Effect of a Concentrated "Inert" Macromolecular Cosolute on the Stability of a Globular Protein with Respect to Denaturation by Heat and by Chaotropes: A Statistical-Thermodynamic Model

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, Bethesda, Maryland 20892-0830 USA

ABSTRACT An equilibrium statistical-thermodynamic model for the effect of volume exclusion arising from high concentrations of stable macromolecules upon the stability of a trace globular protein with respect to denaturation by heat and by chaotropes is presented. The stable cosolute and the native form of the trace protein are modeled by effective hard spherical particles. The denatured state of the trace protein is represented as an ensemble of substates modeled by random coils having the same contour length but different rms end-to-end distances (i.e., different degrees of compaction). The excess or nonideal chemical potential of the native state and of each denatured substate is calculated as a function of the concentration of stable cosolute, leading to an estimate of the relative abundance of each state and substate, and the ensemble average free energy of the transition between native and denatured protein. The effect of the addition of stable cosolute upon the temperature of half-denaturation and upon the concentration of chaotrope required to half-denature the tracer at constant temperature is then estimated. At high cosolute concentration (>100 g/l) these effects are predicted to be large and readily measurable experimentally, provided that an experimental system exhibiting a fully reversible unfolding equilibrium at high total macromolecular concentration can be developed.

INTRODUCTION

The equilibria and rates of a variety of macromolecular reactions, including self- and heteroassociation, condensation, and surface site binding, have been shown to be significantly affected by volume exclusion in solutions of high total macromolecular content, commonly referred to as "crowded" solutions (Minton, 1997; Zimmerman and Minton, 1993). On theoretical grounds, volume exclusion would also be expected to affect macromolecular isomerization in general (Minton, 1981, 1983) and protein denaturation in particular, as an important example of isomerization. Zhou and Hall (1996) (henceforth referred to as ZH) recently presented a statistical-thermodynamic model for the effect of cosolute excluded volume on the denaturation of proteins. According to their model, high concentrations of larger volume-excluding cosolutes would stabilize proteins against denaturation, while high concentrations of smaller cosolutes would destabilize them. The purpose of the present communication is to present an alternative model for the effect of cosolute excluded volume on protein stability, to compare and contrast the results of the new model with that of ZH, and to estimate the magnitude of the excluded volume effect upon experimentally measured parameters of thermal and isothermal denaturation. In the following section we present a simple thermodynamic model for denaturation in nonideal solutions. Next, a molecular structure-based statistical-thermodynamic model is introduced to enable quantitative estimation of the energetic consequences of volume exclusion. Calculations are carried out for a model trace protein undergoing reversible denaturation in the presence of arbitrary concentrations of a second, stable model protein. The effect of volume exclusion on experimentally measurable properties of the model protein solution is estimated, and it is shown that they should be readily measurable. Finally, results of the present model are compared with those obtained from the model of ZH, and the applicability of the present model to the analysis of unimolecular DNA condensation is considered.

THERMODYNAMIC MODEL FOR THE EFFECT OF A COSOLUTE ON THE DENATURATION OF A TEST PROTEIN[1]

Consider a solution containing a test protein at low concentration, together with a single inert cosolute, denoted S, at arbitrary concentration c_s. We model denaturation of the test protein as a reversible transition between a single globular folded or native state and an ensemble of unfolded or denatured states[2]

$$\mathrm{N} \rightleftarrows \mathrm{D}_1 \rightleftarrows \mathrm{D}_2 \rightleftarrows \mathrm{D}_3 \rightleftarrows \ldots,$$

with chemical potentials given by

$$\mu_\mathrm{N} = \mu_\mathrm{N}^0 + RT \ln a_\mathrm{N} = \mu_\mathrm{N}^0 + RT \ln \gamma_\mathrm{N} c_\mathrm{N} \qquad (1)$$

and

$$\mu_{\mathrm{D}_i} = \mu_\mathrm{D}^0 + RT \ln a_{\mathrm{D}_i} = \mu_\mathrm{D}^0 + RT \ln \gamma_{\mathrm{D}_i} c_{\mathrm{D}_i}. \qquad (2)$$

The chemical potential of all denatured states is defined relative to that of a single standard state described below.[3]

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Address reprint requests to Dr. Allen P. Minton, National Institutes of Health, Bldg. 8, Rm. 226, Bethesda, MD 20892. Tel.: 301-496-3604; Fax: 301-402-0240; E-mail: minton@helix.nih.gov.

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Because at equilibrium $\mu_N = \mu_{D_i}$ for all i, we may write the thermodynamic constant for equilibrium between the native and any given denatured species,

$$K^0(T, V) = \exp\left[\frac{-(\mu_D^0 - \mu_N^0)}{RT}\right] = \frac{\gamma_{D_i} c_{D_i}}{\gamma_N c_N}, \quad (3)$$

and define an apparent equilibrium constant,

$$K_i = \frac{c_{D_i}}{c_N} = K^0 \frac{\gamma_N}{\gamma_{D_i}}. \quad (4)$$

Defining the total concentration of denatured protein as the sum over the denatured ensemble, Eq. 4 yields

$$c_D = \sum_i c_{D_i} = \sum_i c_N K_i = c_N K^0 \gamma_N \sum_i \gamma_{D_i}^{-1}. \quad (5)$$

Thus the denaturation model may be regarded as an effective two-state model with

$$K(T, V, \{c\}) = \frac{c_D}{c_N} = \frac{f_D}{(1 - f_D)} = K^0 \frac{\gamma_N}{\gamma_D}, \quad (6)$$

where f_D denotes the fraction of tracer protein that is denatured, as monitored via experiment, and γ_D is an apparent activity coefficient for the denatured "state," given by

$$\gamma_D^{-1} = \sum_i \gamma_{D_i}^{-1}. \quad (7)$$

We may therefore refer to the ensemble of D states as "the D state," keeping in mind that the (ensemble average) properties of the D state so defined are variable. The explicit inclusion of a functional dependence of K on $\{c\}$ serves to remind us that the apparent equilibrium constant, unlike the thermodynamic equilibrium constant, may vary with the concentrations of all solute species present through the composition dependence of γ_N and γ_{D_i}.

STATISTICAL-THERMODYNAMIC MODEL FOR THE DEPENDENCE OF ACTIVITY COEFFICIENTS ON THE CONCENTRATION OF INERT COSOLUTE

According to the McMillan-Mayer theory of solutions (McMillan and Mayer, 1945), the activity coefficient of macrosolute species X may be calculated as functions of the potential of mean force (POMF) acting between a molecule of X and one, two, and higher numbers of other macrosolute molecules of the same and other species in the solution. It has been found that under conditions such that long-range electrostatic interactions between protein molecules are damped out, the POMF acting between native, globular protein molecules is well approximated by a hard particle potential acting between rigid convex bodies, the size and shape of which resemble those of the actual molecule viewed at low resolution (Minton, 1983; Minton and Edelhoch, 1983; Ross et al., 1978; Ross and Minton, 1977). Hard particle models have been found to account quantitatively for the dependence of the thermodynamic activity of compact globular proteins upon solute concentrations up to several hundred grams per liter (Minton, 1983, 1995; Minton and Edelhoch, 1983; Ross et al., 1978). Therefore, for the purpose of the model calculations to follow, we shall represent both the inert cosolute molecule and the native state of the test protein as rigid spheres of fixed radii r_s and r_N, respectively.[4] Denatured states of the test protein will be represented as random coils of varying extent of compaction, either without internal excluded volume (a Brownian walk) or with internal excluded volume (a self-avoiding walk). This model is depicted schematically in Fig. 1.

Activity coefficient of N

The excess chemical potential of a rigid macrosolute species 1 at limiting low concentration, in a solution containing an arbitrary concentration of macrosolute species 2, may be calculated in the hard particle approximation, using the following relation obtained from the scaled particle theory of hard particle fluid mixtures (Boublík, 1974):

$$\ln \gamma_1 = \frac{\Delta A_1}{kT} = -\ln(1 - \rho_2 V_2) + [H_1 S_2 + S_1 H_2 + V_1] \frac{\rho_2}{(1 - \rho_2 V_2)} + [H_1^2 S_2^2 + 2 V_1 H_2 S_2] \frac{\rho_2^2}{2(1 - \rho_2 V_2)^2} + [V_1 H_2^2 S_2^2] \frac{\rho_2^3}{3(1 - \rho_2 V_2)^3}, \quad (8)$$

where ΔA_i is the change in the (Helmholtz) free energy of the system associated with the introduction of a molecule of species 1, k is Boltzmann's constant, ρ_2 denotes the number

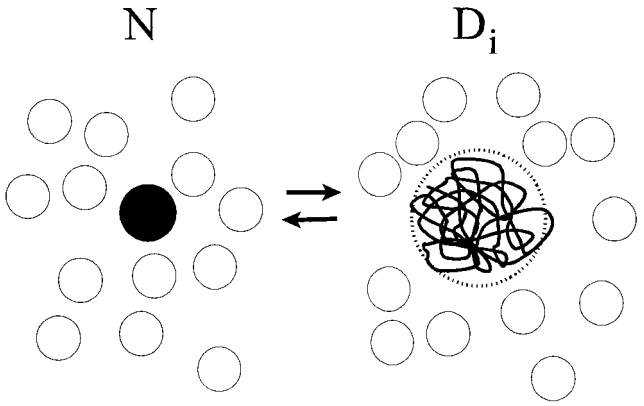

FIGURE 1 Schematic depiction of the equilibrium between the native state N of the tracer (*shaded sphere*) and the unfolded state D_i (*coil*) in the presence of stable cosolute S (*open spheres*). The dashed border around the coil indicates the time-averaged convex hull of the Brownian walk representing the coil, assumed to define an effective hard particle excluding volume to molecules of S.

density of species 2 (proportional to w/v concentration w_2), and H_i, S_i, and V_i denote, respectively, the Kihara supporting function (Kihara, 1953), surface area, and volume of the equivalent convex particle representing species *i*. Because both N and S are represented by equivalent hard spheres, ln γ_N is given by Eq. 8 with $H_1 = r_N$, $S_1 = 4\pi r_N^2$, $V_1 = 4\pi r_N^3/3$, $H_2 = r_s$, $S_2 = 4\pi r_s^2$, and $V_2 = 4\pi r_s^3/3$ (Minton, 1998).

Activity coefficient of D_i

The *i*th denatured "state" is treated as a Brownian or self-avoiding walk with a rms end-to-end distance h_i. We define the standard state of all D_i to be the isolated walk (i.e., solvated, but without intersolute interaction) with a rms end-to-end distance h_0, at unit concentration and standard temperature and pressure. To a first approximation, the nonideal contribution to the chemical potential (or excess chemical potential) of tracer may be partitioned into a term deriving from intermolecular interaction between D_i and S, and a term deriving from the configurational free energy of the isolated molecule of D_i with fixed center of mass:

$$RT \ln \gamma_{D_i} = \mu_{D_i}^{NI}(c_S, h_i) = \mu_{D_i}^{NI,intermol}(c_S, h_i) + \mu_{D_i}^{NI,conf}(h_i). \quad (9)$$

The magnitude of $\mu_{D_i}^{NI,intermol}$, the equilibrium average excess free energy arising from repulsive interactions between a molecule of D_i and all molecules of S, may be approximated by Eq. 8, where the effective particle representing species 1 is the convex hull of the Brownian walk, with $H_1 = (2/3\pi)^{1/2}h_i$, $S_1 = (2\pi/3)h_i^2$, $V_1 = 4(2\pi/3)^{1/2}h_i^3/27$ (Jansons, 1991; Jansons and Phillips, 1990), and H_2, S_2, and V_2 are as given above.[5]

Because the standard states of all D_i are defined as the unperturbed random coil with rms end-to-end distance h_0, $\mu_{D_i}^{NI,conf}$ represents the difference between the equilibrium average free energies of isolated random walks of identical contour length, constrained to rms end-to-end distances h_i and h_0, respectively. It arises from differences in configurational entropy and, if present, intramolecular excluded volume. The magnitude of $\mu_{D_i}^{NI,conf}$ is estimated as follows. Jaeckel and Dayantis (1994a,b) have carried out Monte Carlo calculations of the effect of confinement in a spherical volume of radius *R* upon the rms end-to-end distances of Brownian and self-avoiding walks and upon the entropy per unit volume of the walks. Their results may be expressed in units that are scaled to the rms end-to-end distance of the unrestricted walk h_0. Letting $\lambda = R/h_0$ and $\eta = h/h_0$, the effect of compression on the rms end-to-end distance of the walk is well described by the empirical relation

$$\eta^{-1} = 1 + P_1\lambda^{-1} + P_2\lambda^{-2} + P_3\lambda^{-3} + P_4\lambda^{-4} \quad (10)$$

over the range $0.4 \le \lambda \le 4$, where $P_1 \ldots P_4$ are equal to 0.072713, 0.045978, 0.25152, and −0.062739, respectively, for a Brownian walk, and 0.091936, −0.17012, 0.33811, and −0.076614, respectively, for a self-avoiding walk. The entropy of the walk, relative to that for the unconstrained walk (corrected for change in volume of the confining sphere, hence appropriate for a fixed center of mass), is well represented by the empirical function

$$-\Delta S/k = Q_1 + Q_2\lambda^{-2} - (Q_1 - Q_4)\exp(-\lambda^{-2}/Q_3) \quad (11)$$

over the range $0.4 \le \lambda \le 4$, where $Q_1 \ldots Q_4 = 0.88087$, 1.6725, 1.1652, and 0.3211 for a Brownian walk, and approximately the same for a self-avoiding walk. Assuming that the compression is essentially athermal, one may calculate the dependence of $\mu^{NI,conf}/kT = -\Delta S/k$ on η from Eqs. 10 and 11; this relationship is plotted in Fig. 2. As expected, the self-avoiding walk is less compressible than the Brownian walk because of the presence of intramolecular excluded volume.

Equations 4 and 9 may be combined to yield the probability that a molecule of denatured protein will be present in state D_i at any particular concentration of cosolute S:

$$p_i(\eta_i, c_S) = \frac{\exp[-\mu_{D_i}^{NI}(c_S, \eta_i)]}{\sum_j \exp[-\mu_{D_j}^{NI}(c_S, \eta_j)]}. \quad (12)$$

A "REALISTIC" SIMULATION OF THE EQUILIBRIUM DENATURATION OF A LABILE PROTEIN IN THE PRESENCE OF AN INERT STABLE PROTEIN

As an illustration of the magnitude of excluded volume effects one may expect to observe experimentally, we calculate the effect of the addition of large concentrations of a model protein with the approximate size and shape of ribo-

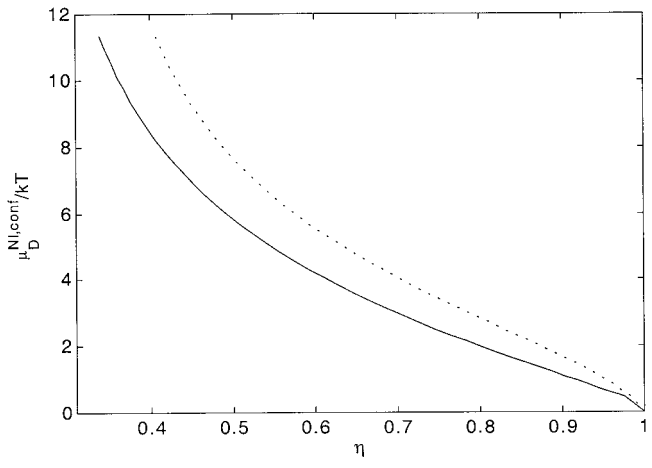

FIGURE 2 The excess chemical potential of an isolated molecule of tracer in state D_i, plotted as a function of η_i. The solid line is calculated for a Brownian walk representation of D_i, the dashed line for a self-avoiding walk representation.

nuclease A (M = 13,000) upon the native-denatured equilibrium of a second, dilute model protein with the approximate size and shape of α-lactalbumin (M = 17,000). The native states of both RNAse A and α-lactalbumin are compact and quasispherical, the maximum dimension of each being no greater than ~1.5 times the minimum dimension (Acharya et al., 1991; Tilton et al., 1992). For purposes of calculating excluded volume, such bodies may be well represented by an equivalent hard sphere (Minton, 1998). The volume of an equivalent convex particle representing a protein is assumed to be equal to the volume of solvent displaced by the protein, which is given by the partial specific or partial molar volume. Thus equivalent hard sphere radii r_S and r_N are evaluated using

$$r_X = \left(\frac{3M_X\bar{v}_X}{4\pi N_A}\right)^{1/3}, \qquad (13)$$

where M_x and $\bar{v}_x$ denote the molar mass and partial specific volume of X, and N_A denotes Avogadro's number. If it is assumed that $\bar{v}_N = \bar{v}_S \approx 0.72$ cm^3/g, a value typical of most compact globular proteins (see Appendix 2 of Attri and Minton, 1983), then r_S = 1.54 nm and r_N = 1.72 nm. The rms end-to-end distance of the unperturbed random coil representing the standard state for all D_i, h_0, is taken to be 7.3 nm = $\sqrt{6}r_g$, where r_g is the experimentally measured radius of gyration of fully denatured α-lactalbumin, 3 nm (Kataoka et al., 1997).

The value of $\ln \gamma_{D_i} = \mu_{D_i}^{NI}/kT$, calculated for both Brownian and self-avoiding walks, using Eqs. 8–11, is plotted in Fig. 3 as a function of $\eta_i = h_i/h_0$ for values of w_s, the w/v concentration of S, ranging between 0 and 400 g/l. The value of $p_i(h_i, c_s)$ was calculated for a high density of discrete states with $0.35 \le \eta \le 1.0$, using Eq. 12 and the results shown in Fig. 2. In the limit of high state density, the value of $p_i(\eta_i, c_s)$ approaches that of the continuous probability distribution $p(\eta, c_s)d\eta$, which is plotted as a function of η and w_s for both Brownian and self-avoiding walks in Fig. 4.[6] Calculated distributions for Brownian and self-avoiding walks do not differ qualitatively, and the quantitative differences between them reflect the greater ease of compression of the Brownian walk noted earlier. As the concentration of S increases above ~200 g/l, the most probable value of η begins to decrease from 1 toward a value of 0.55–0.6 at c_s = 400 g/l.

γ_D was calculated for both Brownian and self-avoiding walks according to Eq. 7. The dependence of log γ_N and log γ_D upon w_s is plotted in Fig. 5. While the addition of S destabilizes both N and D in an absolute sense (as would be expected in the case of pure volume exclusion interactions), the more compact N is stabilized relative to the less compact

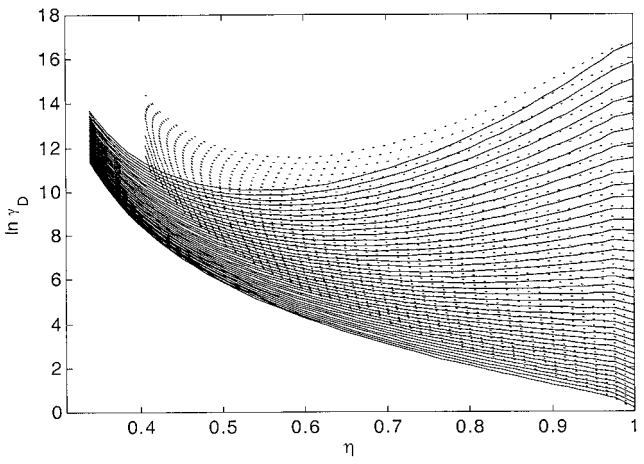

FIGURE 3 The excess chemical potential of D_i plotted as a function of η_i for varying concentrations of stable cosolute S. Solid lines are calculated for a Brownian walk representation of D_i, dashed lines for a self-avoiding walk representation of D_i. Each family of curves represents the calculated dependence for w_s = 0, 10, 20, . . . , 400 g/l from bottom to top.

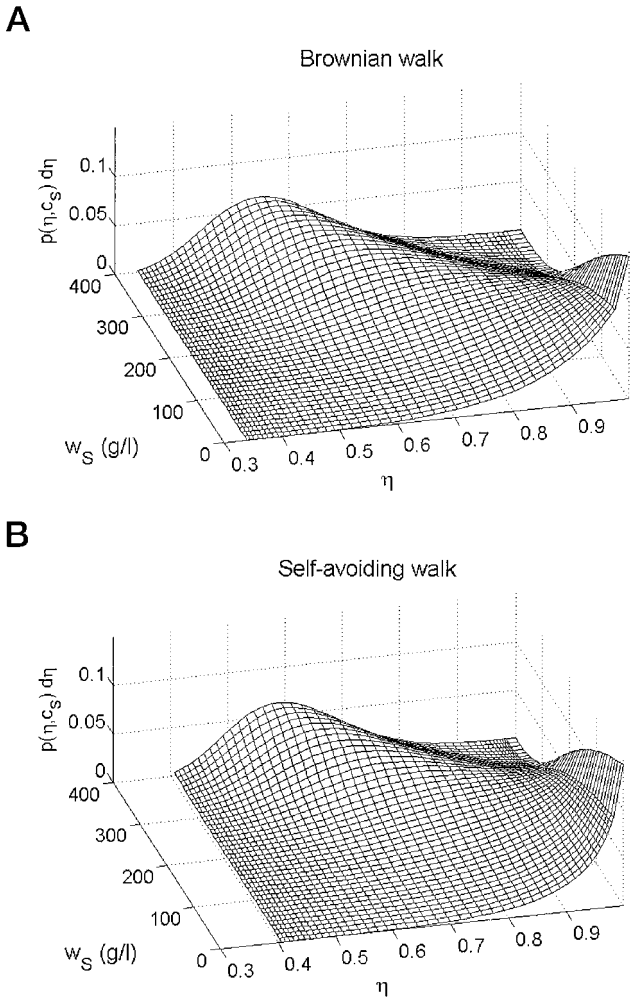

FIGURE 4 Normalized probability distribution of states of D with normalized rms end-to-end distance η at fixed w_s, plotted as a function of η and w_S. Calculations are performed for D represented as a Brownian walk (*A*) and as a self-avoiding walk (*B*).

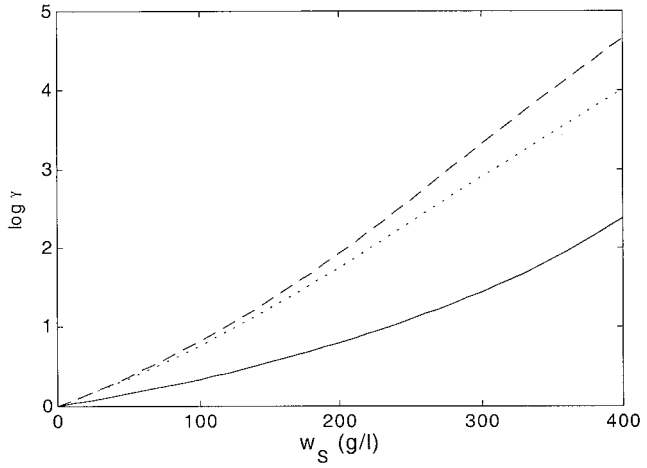

FIGURE 5 Common logarithm of the activity coefficient of tracer in the native state N (*solid line*) and the average denatured state D (Brownian walk representation, *dotted line*; self-avoiding walk representation, *dashed line*) plotted as a function of the concentration of stable cosolute S.

ensemble of D states. The dependence of $\log(K/K^\circ)$ upon w_s, calculated according to Eq. 6, is plotted in Fig. 6.[7]

The basic qualitative conclusion derived from this calculation is that the introduction of sufficiently large concentrations of a stable globular protein of molar mass comparable to that of a labile trace protein can reduce the equilibrium constant for denaturation of the labile protein by between one and two orders of magnitude. Although the specific model presented here is simplified and heuristic rather than rigorous, particularly with respect to treatment of the denatured ensemble, the major excluded volume contributions seem to be taken into account in a reasonable fashion. Thus we believe that the predicted order of magnitude of the excluded volume effect is likely to be correct.

ESTIMATE OF THE MAGNITUDE OF THE EFFECT OF VOLUME EXCLUSION ON THERMAL DENATURATION

Within the context of the effective two-state model for denaturation (Eq. 6), the dependence of the equilibrium constant for unfolding upon temperature may be described by the van't Hoff equation,

$$\frac{\mathrm{d}\ln K}{\mathrm{d}T^{-1}} = \frac{-\Delta H^\circ}{R}, \tag{14}$$

where ΔH° represents the standard state enthalpy change for the transition N $\rightarrow$ D. Consider an interval of temperature within which ΔH° is approximately independent of temperature. It follows from Eq. 14 that an isothermal change in the equilibrium constant $\Delta\log K$ at any temperature within this region will result in a change in T_{50}, the temperature at which the protein is half-denatured ($f_D = 0.5$, $K = 1$), given by

$$\Delta T_{50}^{-1} = 2.303\,\frac{R}{\Delta H^\circ}\,\Delta\log K. \tag{15a}$$

Equation 15a may be generalized to the case of a temperature-dependent ΔH°:

$$\frac{1}{T_{50}^{(2)}} - \frac{1}{T_{50}^{(1)}} = 2.303R\int_{\log K^{(1)}}^{\log K^{(2)}} \frac{d\log K}{\Delta H^\circ[T_{50}(\log K)]}, \tag{15b}$$

where ΔH° is calculated as a function of temperature and heat capacity change ΔC_p via the thermodynamic relation

$$\Delta H^\circ(T) = \Delta H^\circ(T_0) + \Delta C_p(T - T_0). \tag{15c}$$

The dependence of T_{50} on $\Delta\log K$, calculated using Eqs. 15b, c for various values of ΔH° and ΔC_p over ranges spanning the majority of measured enthalpy and heat capacity changes for protein denaturation (Pfeil, 1986), is plotted in Fig. 7. It may be seen that when K is reduced by one to two orders of magnitude, T_{50} is predicted to increase by 5–-20°C.[8]

ESTIMATE OF THE MAGNITUDE OF THE EFFECT OF VOLUME EXCLUSION ON ISOTHERMAL DENATURATION BY CHAOTROPES

The dependence of protein denaturation on chaotrope concentration may best be illustrated by an example taken from the literature. Ogasahara et al. (1993) have reported the fraction of tryptophan synthase α-subunit denatured (f_D) as a function of urea concentration at constant temperature. By means of Eq. 6, these data may be converted into the relation between log K and urea concentration plotted in Fig. 8 *A*.[9]

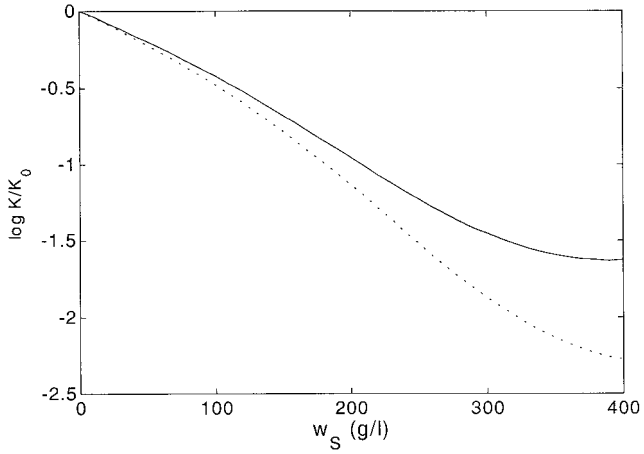

FIGURE 6 Dependence of K, the equilibrium constant for unfolding, upon the concentration of stable cosolute S, calculated for two representations of the average denatured state (Brownian walk, *solid line*; self-avoiding walk, *dotted line*).

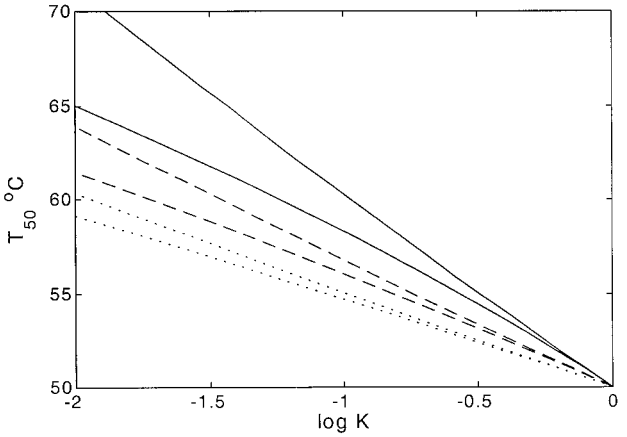

FIGURE 7 Dependence of the temperature of half-denaturation upon the change in K at constant temperature induced by excluded volume, calculated for three values of ΔH° (*solid line*, 200 kJ/mol; *dashed line*, 300 kJ/mol; *dotted line*, 400 kJ/mol). The upper curve of each line type was calculated using Eqs. 15a–c with $\Delta C_p = 0$, and the lower curve was calculated with $\Delta C_p = 10$ kJ/mol-°C.

We define c_{50} to be that concentration of urea at which the protein is half-denatured ($f_D = 0.5$, $K = 1$). Consider the following process. To a solution of labile tracer protein containing stable inert protein S at an initial concentration c_s^{initial}, urea is added to a concentration c_{50}^{initial}. Now a quantity of stable inert protein is added, increasing the concentration to c_S^{final}. The increase in c_s may either stabilize or destabilize the tracer protein; if it stabilizes the tracer, f_D will decrease to a value less than 0.5, and if it destabilizes the tracer protein, f_D will increase to a value greater than 0.5. Now the concentration of urea is adjusted, either upward or downward, to regain the initial state of half-denaturation. Let the new concentration of urea be denoted c_{50}^{final}. Because the protein is once again half-denatured, the total free energy change associated with the addition of stable protein and adjustment of urea concentration must be zero. If we assume that the influences of urea and inert protein are energetically independent, then it follows that $\Delta(\Delta G^\circ_{N\to D})_s$, the change in standard state free energy of denaturation arising from addition of S, must be equal and opposite in sign to $\Delta(\Delta G^\circ_{N\to D})_{\text{urea}}$, and

$$\Delta\log K(c_S^{\text{initial}} \to c_S^{\text{final}}) = -\Delta\log K(c_{50}^{\text{initial}} \to c_{50}^{\text{final}}). \qquad (16)$$

Thus by simple inversion of sign, the results shown in Fig. 8 *A* may be converted to those shown in Fig. 8 *B*, which reveal that the decrease in K of between one and two orders of magnitude expected to result from the excluded volume of large concentrations of S would result in an increase of c_{50} of at least 0.5 M and possibly as much as several M.

COMPARISON WITH THE MODEL OF ZHOU AND HALL

The model presented differs from that of ZH (Zhou and Hall, 1996) primarily in the treatment of the denatured state of the tracer protein. In the present model, the denatured state is assumed to exclude volume to the hard-sphere cosolute as would the convex hull of a Brownian walk, while in the model of Zhou and Hall, the denatured state is assumed to exclude volume to a hard-sphere cosolute as would a chain of tangent hard spheres. The present model is limited to consideration of the effect of hard sphere cosolutes with a mass comparable to or larger than that of the tracer protein, so that the denatured state and the hard sphere cosolute cannot significantly interpenetrate. In this regime, the effect of the hard sphere cosolute is to stabilize the native state relative to the denatured state.

Zhou and Hall's claim that small cosolutes preferentially stabilize the denatured state derives from a severely unphysical assumption regarding the size (volume) of the denatured state. It should be clear from the definition of the potential of mean force (McMillan and Mayer, 1945) that to the extent that the actual potential of average force between solute molecules resembles a hard particle interaction (i.e., consisting of a short-range steric repulsion, without significant attraction or long-range repulsion), the size and shape of an equivalent hard particle representation of a particular macrosolute species should resemble those of the actual molecule at low resolution. Significant attraction or long-range repulsion would result in apparent dimensions of the effective hard particle best reproducing the behavior of the actual macrosolute that are significantly smaller or larger, respectively, than actual molecular dimensions (Minton, 1983, 1995; Minton and Edelhoch, 1983). Because ZH state that the purpose of their model is to investigate the role of excluded volume interactions only, and not other sources of intermolecular interaction, it follows that in the context of such a model, the volume of a hard particle representation of a macrosolute is not a freely variable parameter and must agree with available experimental information regarding the volume of the macrosolute being modeled.

According to the dimensions published in table II of ZH, the model for the denatured state of lysozyme has a volume that is 0.22 times that of the corresponding native state model, and the model for the denatured state of bovine pancreatic trypsin inhibitor has a volume equal to 0.30 times that of the corresponding native state model. Actual solvent-excluded volume changes accompanying protein unfolding may be measured directly (Katz et al., 1973a,b) or calculated from the pressure dependence of the unfolding process (Brandts et al., 1970; Zipp and Kauzmann, 1973). Volume changes for typical globular proteins undergoing unfolding range between ~0 and −50 cm^3/mol for ribonuclease A (Brandts, et al., 1970) and between −50 and −115 cm^3/mol for metmyoglobin (Katz et al., 1973; Zipp and Kauzmann, 1973), depending upon conditions (temperature, pH, etc.). These volume changes represent a fractional change in solvent-excluded volume upon unfolding of less than −12%. If the volume of the denatured state is constrained to be no smaller than 12% less than that of the corresponding

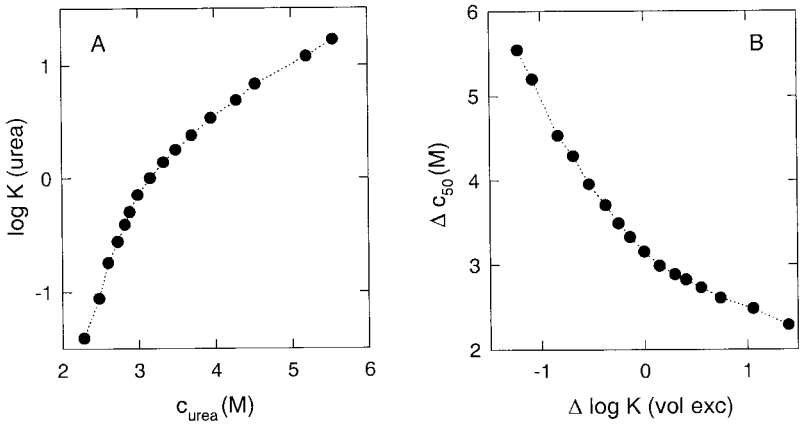

FIGURE 8 (*A*) Dependence of K for the unfolding of the α-subunit of tryptophan synthase upon urea concentration at constant temperature, obtained by transformation of the data of Ogasahara et al. (1993) as described in text. (*B*) Change in the urea concentration of half-denaturation compensating for change in K resulting from volume exclusion, calculated as described in text.

native state, then the model of Zhou and Hall predicts only stabilization of the native state, as was recognized by the authors.[10]

Even in the large cosolute regime, excluded volume effects predicted by the ZH model differ qualitatively from those predicted by the present model. According to figure 3 of ZH, addition of a stable solute comparable in size to the native state of bovine pancreatic trypsin inhibitor may decrease K for unfolding of bovine pancreatic trypsin inhibitor (BPTI) by a factor as large as 10^9.[11] In contrast, the present model predicts a maximum reduction of K by a factor of 10–100. We believe that this large discrepancy results, at least in part, from a fundamental defect in the theory utilized by ZH to calculate the chemical potential of the denatured state in the presence of hard sphere cosolutes.

According to thermodynamic perturbation theory (Ghonasgi and Chapman, 1994) as implemented by ZH, the excess free energy of a molecule modeled as a chain ("necklace") of n tangent hard spheres of radius r_1 ("beads") in a fluid of uniform hard spheres of radius r_s is calculated as the sum of two contributions. The first is the free energy change associated with the placement of n individual beads in the fluid at a great enough distance from each other that they do not interact with each other, which is just n times the excess free energy of placing a single bead in the fluid. The second contribution is the free energy change associated with forming the necklace from the n isolated beads within the hard sphere fluid. This is calculated as $n - 1$ times the free energy of bringing two beads from an infinite distance apart to the contact distance, i.e., a center-to-center distance of $2r_1$, within the fluid. It is the calculation of the second contribution that is flawed in principle. Upon closer inspection one finds that at no point is the entire necklace treated. The only species for which calculations are carried out are the individual beads and bead doublets, or "dumbbells." At no point are correlations between more than two beads considered. Hence the theory used by ZH contains no specification of, or information regarding, chain conformation. It is incapable *in principle* of treating the relationship between chain conformation, configurational entropy, and either intra- or intermolecular excluded volume, factors that are explicitly treated in the present model and shown to be essential determinants of the chemical potential of the denatured state.

COMPARISON WITH EXPERIMENT

The model presented here predicts that excluded volume in concentrated solutions of stable "inert" macrosolutes may serve to stabilize the globular native state of proteins against unfolding by preferentially destabilizing the unfolded state (or ensemble of unfolded states). The model further predicts that the average dimension of the unfolded state will decrease with increasing concentration of inert macrosolute. The magnitude of the changes in experimental observables is predicted to be easily measurable. Why then have such effects not yet been reported in the literature?

The investigation of excluded volume effects in concentrated protein solution is complicated by the likely presence of direct intermolecular interactions in addition to excluded volume interactions, the more so as volume exclusion in concentrated solution is predicted to magnify the consequences of weak interactions that would be negligible in dilute solution (Minton, 1981, 1983; Zimmerman and Minton, 1993). Moreover, the unfolding of a protein exposes hydrophobic residues normally sequestered in the interior of the native protein; such exposure increases the likelihood of interaction of the denatured state of the unstable protein with nonpolar residues on the surface of the stable protein cosolute. In this laboratory we have attempted to characterize the thermal denaturation of individual proteins in mixtures but have repeatedly observed that an unfolding transition that is reversible in dilute solution becomes increasingly irreversible as the concentration of "stable" cosolute protein increases. Such complications make it difficult to test a theory, such as the one presented here, that is based upon the assumption of reversible equilibrium and

treats only one of several possible types of interactions that may be present in any real protein solution.

However, there is a well-studied model system that is in several respects comparable to the native-denatured transition in proteins and may be free of some of the complications noted above. This is the unimolecular condensation of large double-helical DNA, which is known to undergo a collapse to a compact (probably toroidal) form when a sufficient fraction of the negative charge of the phosphate groups is neutralized via interaction with multivalent cations (Bloomfield, 1996, 1997; Minagawa et al., 1991). The effect of an inert nonionic polymer, PEG-10K, on this transition has been studied by Kidoaki and Yoshikawa (1999), who report two interesting phenomena: 1) With increasing concentrations of polyethylene glycol (PEG), the degree of charge neutralization required to attain a condition of half-condensation is lessened. 2) With increasing concentrations of PEG, the average size of uncondensed DNA decreases. Observation 1 indicates that both increasing charge neutralization and increasing PEG concentration stabilize the condensed form of the DNA relative to the open, random-coil form. Hence a condition of 50% condensation ($K = 1$ in the context of the effective two-state model) may be achieved either with greater charge neutralization and a lower concentration of PEG, or by less charge neutralization and a higher concentration of PEG. This observation qualitatively accords with the prediction of the model presented here with respect to isothermal denaturation of proteins by chaotropes, where the addition of chaotrope is energetically equivalent to the removal of a charge-neutralizing ligand. Observation 2 indicates that increasing concentration of the inert cosolute weights the ensemble of uncondensed conformations in favor of more compactly coiled states, as predicted by the model presented here. The analogy between protein folding and unimolecular DNA condensation is of course limited, and a more detailed analysis of the effect of excluded volume on unimolecular DNA condensation is in progress.

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Note added in proof: It has recently come to the author's attention that the temperature at which a characteristic change in intrinsic fluorescence associated with the thermal denaturation of actin is observed was reported to be increased by *ca* 5°C in the presence of 100 g/1 PEG-6000 (Tellam et al, 1983). This effect, similar in magnitude to that predicted by the model presented here (see Figures 6 and 7), was qualitatively attributed by the authors to stabilization via excluded volume of the native state relative to the denatured state.

Endnotes

1. The theory presented here is formally applicable to the constant volume system. Because the actual volume change associated with the unfolding of trace concentrations of a labile protein is miniscule (see below), the numerical results derived therefrom should be semiquantitatively applicable to the constant pressure (laboratory) system.
2. The "linear" diagram presented here has no mechanistic or kinetic implications and is only meant to indicate the presence of equilibria between all species.
3. The use of a single standard state in the definition of the chemical potential of all denatured states does not reflect an assumption of energetic equality of the various denatured states. Instead, it indicates an *arbitrary assignment* of energetic differences between these states to the nonideal part of the chemical potential, manifested in the uniquely defined activity coefficient of each denatured state. The utility of this particular assignment will become evident with the introduction of a specific model for the denatured states in the following section.
4. It is assumed that the volume-excluding cosolute is more stable than the test protein and does not undergo a conformational change under conditions promoting denaturation of the test protein.
5. Because of this approximation, the present treatment is limited to globular cosolutes with molar mass comparable to that of the tracer species; i.e., the molecules are sufficiently large that they do not penetrate into the spatial domain of the random walk.
6. The shape of the plotted probability distribution in the vicinity of $\eta = 1$ is an artifact resulting from truncation of the calculated distribution at $\eta = 1$. In reality, denatured states with $\eta > 1$ do exist, but the calculations of Jaeckel and Dyantis (1994a,b) provide no information on their relative entropies (free energies). It may be readily shown that these states become rapidly depopulated upon the addition of stable solute S because of the large increase in the value of $d\mu_{D_i}^{NI,intermol}/dw_s$ with increasing η_i. Thus neglect of denatured states with $\eta > 1$ will not qualitatively affect our estimates of free energy changes accompanying the addition of S.
7. Although the calculated values of the activity coefficients of the individual N and D species are quite sensitive to the assumed radius of the hard sphere model cosolute, the ratio of calculated activity coefficients is far less so. Thus, for a fixed value of w_s, the absolute value of the slope of the dependence of $\log(K/K^\circ)$ on w_S increases by 10% or less per Å increase in the radius of the hard sphere model cosolute. Hence order-of-magnitude estimates of the effect of excluded volume on the stability of the native relative to the unfolded state will not be affected by a slight uncertainty in the assignment of an effective hard sphere radius to a globular macromolecular cosolute.
8. An additional consequence of Eq. 15 is that in the case of cold denaturation (Privalov, 1990), where the sign of the enthalpy change is negative rather than positive, volume exclusion would be expected to decrease rather than increase T_{50}.
9. For the purpose of this illustrative calculation, it is assumed that tryptophan synthase α-subunit undergoes an effective two-state N $\rightarrow$ D transition, i.e., that intermediate states do not constitute a substantial fraction of total protein at equilibrium. Deviations from strict two-state behavior are not expected to alter the qualitative conclusions.
10. The same result holds for any excluded volume model that models the denatured state as a markedly aspherical hard particle of volume approximately equal to that of the quasispherical native state, as was originally shown by Minton (1981).
11. This result was obtained using a severe underestimate of the actual volume of the denatured state of BPTI, as mentioned above. If the volume of the denatured state had been assumed to be roughly equal to that of the native state, the extent of stabilization calculated by ZH would have been substantially larger.

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