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Quantitative Characterization of the Compensating Effects of Trimethylamine-*N*-oxide and Guanidine Hydrochloride on the Dissociation of Human Cyanmethemoglobin

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Abstract

Dynamic light scattering was used to measure the extent of dissociation of human cyanmethemoglobin (HbCN) $\alpha_2\beta_2$ tetramers into $\alpha\beta$ dimers as a function of HbCN concentration in the presence of varying concentrations of guanidine hydrochloride (GuHCl) and trimethylamine-*N*-oxide (TMAO). It was found that increasing concentrations of GuHCl enhance the dissociation of HbCN, and that GuHCl-induced dissociation is progressively inhibited with increasing concentrations of TMAO. The effects of both cosolutes upon the free energy of HbCN dissociation are shown to be additive. The effect of TMAO on Hb dissociation is largely attributed to steric volume exclusion but is partially compensated by a small attractive interaction between TMAO and the protein.

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Supporting Information: Animations of the 3D figures in Figure 4 rotating around the *z* axis are provided. S1A: Dependence of measured average diffusion coefficient upon the concentrations of HbCN and GuHCl. S1B: Dependence of measured average diffusion coefficient upon the concentrations of HbCN and TMAO in the presence of 0.4 M (red) and 0.8 M (blue) GuHCl. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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INTRODUCTION

Trimethylamine-*N*-oxide (TMAO) is a small organic molecule (MW 75) that is present at high concentration in the blood of sharks and other elasmobranchs.^{1,2} In the pH range from 6 to 8, TMAO is essentially uncharged³ and is of special interest for its ability to preserve protein structure and function under otherwise denaturing conditions.^{4–7} The enhancement of protein stability by TMAO has been attributed to preferential hydration of the protein, which is equivalent to preferential exclusion of TMAO from the immediate vicinity of the solvent-accessible surface of the protein.⁸ We and others have suggested that the preferential exclusion derives primarily from steric repulsion^{9–12} and is therefore relatively nonspecific. Excluded volume theory predicts that the addition of space-filling cosolutes can strongly promote protein association and conversely inhibit protein dissociation.¹³ As a test of this hypothesis, we recently explored the effect of TMAO on the stability of a noncovalent ternary complex of α -chymotrypsin and soybean trypsin inhibitor¹⁴ and found that high concentrations of TMAO do indeed enhance the association of these proteins, even in the presence of subdenaturing concentrations of urea. The present study was conducted to ascertain whether the stabilization of protein complexes by TMAO is general or limited to the particular complex previously studied.

Native human hemoglobin (Hb) exists primarily as a symmetric noncovalent tetramer of two α and two β globin chains. Depending upon Hb concentration and a variety of experimental conditions, the $\alpha_2\beta_2$ tetramer may dissociate to two $\alpha\beta$ dimers, a phenomenon extensively studied for many years as a prototypical ligand-linked protein–protein interaction.^{15,16} Kawahara and Tanford¹⁷ showed long ago that subdenaturing concentrations of urea and guanidine hydrochloride (GuHCl) increase the equilibrium constant for tetramer–dimer dissociation, and under the conditions of their experiments, carboxyhemoglobin (HbCO) could be fully dissociated into dimers without loss of native globin structure in the presence of 1 M GuHCl.

The extent of cyanmethemoglobin dissociation has been monitored by a variety of techniques.^{15,18} In the present study, we chose to monitor dissociation by examining the concentration dependence of the average diffusion coefficient of Hb, as measured by dynamic light scattering (DLS), as described below. The use of a DLS plate reader greatly increased the rate of data collection and enabled a wide variety of conditions to be explored in a reasonable amount of time.

MATERIALS AND METHODS

Human Cyanmethemoglobin (HbCN) Preparation

Packed red blood cells (RBCs) from outdated normal human blood were obtained from the NIH blood bank. About 10 mL of the packed cells was diluted in 20 mL of 0.9% NaCl solution, and centrifuged at 3000g for 15 min. The RBCs were precipitated after the centrifugation and then washed three times with 0.9% NaCl solution. Next, the cells were resuspended in 30 mL of ice-cold pure water to induce lysis by hypoosmotic shock. After 45 min of vigorous stirring, the suspension was centrifuged at 12 000g for 30 min. The supernate was found by size-exclusion chromatography and visible spectrophotometry to

contain essentially pure ferrous oxyhemoglobin, and the concentration was determined from the absorbance at 540 nm ($\epsilon = 11.0 \text{ mM}^{-1} \text{ cm}^{-1}$ per heme¹⁵). Oxyhemoglobin was converted to HbCN by adding 1.2 equiv of potassium ferricyanide and 2 equiv of potassium cyanide per equiv of heme.¹⁹ The protein solution was passed through the Sephadex G-25 desalting columns (17-0851-01, GE, Piscataway, NJ) with phosphate-buffered saline (PBS) as mobile phase to remove unbound ferricyanide and cyanide. Final protein concentration was determined by absorbance at 540 nm. The extinction coefficient is $12.5 \text{ mM}^{-1} \text{ cm}^{-1}$ per heme.¹⁵ Protein was prefiltered through $0.02 \mu\text{m}$ Whatman Anotop filters (Whatman, Germany) before use.

Relative Viscosity Measurement

The time required for 10 mL of aqueous solutions containing different concentrations of GuHCl and TMAO to flow through a capillary tube under the pressure of gravity was measured. The ratio of the flow time of each solution to water is the relative viscosity of the solution, as verified by comparison with concentration-dependent changes of the diffusion coefficient of bovine serum albumin in the solution.

Dynamic Light Scattering Measurement

Buffer solutions containing 0.1, 0.2, 0.4, 0.6, and 1 M GuHCl, and mixtures of 0.4 and 0.8 M GuHCl with 0.76, 1.03, and 1.33 M TMAO were prepared. Serial dilutions of HbCN over a range of concentrations between 10 and 100 μM were prepared in each of these buffers and pipetted into wells of a 384-well black Corning (3450) plate with clear bottom. Four replicate wells of each solution were prepared. After centrifugation at 3000g for 1 min to remove bubbles, DLS data were collected using a Dynapro Plate Reader (Wyatt Technology, Santa Barbara, CA) at 20 °C. The autocorrelation function was acquired 20 times from each well with a 1 s acquisition time.

The averaged scattering intensity autocorrelation function from each well was fit by a user-written MATLAB (Mathworks, Natick, MA) script according to the following equation:²⁰

$$g^{(2)}(\tau) = 1 + \beta e^{-2D_z q^2 \tau} \quad (1)$$

where τ denotes the delay time, β is the amplitude of the correlation function, D_z is the intensity-weighted translational diffusion coefficient, and q is the scattering vector:

$$q = \frac{4\pi n_0}{\lambda_0} \sin\left(\frac{\theta}{2}\right) \quad (2)$$

where n_0 denotes the refractive index of solvent (1.33), λ_0 is the wavelength of incident light (830 nm), and θ is the scattering angle (158°). To compare values of D obtained at different TMAO concentrations, the measured values of D_z were normalized to values in water by multiplying the measured value by the relative viscosity of solvent,²¹ which becomes as large as 1.5 at the highest concentrations of TMAO.

Analysis of the Dependence of Diffusion Coefficient upon the Concentration of HbCN

When HbCN is diluted in the presence of additives, the tetrameric whole protein dissociates into two dimers; under our experimental conditions, the dimers do not dissociate into monomers. The dissociation equilibrium constant K_d is given by:

$$K_d = c_{dim}^2 / c_{tet} \quad (3)$$

and the total concentration of HbCN (tetramer) is given by:

$$c_{tot} = 0.5c_{dim} + c_{tet} \quad (4)$$

Equations 3 and 4 may be solved for the concentrations of c_{dim} and c_{tet} as functions of c_{tot} and K_d . The measured diffusion coefficient is the intensity-average diffusion coefficient:

$$D_z = \frac{\sum_i I_i D_i}{\sum_i I_i} \quad (5)$$

because the scattering intensity of each species (I_i) is proportional to the product of the molar concentration and the square of its molar mass.²¹ In our experiment, the molar mass of the tetramer is twice that of the dimer, so eq 5 reduces to

$$D_z = \frac{c_{dim} D_{dim} + 4c_{tet} D_{tet}}{c_{dim} + 4c_{tet}} \quad (6)$$

where D_{dim} and D_{tet} denote the diffusion coefficients of dimer and tetramer, respectively. Thus given the values of D_{dim} , D_{tet} and K_d , eqs 3, 4, and 6 may be used to calculate D_z as a function of c_{tot} .

RESULTS AND DISCUSSION

The measured intensity-average translational diffusion coefficient of HbCN (D_z) is plotted as a function of total HbCN concentration under various conditions. In Figure 1A, plots are presented for measurements made in the presence of different concentrations of GuHCl specified in the Figure caption. In Figure 1B plots are presented for measurements made in the presence of 0.4 M GuHCl and various concentrations of TMAO specified in the Figure caption. In Figure 1C plots are presented for measurements made in the presence of 0.8 M GuHCl and various concentrations of TMAO specified in the Figure caption. Symbols represent the experimental result (average of four replicates). The solid lines represent the best fit of eqs 3, 4, and 6, where the values of D_{dim} and D_{tet} are constrained to be equal for all solutions and the value of K_d is allowed to float for each solution. The best-fit value of D_{tet} , 6.89×10^{-7} cm²/s, agrees well with literature values;²¹ the best-fit value of D_{dim} is 8.91×10^{-7} cm²/s. Other best-fit parameter values are presented in Table 1.

It may be seen in Figure 1A that in the absence of GuHCl HbCN appears to dissociate only very slightly, even at the lowest concentration of Hb at which we can obtain reliable data. This indicates that the bulk of dissociation occurs at significantly lower Hb concentrations. Thus the data obtained under these conditions cannot be utilized to obtain a reliable estimate

of the dissociation constant and are not taken into account in the following analysis. In Figure 2, the natural logarithm of the best-fit value of the dissociation constant is plotted as a function of the concentration of GuHCl in the absence of TMAO (panel A) and as a function of the concentration of TMAO in the presence of 0.4 M GuHCl and 0.8 M GuHCl (panel B). Plotted for comparison in panel A are the results obtained by Kawahara and Tanford¹⁷ for the GuHCl-induced dissociation of HbCO, indicating that the dissociation of the two ligand states of Hb is similarly affected by GuHCl. It is evident that GuHCl significantly facilitates the dissociation of HbCN into dimeric subunits and that TMAO inhibits the dissociation induced by GuHCl. The logarithm of the dissociation constant appears to be linearly dependent on the concentrations of GuHCl and TMAO, and similar slopes of the dotted lines in Figure 2B seem to indicate that the magnitude of the stabilizing effect of TMAO is approximately independent of the concentration of GuHCl. We therefore write as a first approximation:

$$\ln K_d = -\Delta G_d/RT = -(\Delta G_d^0 + \Delta g_G[\text{GuHCl}] + \Delta g_T[\text{TMAO}])/RT \quad (7)$$

where G_d denotes the standard-state free-energy change of dissociation under the conditions of our experiments, R denotes the molar gas constant, T is the absolute temperature, ΔG_d^0 is the standard state free-energy change of dissociation in the absence of either GuHCl or TMAO, and g_G and g_T are the increments of free-energy change contributed by unit concentration of GuHCl (G) and TMAO (T), respectively. According to this approximation, $\ln K_d$ may be calculated as a function of the concentrations of GuHCl and TMAO with three parameters, G^0 , g_G , and g_T . The dependence of $\ln K_d$ upon the concentrations of GuHCl and TMAO given in Table 1, representing 11 data sets, was modeled using eq 7, and the best fit is plotted together with the data in Figure 3. Agreement between eq 7 and the derived dependence of $\ln K_d$ upon $[\text{GuHCl}]$ and $[\text{TMAO}]$ appears to be within the uncertainty of measurement.

An even more stringent test of this hypothesis is to calculate every measured value of D_z as a function of c_{tot} , $[\text{GuHCl}]$, and $[\text{TMAO}]$ using eqs 3, 4, 6, and 7 and just five parameters, D_{tet} , D_{dim} , G^0 , g_G , and g_T . 80 measured values of D_z were modeled with these four equations, and the combined data are plotted together in Figure 4, with the functions calculated using the best-fit values of the five parameters given in the third column of Table 2. Animations of each of these Figures rotating around the z axis are provided in the Supporting Information (S1). The best-fit values of the five parameters are very close to the values calculated from the previously mentioned “two-step fit”. According to the best-fit parameter values given in Table 2, dissociation induced by the addition of GuHCl may be completely suppressed by the addition of TMAO at a molar concentration approximately four times greater than that of GuHCl.

The hydrodynamic or Stokes radius of dimer and tetramer may be calculated from the respective translational diffusion coefficients according to the Stokes–Einstein relation:

$$r_h = \frac{kT}{6\pi\eta D} \quad (8)$$

where k is the Boltzmann constant, T is the absolute temperature, and η is the viscosity of solution. Using the best-fit values of D_{dim} and D_{tet} obtained from the model, we calculate $r_{\text{dim}} = 2.45$ nm and $r_{\text{tet}} = 3.13$ nm.

For the purpose of making a rough estimate of the excluded volume effect, let us assume that both dimer and tetramer may be represented by spherical particles with radii equal to their hydrodynamic radii. In support of this approximation, we note that the Stokes radius of tetrameric hemoglobin is in reasonably good agreement with the known dimensions of tetrameric hemoglobin.²²

Excluded volume theory^{13,23} predicts that the equilibrium constant for dissociation of a dilute hard spherical tetramer (tet) to two hard spherical dimers (dim) will depend on the concentration of a third hard spherical volume-excluding species (“crowder”, C) according to

$$\ln K_d = \ln K_d^0 + \frac{4\pi N_A}{3} [(r_{\text{tet}} + r_C)^3 - 2(r_{\text{dim}} + r_C)^3] c_C \quad (9)$$

where N_A denotes Avogadro’s number and r_C is the radius of “crowder”. Taking the effective hard spherical radius of TMAO to be 0.27 nm,^{11,12} we use eq 9 to estimate the steric contribution to $d\ln K_d/dc_C$ at around -2.4 . This is ca. 30% larger than the best-fit experimental value of $-g_T/RT = -1.9$, indicating that while the overall inhibiting effect of TMAO upon HbCN dissociation is dominated by steric repulsion, the effect is attenuated by a small amount of attractive interaction between TMAO and the surface of HbCN that tends to promote dissociation.²⁴

Our present finding that TMAO inhibits the dissociation of HbCN, together with our previous finding that TMAO inhibits the dissociation of the α -chymotrypsin–soybean trypsin inhibitor complex,¹⁴ supports the general proposition that the effect of TMAO on protein self-assembly is primarily due to nonspecific volume exclusion but subject to species-specific modulation arising from the presence and magnitude of attractive interactions between TMAO and certain regions of the protein surface.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Dependence of measured diffusion coefficient upon the concentration of HbCN. Symbols are experimental data; curves are the best fit of eqs 3, 4, and 6, as described in the text. (A) Various GuHCl concentrations: 0 (black), 0.1 (red), 0.2 (blue), 0.4 (green), 0.6 (yellow), and 1.0 M (purple). (B) Various TMAO concentrations in the presence of 0.4 M GuHCl: 0 (green), 0.76 (blue), 1.03 (red), and 1.33 M (black). (C) Various TMAO concentrations in the presence of 0.8 M GuHCl: 0.76 (blue), 1.03 (red), and 1.33 M (black).

Figure 2.

(A) Dependence of $\ln K_d$ upon the concentrations of GuHCl ($\bullet$); results of Kawahara and Tanford's¹⁷ measurement of HbCO dissociation as a function of GuHCl concentration (Δ). (B) dependence of $\ln K_d$ upon the concentrations of TMAO in the presence of 0.4 M GuHCl ($\bullet$) and 0.8 M GuHCl ($\square$). Dotted lines indicate the best fit of straight lines to each set of data.

Figure 3.

Dependence of $\ln K_d$ upon the concentrations of GuHCl and TMAO. Surface is calculated according to eq 7 with the best-fit values presented in the second column of Table 2.

Figure 4.

Dependence of measured average diffusion coefficient upon the concentrations of HbCN and GuHCl (A), TMAO in the presence of 0.4 M GuHCl (B), and TMAO in the presence of 0.8 M GuHCl. Surfaces are calculated according to eqs 3, 4, 6, and 7 using the best-fit parameter values presented in the third column of Table 2. Animations of each of these Figures rotating around the z -axis are provided in S1.

Table 1

Best-Fit Values of $\ln K_d$ Obtained by Least-Squares Fitting of Equations 3, 4, and 6 to the Measured Dependence of Diffusion Coefficient upon the HbCN Concentration in the Presence of Additives

TMAO (M)	Gdn (M)	$\ln K_d$					
		0	0.1	0.2	0.4	0.6	0.8
0	-14.54	-12.01	-11.06	-9.45	-8.01		-5.59
0.76				-11.14		-7.76	
1.03				-11.34		-8.30	
1.33				-12.14		-9.19	

Table 2

Best-Fit Parameter Values from the Two-Step Fitting and the Global Fitting

parameters	two-step fit	global fit
$D_{\text{tet}} (\text{cm}^2 \text{s}^{-1})$	6.89×10^{-7}	6.85×10^{-7}
$D_{\text{dim}} (\text{cm}^2 \text{s}^{-1})$	8.91×10^{-7}	8.77×10^{-7}
$G^0 (\text{kcal mol}^{-1})$	7.31	7.17
$g_G (\text{kcal mol}^{-1} \text{M}^{-1})$	-4.28	-4.49
$g_T (\text{kcal mol}^{-1} \text{M}^{-1})$	1.03	1.10