FOR THE RECORD

The failure of simple empirical relationships to predict the viscosity of mixed aqueous solutions of guanidine hydrochloride and glucose has important implications for the study of protein folding

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Abstract: Viscosities of aqueous solutions of guanidine hydrochloride (GuHCl) were measured in the presence of varying amounts of glucose. At high concentrations of glucose or GuHCl, the measured viscosities showed significant deviation from the values computed using a method proposed by Tanford (1966, *J Biol Chem* 241:3228–3232). This method was originally derived to allow the calculation of the effects of buffer or low concentrations of salts and other additives on the density and viscosity of aqueous solutions of urea or GuHCl. Recently it has been used to estimate the viscosity of denaturant solutions that contain high concentrations of viscogens. Our results show that the extrapolation of this approach to solutions of highly concentrated viscous co-solutes leads to significant errors. The implications for experimental studies of the viscosity dependence of conformational transitions in proteins is discussed.

Keywords: glucose; guanidine hydrochloride; protein folding; viscosity

In recent years there has been a resurgence of interest in the viscosity dependence of protein folding (Chrnyk & Matthews, 1990; Waldburger et al., 1996; Creighton, 1997; Jacob et al., 1997, 1999; Plaxco & Baker, 1998; Bhattacharyya & Sosnick, 1999; Jacob & Schmid, 1999; Ladurner & Fersht, 1999). A dependence of folding rates upon viscosity is expected if the rate-determining step for folding is coupled with large motions of the protein chain that involve significant displacement of solvent molecules. Experimental investigations of the viscosity dependence of conformational changes in proteins typically involve the use of small molecule viscogens to vary solvent viscosity. One complication that arises,

particularly in studies of protein folding, is that most viscogens increase the stability of proteins (Arakawa & Timasheff, 1982). An approach for circumventing this problem is to measure the viscosity dependence of folding rates under “iso-stability conditions” (Chrnyk & Matthews, 1990; Jacob et al., 1997; Plaxco & Baker, 1998). This is typically achieved by the addition of varying amounts of urea or guanidine hydrochloride (GuHCl) to compensate for the stabilizing effects of the small molecule viscogen. One of the obstacles to these studies is that no detailed compilation of data has been published for the viscosity of denaturant–viscogen mixtures. There are also no analytical expressions for describing the viscosity of these solutions, and it is known that simple additivity relations are not valid. More than 30 years ago, Tanford proposed a simple method for estimating the viscosity of aqueous solutions of urea or GuHCl that contained buffers or low concentrations of salts or other additives. This approach has been used in some recent studies of the viscosity dependence of folding rates (Plaxco & Baker, 1998). Tanford’s method works well for modest concentrations of co-solutes, but he cautioned that it might not be suitable for all solutions, and it is not clear if the method can be applied to highly viscous co-solutes (Kawahara & Tanford, 1966). In this work, we demonstrate that it is not valid to extend the original Tanford approach to highly viscous solutions of co-solutes. We show that the use of this approach can lead to a significant underestimation of the viscosity, and this, in turn, can lead to significant changes in plots of the inverse of the folding rates vs. the viscosity of solvent.

Results and discussion: The measured viscosities of aqueous solutions of GuHCl and glucose are plotted as a function of denaturant and viscogen concentration in Figure 1A. The measured viscosities of GuHCl solution in the presence of 2.0 M glucose are plotted in Figure 1B, together with viscosities computed using the Tanford method. One obvious feature of the data is the significant nonlinearity in the plots. The strong curvature in the plot makes the

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Fig. 1. A: The measured viscosities of aqueous solutions of GuHCl in the presence of glucose are plotted as a function of GuHCl and glucose. Viscosities of GuHCl solutions in 0 M glucose were taken from Tanford (Kawahara & Tanford, 1966). The mesh plot is included as a visual aid. **B:** The measured viscosities of GuHCl solutions in 2.0 M glucose are plotted together with computed viscosities using the Tanford method. Solid circles are measured values, and the open circles are the predicted values. All measurements were made at 25.0 °C.

prediction of viscosities by simple additivity relationship impossible. The Tanford method works relatively well at low concentrations of GuHCl (<2 or 3 M) and low concentrations of glucose (<1 M) (data not shown). Predicted viscosities, however, show significant deviations from measured values, especially at high concentrations of glucose as shown in Figure 1B. For example, a solution of 5.4 m (3.0 M) GuHCl and 2.0 M glucose has a predicted viscosity that deviates from the actual value by 20%. At 16.5 m (5.8 M) GuHCl and 2.0 M glucose, the deviation is 55%. (Note M is used for molar concentrations and m for molal concentrations.)

The Tanford method for predicting densities and viscosities of mixtures was originally derived using low concentrations of buffer, salts, or alcohols (typically in mM concentrations) in ~2 M to 6 M GuHCl solutions. The prediction of the densities of the multicomponent systems was relatively easy. The relative densities of solutions of urea or GuHCl are well approximated as a linear function of the molar concentrations of denaturant, and the increment in density resulting from multiple solutes is reasonably approximated by a linear additivity relationship. In contrast, the viscosities of an

aqueous solutions of urea or GuHCl are strongly nonlinear with solute concentrations, and linear additivity cannot be used to describe the viscosity of multicomponent systems. The Tanford procedure for the estimation of the viscosity relies on calculation of an effective concentration of GuHCl, m^* , that would give the same viscosity as the solute alone gives in the absence of denaturant. The viscosity of the mixture is then calculated using the apparent concentration of GuHCl, which is a sum of m^* and the actual concentration of GuHCl. For example, the viscosity of a 10.4 m (5.9 M) GuHCl and 40 mM NaCl solution is estimated in the following way. The relative viscosity of an aqueous solution of 40 mM NaCl is 1.012, and a 0.4 m (0.4 M) solution of GuHCl has the same relative viscosity, so $m^* = 0.4$ m. The apparent GuHCl concentration of the mixture is then 10.8 m (10.4 m + 0.4 m). The relative viscosity of the mixture is predicted to be 1.639 using this effective concentration. The actual relative viscosity is 1.627. Although this method works well for low concentrations of co-solutes, a relatively large deviation was reported when the co-solute alone produces a relatively high viscosity in water (Kawahara & Tanford, 1966).

For the mixtures of 2.0 M glucose with various concentrations of GuHCl, part of the deviation probably arises from the long extrapolation of the curved viscosity vs. concentration relation. The effective concentration of a mixture of 5.4 m (3.0 M) GuHCl and 2.0 M glucose is 33.0 m, which is significantly higher than the highest concentration of GuHCl (15.9 m or 7.4 M in water) that was examined. For higher concentrations of GuHCl, even higher effective concentrations of GuHCl will be required and even greater deviations will be observed. The results reported here indicate that the method cannot predict viscosities of the mixtures of GuHCl and glucose under the conditions where effective concentrations of GuHCl exceed experimentally accessible concentrations of GuHCl. Our measurements were made at 25.0 °C. We have made some additional measurements at a higher temperature (33 °C) and also observed that the experimental viscosity is not well predicted by the Tanford approximation.

The results of this study clearly show that the viscosities of solutions of denaturants and viscosogens are not well predicted by the Tanford relationship. It is important to stress that there is no reason that they should be, and indeed, Tanford cautioned about using the empirical procedure for solutions with high concentrations of viscous co-solutes (Kawahara & Tanford, 1966).

Our results have clear implication for viscosity dependent studies of protein folding. We illustrate this point using data collected with the N-terminal domain of L9. We have been using this 56 residue, mixed α - β domain as a model system for studies of folding and stability (Kuhlman et al., 1998, 1999; Sato et al., 1999). Extensive studies have shown that it folds in a two-state fashion. In Figure 2, we plot the inverse of the folding rate k_f^{-1} vs. the solvent viscosity η . The folding rates in Figure 2 were measured under an isostability condition where the Gibbs free energy for unfolding is 3.0 kcal mol⁻¹. For many reactions in condensed matter, such a plot is found to be linear, as predicted by the simple one-dimensional Kramers' model (Kramers, 1940). A number of protein folding reactions have been shown to follow a Kramers' dependence of rate upon viscosity (Waldburger et al., 1996; Jacob et al., 1997, 1999; Plaxco & Baker, 1998; Bhattacharyya & Sosnick, 1999). The open circles show the variation of k_f^{-1} with η using values of η predicted by the Tanford procedure. The solid circles show the dependence of k_f^{-1} with η using the experimentally determined values of η . The plot generated using the pre-

Fig. 2. Plots of the inverse of the folding rate constant k_f^{-1} vs. the solvent viscosity η for the N-terminal domain of L9. Solid circles, k_f^{-1} vs. measured viscosity; open circles, k_f^{-1} vs. viscosity predicted by the Tanford relationship. The predicted and measured values are essentially superimposable below 1.5 cP, and only the open circles are shown for clarity. The line represents a linear regression to the plot generated using the predicted viscosities.

dicted values of η comes closer to following a Kramers' type relationship, while the plot generated using the actual values of η shows clear deviations. This study provides a dramatic example of the problems that arise if predicted viscosities are used instead of actual viscosity and stresses the importance of direct measurements of the viscosity.

Materials and methods: Viscosities of aqueous GuHCl solutions in the presence of 0.25 to 2.0 M glucose were measured in Ostwald-type capillary viscometers with flow times for water of 33 and 87 s. Each measurement was repeated three or four times. Corrections for kinetic energy effects were included. Viscosities of GuHCl solutions in 0 M glucose were taken from Tanford (Kawahara & Tanford, 1966). Viscometers were calibrated against water and pure ethylene glycol. Densities were measured in a pycnometer with a bulb capacity of 25.0 mL. Measurements were made at 25.0 (± 0.1) °C. The temperature was controlled by a constant-temperature water bath. For each glucose concentration, two solutions were prepared volumetrically with an identical concentration of glucose; one contains no GuHCl and the other contains a large amount of GuHCl. The concentrations of solutions of glucose alone were

confirmed by measuring the refractive index. The mixtures with identical concentrations of glucose and varying amounts of GuHCl were prepared by mixing the two solutions with appropriate ratios. The concentrations of GuHCl in the mixtures were determined by measuring the refractive index.

Supplementary material in the Electronic Appendix: The measured viscosities of aqueous GuHCl solutions in the presence of 0.25 to 2.0 M glucose are available in a tab-separated format.

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