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Compensating Effects of Urea and Trimethylamine-N-Oxide on the Heteroassociation of α -Chymotrypsin and Soybean Trypsin Inhibitor

Di Wu and Allen P. Minton*

Section on Physical Biochemistry, Laboratory of Biochemistry and Genetics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, U.S. Department of Health and Human Services, Bethesda, Maryland 20892, United States

Abstract

An assay for the determination of the equilibrium constant for heteroassociation of α -chymotrypsin and soybean trypsin inhibitor via fluorescence depolarization is described. Results obtained at neutral pH in saline buffer were consistent with prior determinations via sedimentation equilibrium and static light scattering. The dependence of the association equilibrium constant upon the concentrations of urea and trimethylamine-N-oxide (TMAO) added individually and in mixtures was determined at several temperatures. It was found that subdenaturing concentrations of urea decrease the extent of heteroassociation and that added TMAO increases the extent of heteroassociation. The effects of both cosolutes in mixtures upon the equilibrium heteroassociation of α -chymotrypsin and soybean trypsin inhibitor appear to be additive. A thermodynamic analysis of the combined results is presented.

INTRODUCTION

The structure and function of proteins have been found to be significantly affected by a class of small organic compounds termed osmolytes that are synthesized in cells to protect proteins and other macromolecules from the effect of osmotic stress.¹ One of these compounds, trimethylamine N-oxide (TMAO), is accumulated at high concentration by marine organisms.² The molecule, which is essentially uncharged in the pH range 6–8,³ is noted for its ability to preserve protein structure and function under otherwise denaturing conditions.^{4,5} Previous studies indicated that the stabilizing effect of TMAO is caused by

preferential exclusion of the osmolyte from the immediate vicinity of the protein backbone.^{6,7} It has been found that the effect of concentrated TMAO on the chemical potential of several native proteins may be accounted for quantitatively by a model in which the interaction between TMAO and each protein is described as a purely steric repulsion between equivalent hard spherical particles representing a particular protein and TMAO respectively.⁸ The effect of concentrated TMAO upon a functionally related conformational equilibrium in adenylate kinase may also be accounted for quantitatively by assuming that TMAO acts as an inert spherical particle that interacts with the protein solely via steric repulsion.⁹

A second class of small molecule cosolutes, typified by urea, acts to destabilize the native structures of proteins.¹⁰ The destabilizing effect of urea is attributed to attractive interactions with the exposed interior of an unfolded protein.¹¹

Prior studies have shown that subdenaturing concentrations of urea can enhance the dissociation of multisubunit proteins.^{5,12,13} We are unaware of prior quantitative studies of the effect of TMAO upon self- or heteroassociation equilibria. The study reported here was therefore undertaken for two reasons: (1) to develop and validate a novel relatively high throughput method for assaying quantitatively the effect of additives upon the strength of macromolecular association equilibria, and (2) to determine whether TMAO can stabilize noncovalent oligomeric complexes in solution relative to their separated constituent species, and whether TMAO can compensate for the dissociating effect of urea.

In the present study, the strength of heteroassociation equilibria was determined via measurement of the influence of varying concentration of an unlabeled protein upon the fluorescence anisotropy of a trace concentration of a fluorescently labeled protein with which the unlabeled protein is presumed to bind. The measured anisotropy is a measure of the rate of rotational diffusion of the labeled protein and therefore its equilibrium average state of association.^{16,23,24} This method was selected due to the availability of automated instrumentation that greatly facilitated the collection of the large amounts of data required to enable the analysis presented below.

The particular association equilibrium selected to be studied is that of α -chymotrypsin and soybean trypsin inhibitor (STI), which has been previously characterized by both sedimentation equilibrium¹⁴ and static light scattering.¹⁵ These prior studies established that STI has two independent sites for binding of chymotrypsin with affinities that are equal to within experimental uncertainty, and may be represented by a single equilibrium association constant.

Following the description of materials and preparation presented below, we describe the fluorescence depolarization assay used in the present study. The method was validated by establishing that the equilibrium constant determined using this technique is equal to within experimental uncertainty to that obtained in the prior studies under comparable conditions. Next, the measured dependences of the equilibrium constant for heteroassociation upon temperature and the concentrations of urea and TMAO are presented, and analyzed

thermodynamically. The results may be accounted for quantitatively assuming that the effects of the two cosolutes, although acting in opposite directions, are additive.

MATERIALS AND METHODS

Chemicals and Reagents

α -Chymotrypsin (MW 25K), soybean trypsin inhibitor (MW 21.5K), and ovalbumin (MW 45K) were obtained from Worthington Biochemical Corporation (LS001450, LS003571, and LS003048, respectively). The fluorescent dye Alexa Fluor488 and Trimethylamine-N-oxide (TMAO) were obtained from Sigma-Aldrich (A20100 and T0514, respectively). Urea was obtained from Invitrogen (15505–050). Dimethylsulfoxide (DMSO) was obtained from Fisher Scientific (D128). Proteins, TMAO, and urea were used without additional purification. Protein concentrations were determined by absorbance at 280 nm. The standard values of absorbance of each protein in optical units per centimeter path length for 1 g/L were 2.04 for chymotrypsin,¹⁷ 0.94 for STI,¹⁸ and 0.75 for ovalbumin.¹⁹ Stock solutions of TMAO at 4 M and urea at 8 M in phosphate-buffered saline (PBS) were prepared, and the final pH of each solution was adjusted to 7.4.

Labeling of STI with Alexa Fluor488

The protein was dissolved in 0.1 M sodium bicarbonate buffer at pH 8.3 to a final protein concentration of about 10 mg/mL. The amine-reactive dye was dissolved in DMSO at a concentration of about 10 mg/mL. Approximately 5 equiv of dye was slowly added into the protein solution, with continuous stirring. The reaction mixture was incubated at room temperature for 1 h, and the labeled protein was dialyzed against PBS.

Fluorescence Anisotropy Measurement

The labeled STI as tracer at 10^{-8} M was titrated by unlabeled chymotrypsin up to about 10^{-5} M. The titration was also carried out in the presence of TMAO and urea at different concentrations separately or together. Samples were prepared using a NIMBUS pipetting robot (Hamilton, Reno, NV) that dispensed programmed amounts of labeled STI, chymotrypsin, TMAO, and urea stock solutions into wells of a black 96-well Corning microplate (3677) according to protocols written by the authors. The samples were mixed using a microplate stirrer and then spun in a centrifuge equipped with a microplate rotor at 1600g for 3 min to remove bubbles and residual particulates. Then the sample plates were incubated at 4 °C, 16 °C, 25 °C, or 37 °C for one hour prior to measurement. The fluorescence anisotropy and intensity of each well ($\lambda_{\text{ex}} = 485$ nm, $\lambda_{\text{em}} = 525$ nm) were read using a SpectraMax M5 Microplate Reader (Molecular Devices, Sunnyvale, CA). Measurements of sample temperature within a microplate well using a thermocouple probe established that the temperature of solutions in the microplate set prior to reading did not vary significantly during the duration of the read cycle.

Analysis of the Dependence of Fluorescence Anisotropy of Labeled STI upon the Concentration of α -Chymotrypsin

In accordance with prior results, we postulate that STI can bind chymotrypsin at two independent sites with approximately equal affinity, and that under our experimental

conditions, chymotrypsin does not self-associate significantly.^{14,15,20–22} Denoting chymotrypsin by C and STI by S , it follows that at equilibrium

$$[SC] = 2K[S][C]$$

$$[SC_2] = K^2[S][C]^2 \quad (1)$$

We note that, in the present experiments, the molar concentration of fluorescently labeled STI is so small that it may be safely assumed that the concentration of free C is essentially equal to the total added concentration of C . It follows that the mole fractions of STI binding zero, one, and two molecules of chymotrypsin at equilibrium are then given respectively by

$$f_S = \frac{[S]}{[S]_{\text{tot}}} = \frac{1}{(1 + K[C])^2} \quad (2a)$$

$$f_{SC} = \frac{[SC]}{[S]_{\text{tot}}} = 2K[C]f_S \quad (2b)$$

$$f_{SC_2} = \frac{[SC_2]}{[S]_{\text{tot}}} = K^2[C]^2 f_S \quad (2c)$$

The equilibrium average anisotropy is given by

$$\langle A \rangle = \sum_i f_i A_i \quad (3)$$

where A_i denotes the fluorescence anisotropy of the i th species, equal to

$$A_i = A_0 / (1 + \tau_F / \phi_i) \quad (4)$$

where A_0 denotes the anisotropy of a static dye, τ_F denotes the fluorescence lifetime and ϕ_i denotes the rotational relaxation time of species i , which is proportional to the effective hydrodynamic volume of the species.^{23,24} To simplify the model, we assume that all of the labeled species behave as effectively spherical particles (i.e., rotate isotropically) of nearly identical density, so that the relaxation time of the i th species is proportional to the molar mass. Equation 4 may then be rewritten as follows:

$$A_i = A_0 / (1 + \alpha / M_i) \quad (5)$$

where M_i denotes the molar mass of species i , and α is a constant of proportionality. It follows that given independently determined values for the molar masses of chymotrypsin and STI, the dependence of $\langle A \rangle$ upon the total concentration of chymotrypsin may be calculated as functions of A_0 , α , and K using eqs 2a–2c, 3, and 5.

RESULTS

The measured fluorescence anisotropy of tracer Alexa Fluor488 - labeled STI at a constant concentration (10^{-8} M) is plotted in Figure 1 as a function of the concentration of two added unlabeled proteins, ovalbumin and STI, measured in at 25 °C in PBS without urea or TMAO. The observed lack of systematic dependence of measured anisotropy upon ovalbumin concentration indicates the absence of both binding and any nonspecific quenching effect due to added unlabeled protein. Also plotted in Figure 1 is the dependence of anisotropy calculated using the equilibrium binding model (eqs 2a–2c, 3, and 5) with the best-fit parameter values given in the figure caption. The best-fit value of the association equilibrium constant agrees with that obtained in a previous study carried out under the same conditions using composition gradient light scattering.¹⁵

The influence of various concentrations of TMAO and urea on the association of STI with chymotrypsin was determined by titrations of STI with chymotrypsin carried out in buffers containing urea and/or TMAO at a variety of concentrations and at four temperatures. The dependence of the natural logarithm of the association equilibrium constant upon the concentrations of each additive at four temperatures is plotted in the top and middle rows of Figure 2. It is evident that TMAO significantly enhances and urea significantly reduces the association at all four temperatures. The logarithm of the association constant appears to depend linearly upon the concentrations of both additives, to within the uncertainty of measurement.

To assess the effect of mixed additives, titration was performed in the presence of urea at a fixed concentration (1 M) and TMAO at various concentrations. The dependence of $\ln K$ on the concentrations of TMAO is plotted in the bottom row of Figure 2. The results indicate that, even in the presence of urea, TMAO enhances the association of STI with chymotrypsin as well at all temperatures. In Figure 3, the fractional change of anisotropy (f_A) of labeled STI at 25 °C is plotted as a function of chymotrypsin concentration for four solution compositions: in the absence of either additive (black), in the presence of 1.9 M TMAO (blue), in the presence of 1 M urea (red), respectively, and in the presence of both additives (green). Plotted together with the data is the best-fit of the binding model to each data set, calculated using the parameter values presented in Table 1. Whereas the addition of each cosolute individually results in a substantial increase or decrease in the value of the equilibrium association constant, the simultaneous addition of both results in essentially no change, i.e., the two effects appear to cancel each other.

DISCUSSION

The standard state Gibbs free energy change (ΔG) associated with the binding of one mole of chymotrypsin to each of the two independent and equivalent binding sites on STI at constant temperature and pressure, and the enthalpic and entropic contributions to the free energy change, may be written as follows:

$$\ln K = \frac{-\Delta G}{RT} = \frac{-\Delta H}{R} \left(\frac{1}{T} \right) + \frac{\Delta S}{R} \quad (6)$$

where R denotes the molar gas constant and T denotes the absolute temperature. H and S , respectively, denote the standard state enthalpy and entropy changes accompanying this process. The total enthalpy and entropy changes of the association of STI with chymotrypsin can be divided into three parts: the changes associated with site binding in the absence of cosolutes and the differential changes associated with each cosolute. As a first approximation, we assume that the differential changes in standard state enthalpy and entropy are linear in the concentrations of each cosolute, and that the contributions of each cosolute are additive, i.e., independent of the other. In addition, we assume that the effect of TMAO is purely entropic, consistent with previous findings that the interaction of TMAO with several proteins is dominated by steric exclusion from the backbone surface.^{6,7} In accordance with these approximations and assumptions, we write

$$\Delta H = \Delta H_0 + \Delta h_U c_U \quad (7)$$

$$\Delta S = \Delta S_0 + \Delta s_U c_U + \Delta s_T c_T \quad (8)$$

where H_0 and S_0 , respectively, denote the enthalpy and entropy changes accompanying the site binding in the absence of cosolutes, and h_U , s_U , and s_T , respectively, denote the increments of enthalpy and entropy change contributed by unit concentrations of urea (U) and TMAO (T).

Given the values of five parameters (H_0 , S_0 , h_U , s_U , and s_T), the value of $\ln K$ may be calculated as a function of the temperature and the concentrations of urea and TMAO using eqs 6–8. The data collected at all additive compositions and all temperatures were modeled simultaneously using these equations to obtain best-fit values of the five thermodynamic parameters listed above. These data are plotted in Figure 4A–C together with the corresponding functions calculated according to eqs 6–8 with the best-fit parameter values presented in Table 2. It may be seen that the simple model presented above can account quantitatively for an extensive body of data representing the combined results of 63 independent titration experiments to within the uncertainty of measurement. Our results indicate with a high likelihood that urea diminishes the tendency to associate due to a positive enthalpy increment, and that the dissociating effect of urea lessens somewhat with increasing temperature as the enthalpic contribution diminishes relative to the temperature-independent entropic contribution favoring association. TMAO, on the other hand, with an exclusively entropic interaction increment, favors association at all temperatures.

The results further indicate that TMAO can cancel the inhibiting effect of urea on the heteroassociation of chymotrypsin and STI. Utilizing the best-fit values of h_U , s_U , and s_T , we may use eqs 6–8 to calculate the molar ratio of added TMAO to added urea that will result in a null effect, i.e., an equilibrium association constant in the presence of both additives that is equal to that in the absence of both additives. The result of this calculation is plotted in Figure 5. On the basis of earlier studies of compensating effects of urea and TMAO on protein stability, it has been suggested that compensation is due to the formation of urea–TMAO complexes with a specific stoichiometry.²⁵ In contrast, the present model can account for compensating effects of urea and TMAO on protein association without

proposing any interaction between urea and TMAO (relative to that between water and TMAO), in qualitative accord with the conclusions of Rösger and Jackson-Atogi.²⁶

Finally, we note that the observed enhancement of heteroassociation by TMAO is rather small in thermodynamic terms, amounting to a decrease in the standard state free energy of association of 0.8 kcal per mole of site, or 1.6 kcal per mole of STI, in 1 M TMAO at 25 °C. However, that does not imply that the effect is inconsequential, as it can result in a substantial change in the fractional extent of binding of chymotrypsin to STI near the midpoint of the binding isotherm. The effect of TMAO and other presumably inert cosolutes upon other reversible protein associations should be studied, and if the effect proves to be general, it could be an important factor in the regulation of enzymatic activity, signaling, gene expression, and other biological processes involving reversible macromolecular associations.

Measurements of fluorescence depolarization have been used previously to monitor the extent of association of labeled and unlabeled macromolecules.¹⁶ The novelty of the present implementation derives from the incorporation of automated sample preparation within microplate wells and concurrent measurement of fluorescence anisotropy and intensity of multiple samples within microplate wells. These features have enabled the collection of an unprecedentedly large body of titration data over a significant range of sample compositions and temperatures. Without such automation, collection of a comparably comprehensive body of data via conventional means would have required far greater amounts of time, labor, and protein, rendering unfeasible the global analysis of results described here.

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Figure 1.

Fluorescence anisotropy of the titration of Alexa Fluor488 labeled soybean trypsin inhibitor as tracer at constant concentration with unlabeled α -chymotrypsin (dots) and ovalbumin (triangles) as titrant at variety of concentrations. The error bars represent two standard deviations of measurement (95% confidence limits). Solid curve is calculated using eq 3 with the following best-fit parameter values: $A_0 = 0.13$, $\alpha = 12.5$, $\ln K = 14$.

Figure 2.

Dependence of the natural logarithm of the association equilibrium constant of soybean trypsin inhibitor with α -chymotrypsin as a function of TMAO concentration (top row), urea concentration (middle row), and TMAO concentration in the presence of 1 M urea (bottom row). Dotted lines indicate the best-fit of a straight line to each set of data. The error bars represent one standard deviation.

Figure 3. Dependence of normalized fluorescence anisotropy (f_A) of labeled soybean trypsin inhibitor upon unlabeled α -chymotrypsin concentration in the absence (black) or presence of 1.9 M TMAO (blue) and 1 M urea (red), separately or together (green). Solid curves are calculated using eqs 2a–2c, 3, and 5, using the best-fit parameter values presented in Table 1.

Figure 4.

Dependence of $\ln K$ upon the concentration of TMAO and inverse temperature (panel A), the concentration of urea and inverse temperature (panel B), and the concentration of TMAO and inverse temperature in the presence of 1 M urea (panel C). Surfaces are calculated according to eqs 6–8 using the best-fit parameter values presented in Table 2.

Figure 5. Temperature dependence of the ratio of TMAO to urea concentrations required to achieve a null effect on the equilibrium association constant, calculated as described in the text.

Table 1

Best-Fit Parameter Values Obtained by Nonlinear Least Squares Fitting of Text Eqs 2a–2c, 3, and 5 to the Measured Dependence of $\langle A \rangle$ upon the Total Concentrations of α -Chymotrypsin in the Presence of Additives Plotted in Figure 3

additive concentration (M)		A_0	α	$\ln K$
TMAO	urea			
0	0	0.133	12.5	14.0
1.9	0	0.15	15.9	14.9
0	1	0.12	12.3	13.3
1.9	1	0.15	13.5	14.0

Table 2

Best-Fit Parameter Values Obtained by Nonlinear Least Squares Fitting of Text Eqs 6–8 to the Measured Dependence of $\ln K$ upon Temperature and the Concentrations of TMAO and Urea

parameter	best-fit value ^a
H_0	3.0 ± 1.0 (kcal mol ⁻¹)
S_0	38 ± 3 (cal K ⁻¹ mol ⁻¹)
s_T	0.8 ± 0.1 (cal K ⁻¹ mol ⁻¹)
h_U	1.6 ± 1.4 (kcal mol ⁻¹)
s_U	4.0 ± 4.7 (cal K ⁻¹ mol ⁻¹)

^a Indicated uncertainties represent 95% confidence limits.