. Poland, H.A. Scheraga, Thermodynamic data from fluorescence spectra. I. The system phenol-acetate, J. Phys. Chem. 69 (1965) 2960–2966.
[50] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, third ed. Springer, New York, 2006.
[51] B.J. Siwick, M.J. Cox, H.J. Bakker, Long-range proton transfer in aqueous acid–base reactions, J. Phys. Chem. B 112 (2008) 378–389.
[52] M. Namazian, S. Halvani, Calculations of pKa values of carboxylic acids in aqueous solution using density functional theory, J. Chem. Thermodyn. 38 (2006) 1495–1502.
[53] D. Wu, A.P. Minton, Compensating effects of urea and trimethylamine-N-oxide on the heteroassociation of alpha-chymotrypsin and soybean trypsin inhibitor, J. Phys. Chem. B 117 (2013) 3554–3559.