Viscosity Dependence of the Folding Kinetics of a Dimeric and Monomeric Coiled Coil†

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ABSTRACT: We measured whether solvent viscosity, and hence chain diffusion, plays a role in the rate-limiting step of the folding reactions of GCN4-p2′, a simple α-helical coiled coil derived from the leucine zipper region of bZIP transcriptional activator GCN4. To deconvolute the dual effects of viscosogenic solvents on both viscosity, η, and stability, earlier attempts assumed that the cosolvent and denaturant interact to the same degree in the transition state. Applying this analysis to GCN4-p2′ yielded a nearly $1/\eta$ dependence between folding rates and viscosity for both the dimeric and the cross-linked, monomeric versions of the coiled coil, but it revealed no such coherent relationship for cytochrome *c*. We also developed a method to determine the relative viscosity dependence of the dimeric and monomeric forms of the coiled coil independent of the assumption concerning the transition state's relative interaction with cosolvents and denaturants. Application of this method indicated that the effect of viscosity on both the folding and the unfolding rates was the same for the dimeric and monomeric versions, further supporting the view that the folding of the dimeric version is folding-limited rather than encounter-limited. The finding that GCN4-p2′ folding appears to exhibit a $1/\eta$ viscosity dependence implies that the rate-limiting step in folding is opposed predominantly by solvent-derived rather than internal frictional forces. These results are interpreted in relation to various models for protein folding.

Does the rate of protein folding depend on the speed at which the polypeptide chain diffuses around in solution? The general issue of the diffusion dependence of reaction rates for activated processes in solution was first treated extensively by Kramers (*1*) and subsequently by others (*2*−*4*). Kramers proposed that diffusion plays a role in activated processes, and that reaction rates are inversely proportional to solvent viscosity, η, for flat barrier crossings in the high friction limit (which includes aqueous solvents). Based upon this theory, protein folding reactions limited by diffusional events through solution are likely to exhibit a $1/\eta$ or unitary viscosity dependence, while those reactions limited by internal rearrangements should be independent of solvent viscosity.

A recent simulation of off-lattice protein folding utilizing Langevin dynamics found the folding rate for a two-state folding model to be proportional to $1/\eta$ at solvent viscosities near that of water (*5*). However, experimental determination of the viscosity dependence of protein folding has proven more challenging than expected (*6*). Generally, the test involves comparing the change in folding rate with the change in solvent viscosity mediated by the addition of viscosogenic agents, typically glycerol, ethylene glycol, sucrose, glucose, or other polyhydroxylated molecules. Complications arise in such studies because these viscosogenic agents modulate protein stability as well as solvent viscosity, and these two effects are difficult to separate experimentally. A priori, a change in the kinetic prefactor, attributable to a change in viscosity, is kinetically indistinguishable from a change in the absolute barrier height, attributable to transition state stabilization. For example, even assuming a Kramers-like $1/\eta$ viscosity dependence, folding rates may still increase upon the addition of a viscosogenic agent if the acceleration due to the stabilization of the transition state exceeds the deceleration due to the increase in viscosity.

Early attempts at determining the viscosity dependence of folding failed to account for this stabilizing effect of the viscosogenic cosolvent (*7*). Other studies were performed on slow folding systems (*7*, *8*) in which the rate-limiting process represented the rearrangement of misfolded structures in a multistate refolding process rather than any intrinsic folding step that limits the two-state folding behavior now observed in numerous small proteins (*9*).

Recently, several other groups have attempted to deconvolute stability and viscosity effects by comparing folding rates at points of equivalent protein stability in different cosolvents (*8*, *10*, *11*). This analysis yielded a nearly $1/\eta$ viscosity dependence, but it relied upon the assumption that the viscosogenic cosolvent and the denaturant interact to the same degree with the transition state relative to their interaction with the folded state. Although this assumption may be valid for those systems, α subunit of *E. coli* tryptophan synthase (*8*, *10*) and CspB (*11*), and perhaps for the coiled coil as well, it is not for equine cytochrome *c* (cyt *c*).[1]

To circumvent the problem associated with the cosolvent's dual effect on both viscosity and stabilization, we developed a method for measuring the relative effects of viscosity upon

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the folding rates of monomeric (linked) and dimeric (unlinked) versions of the GCN4-p2′ coiled coil. Since each variant exhibits the same change in stability and in kinetic surface burial parameters upon the addition of glycerol, the effects of cosolvent-mediated stabilization upon folding kinetics should be identical for both. Any difference in the relative rates of the monomeric and dimeric species following the addition of glycerol should therefore be attributable to divergent effects of a change in viscosity upon rates. Using this analysis, we find that both the folding and unfolding rates for the monomeric and dimeric species exhibit identical viscosity dependence. The data also indicate that the folding of the dimeric species is folding-limited rather than encounter-limited.

MATERIALS AND METHODS

Chemicals. Ultrapure guanidinium chloride (GdmCl) was purchased from ICN Biomedicals (Aurora, OH); concentrations were verified by refractometry using a Bausch and Lomb refractometer. Equine cytochrome *c* (cyt *c*, mass = 12 384 g/mol) was type VI from Sigma Chemical Co. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was obtained from Pierce (Rockford, IL). All other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ).

Peptide Synthesis. GCN4-p1′ and GCN4-p2′ peptides were prepared and characterized as described *(12)*. GCN4-p1′ (Ac-RMKQLEDKVEELLSKNWHLENEVARLKKLVGER-NH_2) includes a tryptophan in place of the tyrosine at position 17 to allow for the monitoring of folding and unfolding by fluorescence spectroscopy. GCN4-p2′ contains an additional Cys-Gly-Gly tripeptide at the amino terminus to allow for studies of both monomeric and dimeric forms. Monomeric GCN4-p2′ is formed by oxidation, carried out by bubbling oxygen into a peptide solution under native conditions at room temperature for 2 h. Dimeric GCN4-p2′ is formed by reduction with 10-fold molar excess of TCEP at room temperature. Peptide concentrations were determined using an extinction coefficient of 5700 M^{-1} cm^{-1} for tryptophan at 280 nm (*13*).

Equilibrium Measurements. Equilibrium free energies of folding, $\Delta G°$, of GCN4-p2′ in various cosolvents were determined from guanidinium chloride (GdmHCl) denaturation profiles monitored by circular dichroism spectroscopy at 222 nm (a probe of α-helicity) using a Jasco J-715 spectropolarimeter with a computer-interfaced titrator. Measurements were conducted with 2 nm resolution and a 1 cm path length cuvette. Peptide concentrations ranged from 2 to 10 μM, and experiments were carried out in 20 mM sodium acetate at pH 5.5, 10 °C.

For the dimeric version of the coiled coil, the thermodynamic parameters were obtained assuming a two-state equilibrium between unfolded monomers and folded dimers and a linear dependence of $\Delta G°$ on GdmHCl concentration:

$$\Delta G°([\mathrm{GdmHCl}]) = -RT \ln K_U([\mathrm{GdmHCl}]) = \Delta G°_{H_2O} - m°[\mathrm{GdmHCl}] \quad (1)$$

where K_U is the equilibrium constant and the slope $m°$ is a measure of the denaturant-sensitive surface area exposed upon unfolding. Thermodynamic parameters of the monomeric and dimeric versions of the coiled coil GCN4-p2′ were obtained to according to (*14*).

Similar measurements were made on equine cytochrome *c* using tryptophan fluorescence as a probe for folding (excitation at 285 nm with 10 nm bandwidth, emission detected from 300 to 400 nm). These studies were conducted using the Jasco J-715 spectropolarimeter and a 1 cm path length cuvette. Protein concentrations ranged from 10 μM to 20 μM, and experiments were carried out in 50 mM imidazole at pH 7.0, 10 °C.

Stopped-Flow Spectroscopy. Rapid mixing fluorescence experiments used a Biologic (Grenoble, France) SFM-4 stopped-flow apparatus. The SFM-4 was interfaced with a Jasco Model 715 CD spectropolarimeter and utilized a 0.8 × 0.8 mm^2 square (FC/08) cuvette. Fluorescence spectroscopy used excitation and emission wavelengths of 280−290 and 300−400 nm, respectively. Temperature control of the sample syringes and the observation cuvette was maintained using a circulating water bath. The dead-time of the stopped-flow measurements was 1−2 ms depending upon cuvette type, syringe speed, and dilution ratio, calibrated using a dye quenching reaction. GCN4-p2′ concentrations ranged from 5 μM to 35 μM, while cytochrome *c* concentrations ranged from 30 to 50 μM.

Kinetic data were analyzed using a Simplex algorithm as implemented in the Biologic Biokine software. Kinetics of cytochrome *c* folding and unfolding, monomeric GCN4-p2′ folding and unfolding, and reduced (dimeric) GCN4-p2′ unfolding were fit as first-order exponential processes. Kinetic data for reduced (dimeric) GCN4-p2′ folding were fit using Biokine's second-order (bimolecular) fitting algorithm to account for the dependence of this reaction upon peptide concentration, and extracted rate constants were scaled to a single peptide concentration.

RESULTS

Folding of GCN4-p2′ and cyt c. To separate the stabilizing and viscosogenic effects of cosolvents upon folding rates, we took advantage of the ability of a modified version of the GCN4-p1′ coiled coil molecule to fold either as a dimer or as a cross-linked monomer. This modified species, GCN4-p2′, consists of a complex of two 36-mers, each with the normal 33 residue GCN4-p1′ sequence plus an additional amino-terminal Cys-Gly-Gly linker. Under reducing conditions, the cross-link is not formed, and the folding behavior is the same as for the tetherless version (results not shown). The monomeric molecule forms upon oxidation with the formation of a disulfide bond between the terminal Cys residues.

Equilibrium stability of GCN4-p2′ is measured in 20 mM sodium acetate, pH 5.5, using circular dichroism at 222 nm (helix formation) in buffer ($\eta_{rel} = 1.0$) and the presence of 15.2% (w/w) glycerol ($\eta_{rel} = 1.50$). Equilibrium stability of equine cyt *c* is measured in 50 mM imidazole, pH 7.0, using fluorescence spectroscopy at $\eta_{rel} = 1.0$ in buffer and at $\eta_{rel} = 2.0$ (*15*) in ethylene glycol (27% w/w), glucose (21% w/w), and glycerol (25% w/w). In cyt *c*, the fluorescence of Trp59 is essentially fully quenched by Förster energy transfer to the heme group upon collapse during folding. The equilib-

[1] Abbreviations: CD, circular dichroism; cyt *c*, cytochrome *c*; GdmHCl, guanidinium chloride; TFE, 2,2,2-trifluoroethanol; TCEP, tris(2-carboxyethyl)phosphine hydrochloride.

Table 1: Parameters of GCN4 Folding in the Presence of Cosolvents[a]

		equilibrium		kinetics					
solvent	species	ΔG°	m°	$RT \ln k_f$	$RT \ln k_u$	$-m_f$	m_u	$\Delta G^\circ = \Delta G^\ddagger_u - \Delta G^\ddagger_f$	$m^\circ = m_u - m_f$
buffer	dimeric	-8.35 ± 0.1	1.88 ± 0.04	1.35 ± 0.12	-1.04 ± 0.06	0.60 ± 0.13	0.90 ± 0.02	-8.40 ± 0.13	1.50 ± 0.13
buffer	monomeric	-6.5 ± 0.2	1.59 ± 0.05	5.11 ± 0.29	-0.96 ± 0.40	0.82 ± 0.10	0.75 ± 0.06	-6.07 ± 0.13	1.57 ± 0.13
9% glycerol, $\eta_{rel} = 1.25$	dimeric	(nd)	(nd)	2.34 ± 0.11	-2.33 ± 0.19	0.91 ± 0.14	1.11 ± 0.06	-11.45 ± 0.22	2.01 ± 0.15
9% glycerol, $\eta_{rel} = 1.25$	monomeric	-9.0 ± 1	2.0 ± 0.2	5.14 ± 0.29	-3.6 ± 1.5	0.74 ± 0.08	1.19 ± 0.30	-8.76 ± 1.5	1.93 ± 0.31
15.2% glycerol, $\eta_{rel} = 1.5$	dimeric	-10.4 ± 0.1	2.09 ± 0.04	2.47 ± 0.30	-2.36 ± 0.19	1.02 ± 0.28	1.04 ± 0.07	-10.37 ± 0.22	2.06 ± 0.29
15.2% glycerol, $\eta_{rel} = 1.5$	monomeric	-9.2 ± 0.1	2.1 ± 0.4	6.09 ± 0.30	-3.14 ± 0.09	0.96 ± 0.08	1.04[b]	-9.22 ± 0.31	2.03 ± 0.08

[a] Data are taken in 20 mM sodium acetate, pH 5.5, 10 °C. The equilibrium and activation free energies (units of kcal/mol) are extrapolated to zero denaturant and, for the dimeric species, 1 M standard state peptide concentration. Dimeric refolding parameters are extrapolated to 34.1 μM peptide concentration. Units of m are kcal mol^{-1} M^{-1}. [b] Set to the m_u value for the dimeric species.

Table 2: Parameters of cyt *c* Folding in the Presence of Cosolvents at $\eta_{rel} = 2$[a]

solvent	ΔG°	m°	k_f	$-m_f$
buffer	-8.1 ± 0.3	2.7 ± 0.1	2.88 ± 0.15	1.12 ± 0.03
27% (w/w) ethylene glycol	-7.8 ± 0.2	2.7 ± 0.1	5.07 ± 0.22	1.06 ± 0.03
21% (w/w) glucose	-10.3 ± 1.1	2.7 ± 0.3	1.39 ± 0.06	1.07 ± 0.02
25% (w/w) glycerol	-9.4 ± 0.3	2.9 ± 0.1	1.88 ± 0.06	1.04 ± 0.02

[a] Data are taken in 50 mM imidazole at pH 7.0, 10 °C. The ΔG° values (units of kcal/mol) are extrapolated to zero denaturant. Refolding rate (units of s^{-1}) are calculated at the vertex of the chevron where $K_{eq} = 1$. Units of m are kcal mol^{-1} M^{-1}.

rium parameters of GCN4-p2′ and cyt *c* in various cosolvents are summarized in Tables 1 and 2.

Folding kinetics were studied by stopped-flow fluorescence spectroscopy in various cosolvents in order to investigate the effects of viscosity on the folding of GCN4-p2′ (probing Trp17 burial) and cyt *c* (probing collapse). Monomeric (oxidized) GCN4-p2′ exhibited first-order folding and unfolding behavior, which is indicative of unimolecular processes. Dimeric (reduced) GCN4-p2′ exhibited second-order folding and first-order unfolding consistent with bimolecular folding and unimolecular unfolding. The bimolecular nature of the folding reaction of this dimeric species is further verified by establishing a linear dependence of folding rates upon protein concentration with zero intercept [data not shown and refs (*16, 17*)].

At neutral pH, the folding of oxidized equine cyt *c* is complicated by the presence of two surface histidines (His26 and His33) which are capable of ligating the heme in the denatured state. When these non-native ligations are formed, an intermediate containing the amino- and carboxy-terminal helices accumulates and folding occurs in a 3-state manner *(9)*. However, when cyt *c* is folded in the presence of 50 mM imidazole, pH 7.0, the non-native His−heme ligations do not form in the denatured state and folding is essentially two-state (*18*). Under these conditions, the fluorescence-monitored folding kinetics exhibit essentially single-exponential behavior.

The denaturant dependence of kinetic folding and unfolding reactions produces a "chevron" or v-shaped curve with its vertex at the midpoint of the equilibrium transition (*19*) (Figures 1 and 2). The dependence of the unfolding equilibrium constant, K_U, and stability, ΔG°, on the concentration of GdmHCl is commonly described by the linear relationship shown in eq 1 where the slope, m°, is a measure of the denaturant-sensitive surface area exposed upon unfolding. Equation 2 describes the analogous linear dependence of the

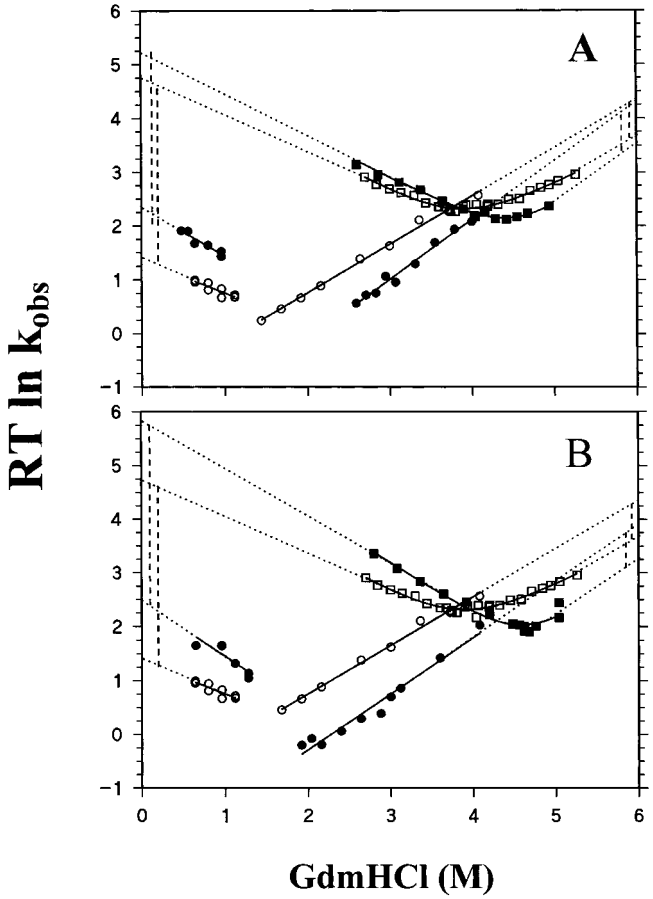

FIGURE 1: Strategy for deconvoluting viscosity and stability from dimeric (circles) and monomeric (squares) folding in water (open symbols, data duplicated in panels A and B) and in the viscosogenic solvents (closed symbols) 9% glycerol (A) and 15.2% glycerol (B). The pairs of vertical lines (long dash) connecting the parallel extrapolations of folding data (short dashed lines) are nearly equal in length, providing a reference that corresponds to the differences in the exponential terms of eq 4d (see text). The equality of the lengths for both the folding and unfolding arms indicates that the viscosity dependence of both the folding and unfolding reactions is the same for the monomeric and dimeric species. The measured bimolecular folding rates ($k_{obs} = [\text{protein}]k_f$) have been scaled to conform to a uniform 34.1 μM protein concentration.

activation free energy, $\Delta G^\ddagger$, for kinetic folding (f)

$$\Delta G^\ddagger_f([\text{GdmHCl}]) = -RT \ln k_f^{H_2O} - m_f[\text{GdmHCl}] + \text{constant} \quad (2a)$$

and unfolding (u) reactions

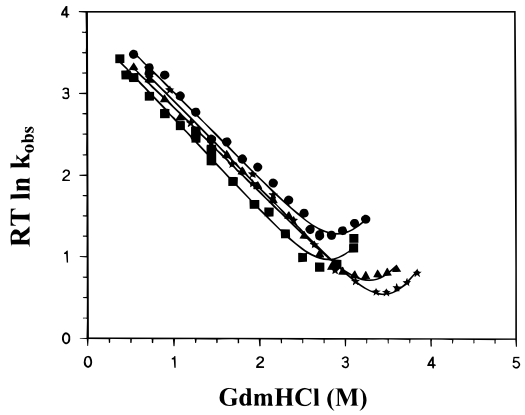

FIGURE 2: cyt *c* folding measured by fluorescence quenching of Trp59 in the presence of viscosogenic cosolvents at $\eta_{rel} = 2.0$ as a function of denaturant. cyt *c* in water (■), 21% (w/w) glucose (★), 25% (w/w) glycerol (▲), and 27% (w/w) ethylene glycol (●) is folded in the presence of 50 mM imidazole, pH 7.0, 10 °C, which prevents non-native His−heme contacts in the unfolded state that would lead to the formation of a kinetic intermediate (see text). Solid lines are a fit to the data using the chevron formalism (eqs 4a,c) and equilibrium-derived parameters for $\Delta G°$ and $m°$.

$$\Delta G^{‡}_{u}([\text{GdmHCl}]) = -RT \ln 2k_u^{H_2O} - m_u[\text{GdmHCl}] + \text{Constant} \quad (2b)$$

$$\Delta G^{‡}_{u}([\text{GdmHCl}]) = -RT \ln k_u^{H_2O} - m_u[\text{GdmHCl}] + \text{Constant} \quad (2c)$$

where eqs 2b and 2c apply to dimeric *(20)* and monomeric unfolding data, respectively. The denaturant dependencies of the activation energies for folding and unfolding reactions are given by m_f and m_u, respectively. These *m* values, or denaturant response parameters, represent the change in activation energy per unit change in denaturant concentration for the folding and unfolding reactions, respectively. The m_f parameter represents the amount of denaturant-sensitive surface buried going from the unfolded state to the transition state, while $-m_u$ represents the amount buried going from the transition state to the native state.

When equilibrium and kinetic folding reactions are effectively two-state, and are limited by the same activation barrier, it is possible to calculate the equilibrium values for the change in free energy and surface burial from kinetic measurements according to: $\Delta G°_{H_2O} = \Delta G^{‡}_{u} - \Delta G^{‡}_{f}$ and $m° = m_u - m_f$, derived from eqs 1 and 2. Table 1 shows values determined for the GCN4-p2′ variants under the conditions used here. The equivalence of $\Delta G°$ and $m°$ values from equilibrium measurements with the values determined independently from kinetic experiments both here and in previous work demonstrates the applicability of a two-state model for GCN4-p2′ (*16, 17*) and cyt *c* (*18, 21*) folding under the measured solvent conditions. This also confirms the validity of the fluorescence probe for measuring GCN4-p2′ folding rates, since every probe measures the same rate in an all-or-none, two-state reaction.

Viscosity Dependence. The major difficulty in determining the viscosity dependence of a folding reaction is the effect of the viscosogenic agent on both solvent viscosity and protein stability. Our analysis assumes the effect of glycerol can be factored as $k_f^{water} = \sigma_f\ \upsilon_f\ k_f^{glycerol}$ where σ_f and υ_f represent the effects of stability and viscosity, respectively. The value of υ_f may range from 1 (no viscosity dependence of folding rates) to $1/\eta$ (unitary viscosity dependence) as per Kramers' Theory. This decomposition yields an expression for the stabilizing effect on the dimeric folding rate:

$$\sigma_f^{dimer} = \nu_f^{dimer} \frac{[k_f^{dimer,glycerol}(\text{Gdm})]}{[k_f^{dimer,water}(\text{Gdm})]} \quad (3a)$$

where rates are explicitly expressed as a function of denaturant concentration, which includes any change in *m*-values upon addition of glycerol. The effects of cosolvent stabilization alone on monomeric folding are expressed in analogous fashion as

$$\sigma_f^{monomer} = \nu_f^{monomer} \frac{[k_f^{monomer,glycerol}(\text{Gdm})]}{[k_f^{monomer,water}(\text{Gdm})]} \quad (3b)$$

The near-identity of both the m_f and m_u values for the dimeric and monomeric species indicates that their transition states have a similar amount of surface burial. Thus, the transition states for both species should be stabilized to a similar extent by glycerol, and the factors σ_f^{dimer} and $\sigma_f^{monomer}$ can be equated. The covariation of *m*-values (see Table 1) further supports this view, and the explicit denaturant dependence present in the ratios in eqs 3a,b can be dropped. The ratio of the viscosity dependencies of the monomeric and dimeric species then can be solved for independent of the stability factors.

$$\sigma_f^{dimer} = \sigma_f^{monomer} \quad (4a)$$

$$\nu_f^{dimer} \frac{k_f^{dimer,glycerol}}{k_f^{dimer,water}} = \nu_f^{monomer} \frac{k_f^{monomer,glycerol}}{k_f^{monomer,water}} \quad (4b)$$

$$\frac{\nu_f^{monomer}}{\nu_f^{dimer}} = \frac{k_f^{dimer,glycerol}\ k_f^{monomer,water}}{k_f^{dimer,water}\ k_f^{monomer,glycerol}} \quad (4c)$$

$$\frac{\nu_f^{monomer}}{\nu_f^{dimer}} = e^{\{[(RT \ln k_f^{monomer,water} - RT \ln k_f^{dimer,water}) - (RT \ln k_f^{monomer,glycerol} - RT \ln k_f^{dimer,glycerol})]/RT\}} \quad (4d)$$

Equation 4 expresses $\nu_f^{monomer}/\nu_f^{dimer}$, the ratio of the viscosity dependence of the folding rate of the monomeric to the dimeric protein as a function of experimentally determined parameters.

An analogous expression to eq 4d can be written for the ratio of the viscosity dependence of the unfolding reactions for the monomer and dimer. Since viscosity is a kinetic parameter that does not affect thermodynamics, the viscosity dependence of the folding and the unfolding rate should be equal for a two-state reaction. This notion is verified in various traditional studies of the effects of viscosity on reactions in solution (*22*). Therefore, comparing the independent results of the relative viscosity dependencies for the folding and the unfolding reactions offers an internal control of our method.

We are able to carry out this analysis in solutions with $\eta_{rel} = 1.25$ and 1.5, corresponding to 9% and 15.2% w/w

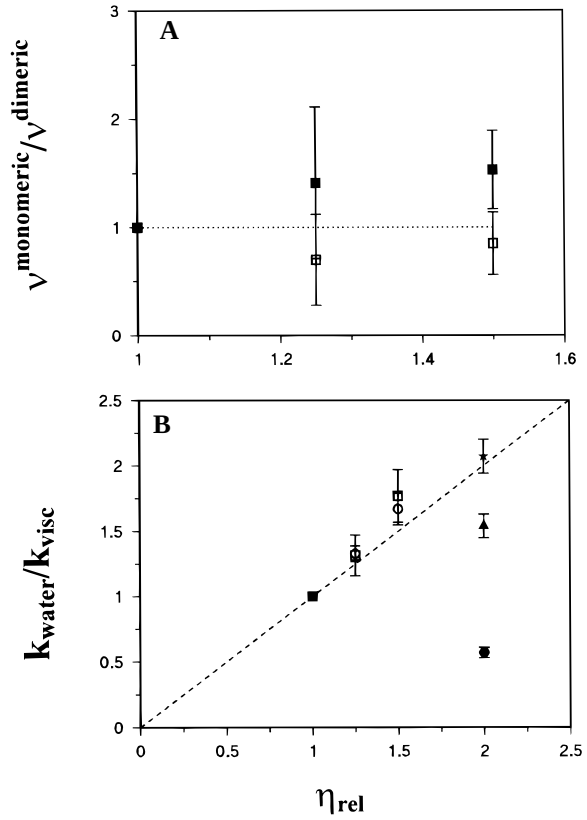

FIGURE 3: Viscosity dependence of folding. (A) Relative viscosity dependence of folding (■) and unfolding (□) rates for the monomeric versus the dimeric version of coiled coil. The unitary value for the ratio $\upsilon^{monomeric}/\upsilon^{dimeric}$ indicates that both systems have the same relative dependence upon solvent viscosity. Values are obtained as described by eqs 4a−d at 2 and 5 M GdmCl for the folding and unfolding values, respectively. (B) Viscosity dependence of folding rates using chevron-shift analysis for dimeric (○) and monomeric (□) GCN4-p2′ in 9 and 15% glycerol; and for cyt *c* in 21% glucose (★), 25% glycerol (▲), and 27% ethylene glycol (●). All rates are normalized with respect to the same protein in water (■).

glycerol. Figure 1A,B displays overlaid chevrons of the monomeric and dimeric species of GCN4-p2′, which can be analyzed graphically as follows. The folding and unfolding arms are extrapolated for both dimeric and monomeric species (dotted lines). The vertical distances between the extrapolated lines for the monomeric and dimeric species in aqueous and in glycerol solutions (represented in Figure 1 by pairs of vertical dashed lines of equal length) correspond to the differences in the exponential terms of eq 4. In the case where these differences ($RT \ln k_f^{monomer,water} - RT \ln k_f^{dimer,water}$ and $RT \ln k_f^{monomer,glycerol} - RT \ln k_f^{monomer,water}$) are equal, eq 4 reduces to $\upsilon_f^{monomer}/\upsilon_f^{dimer} = e^{(0/RT)} = 1$. In this circumstance, folding rates of the monomeric and dimeric forms have identical viscosity dependencies.

The results of this analysis for $\upsilon_f^{monomer}/\upsilon_f^{dimer}$ using extrapolated rates are shown in Figure 3A. These results indicate that both folding and unfolding rates of the monomer and dimer exhibit the same viscosity dependence within experimental error.

Chevron-Shift Analysis. Earlier works examining the viscosity dependence of folding have suggested that the stabilizing effects of a viscosogenic cosolvent can be accounted for by comparing rates at points of equivalent protein stability (*8, 10, 11*). The validity of this analysis

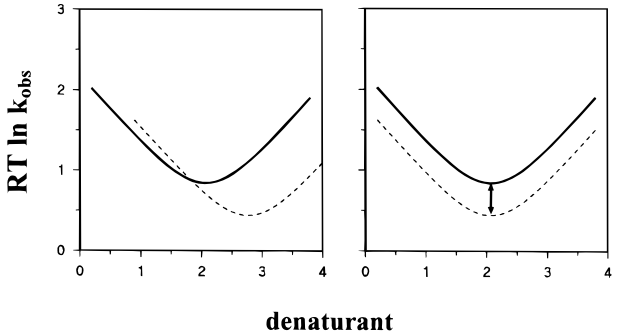

FIGURE 4: Chevron-shift analysis. (A) To account for the increased stability of the protein due to the viscosogenic solvent, the folding chevron (dashed line) in cosolvent is translated horizontally until it is underneath the folding chevron in aqueous solvent (solid line). (B) Upon this shift, a comparison of the folding rate at the vertex (or any given denaturant concentration) is at a point of equal stability. The height of the double headed arrow reflects the effect on folding due to a change in viscosity after correction for the stabilizing effect of the cosolvent. This analysis assumes the transition state has the same relative degree of interaction with the viscosogenic cosolvent as with denaturant.

requires that viscosogenic cosolvent and the denaturant interact to the same degree with the transition state relative to their interaction with the folded state (*8*), a point that will be considered in more detail under Discussion. Under this assumption, the altered stability of the protein due to the cosolvent is manifested in a purely horizontal translation of the chevron (a "chevron-shift", Figure 4). The vertical displacement of the new, shifted chevron compared with the chevron in the absence of the cosolvent represents the difference in folding rates at points of equivalent stability. This difference is attributed to the change in the folding rates resulting from only the increase in solvent viscosity. Such a treatment has yielded a nearly linear dependence of folding rates upon viscosity over a limited range of viscosities (including the range used in this study) for the particular folding systems used, though this dependence deviates from linearity at higher viscosities (*11*).

To test its feasibility as a quantitative solution to the problem of separating these effects, we performed this same chevron-shift analysis on coiled coil and cyt *c* in different cosolvents. As shown in Figures 1 and 2, the stabilizing effect of these cosolvents upon the proteins is reflected by the translation of the midpoints of the chevrons toward higher denaturant concentration. Treating the vertex of each chevron where folding and unfolding rates are equal as the reference point of equal stability (i.e., at $K_{eq} = k_u/k_f = 1$ for monomeric GCN4-p2′ and cyt *c* and $K_{eq} = 2k_u/k_f = 2$ for dimeric GCN4-p2′), we plotted the (un)folding rates at this point against the relative viscosity of the solution (Figure 3B). The chevron shift did produce a nearly $1/\eta$ dependence for both forms of the coiled coil in glycerol. For cyt *c*, however, no coherent relationship between viscosity and folding rates is observed for folding in ethylene glycol, glycerol, and glucose.

To further examine the validity of the chevron-shift analysis, we applied it to the kinetic data for dimeric GCN4-p1′ in a different cosolvent, the helix-stabilizing monohydric alcohol 2,2,2-trifluoroethanol (TFE) *(14)*. Interestingly, the stabilizing effect of TFE is only manifested in an increase in folding rates. The chevron-shift analysis with a $1/\eta$ viscosity dependence would predict a slight depression in the folding rate ($\eta_{rel} = 1.2$ for 5% ethanol), but a 2.4-fold

increase in speed is actually observed at the chevron vertexes. This result further demonstrates that the applicability of the chevron-shift treatment is not universal, but rather may apply only in cases where the cosolvent interacts with a protein along its folding pathway in a manner similar to denaturant.

DISCUSSION

To characterize the folding transition state, we have chosen to study the effects of a change in microscopic diffusion rates, mediated by a change in solvent viscosity, upon the rate of protein folding. If an increase in viscosity is matched by a proportional decrease in folding rate, then the rate-limiting step depends on microscopic diffusion through solvent. However, if no change in rate is observed, then folding is limited by a step that is independent of diffusion through solution.

Viscosity Dependence of Folding and Unfolding. We determined the relative viscosity dependence of two simple two-state folding reactions: those of a monomeric and a dimeric version of a 10-turn α-helical coiled coil. By taking advantage of the ability of the GCN4-p2′ folding system to fold in solution either as a dimer or as a disulfide-tethered monomer, we are able to separate the effects of cosolvent-mediated stabilization and viscosity changes. The folded state of GCN4-p2′ is identical in the monomeric and dimeric forms, and the degree of denaturant-sensitive surface buried in the transition state, as reflected in the m_f and m_u parameters, is the same for each type. Further, the correlation of the variations in m_f and m_u at each different glycerol concentration indicates that the added cosolvent stabilizes the two transition states to the same extent. This equivalency enables one to factor out the stabilizing influence of glycerol and thus extract the comparative dependence of monomeric and dimeric folding kinetics upon viscosity. Using this procedure, the folding rates of these two forms are seen to exhibit the same viscosity dependence.

In addition to having acquired data about the viscosity dependence of folding at several different viscosities, we also independently determined the viscosity dependence of unfolding at each viscosity. The dependence of each process upon viscosity must be equivalent, since viscosity is inherently a kinetic parameter. Therefore, a change in viscosity alone cannot affect the equilibrium of a reaction, and changes in viscosity must affect both forward and reverse rates to the same extent. Jacob et al. also argued that the principle of microscopic reversibility dictates that the viscosity dependence of a barrier-crossing process should be independent of the direction of crossing (*11*). Since we were able to carry out our analysis on the folding and unfolding processes separately, each determination provided independent confirmation of the relative viscosity dependencies of the processes.

However, this method does not allow one to determine the absolute viscosity dependence of either folding process. One can only conclude that the dimeric and monomeric folding processes have the same viscosity dependence. Although it might seem that the folding of the dimeric protein necessarily would be viscosity dependent since its refolding rate depends linearly upon peptide concentration, in fact this is not necessarily the case. Unless every encounter in solution is productive (as is the case for an "encounter-limited" reaction), the viscosity dependence of the initial collision portion of the reaction can cancel out for a "folding-limited" reaction (*22*). A preequilibrium can be established between separated chains and an encounter complex in which both the forward and the reverse reactions exhibit a $1/\eta$ viscosity dependence (*23*). The resulting equilibrium constant, given by the ratio of these rates, is then independent of viscosity, although it still depends on protein concentration. The overall bimolecular folding rate is the product of this concentration-dependent equilibrium constant and the forward rate of the remaining folding steps. Thus, the linear increase in folding rates with protein concentration dependence can stem from the concentration dependence of the preequilibrium step rather than explicitly from an increase in collision frequency.

Therefore, the viscosity dependence of the entire dimeric folding reaction can be independent of the Smoluchowski-derived $1/\eta$ dependence for the collision step, except in the extreme case that every encounter is productive. The slow speed ($\sim 10^7$ M^{-1} s^{-1} extrapolated to zero denaturant) and considerable surface burial ($m_u/m^\circ \sim 0.5$) argue against encounter-limited folding in the dimeric coiled coil (see below). Thus, the absolute viscosity dependence of the dimeric system, and hence the identical dependence of the monomeric system, cannot be determined from this analysis.

Chevron-Shift Analysis. The chevron-shift treatment has been used to account for the cosolvent stabilization of the transition state in CspB (*11*) and in the α subunit of *E. coli* tryptophan synthase (*8*, *10*). For these proteins, this analysis indicates that that folding (*11*) or unfolding (*10*) displays a $1/\eta$ viscosity dependence. Furthermore, this dependence is observed in a variety of viscosogenic cosolvents. This treatment also produces the same result for the dimeric and monomeric forms of the coiled coil in glycerol. However, our studies on cyt *c* failed to reveal any coherent viscosity dependence for folding in glycerol, ethylene glycol, and glucose. Further, this analysis actually produced a 2.4-fold increase in folding rates as a result of a ~1.2-fold increase in viscosity for GCN4-p2′ folding in TFE (*14*). Potentially, the underlying assumption that denaturant and the viscosogenic agent interact with the transition state to the same degree is applicable in some cases but is not generally valid. In any case, it seems likely that folding is viscosity-dependent based upon our studies with monomeric and dimeric GCN4-p2′ and the studies on CspB (*11*) and the α subunit of *E. coli* tryptophan synthase (*10*).

The chevron-shift analysis lends another important interpretation of our folding data for dimeric and monomeric GCN4-p2′. Analysis of the folding of both species in glycerol shows that the chevron-shift treatment yields the same result for each, namely, that stability-adjusted folding rates scale inversely with solvent viscosity. Even if this treatment were an inappropriate means of accounting for the effects of the cosolvent upon stability, it would certainly account for cosolvent stabilization in the same manner and to the same extent for each species. Therefore, the chevron-shift analysis, independent of its ability to account rigorously for the absolute magnitude of cosolvent-mediated stabilization, verifies our result that the viscosity dependencies of the folding and unfolding rates of monomeric and dimeric versions of GCN4-p2′ are identical.

Reaction-Rate Theory and Protein Folding. The application of reaction rate theory to macromolecular reactions in

solution, in particular to intramolecular protein folding processes, has proven to be a difficult task. Although frequently applied to protein folding reactions, the canonical transition state theory, expressed by $k^{TST} = k_B T/h \, exp(-\Delta G^{\ddagger}/k_B T)$, offers only a first approximation, predicting an upper limit for the rates of barrier crossing steps. The quantum mechanical prefactor ($k_B T/h \sim 10^{13}$ Hz) overestimates by many orders of magnitude the attempt frequencies of a polypeptide moving in high-friction media such as aqueous solutions (*5*, *24*, *25*). The predicted rate derived from transition state theory can then be modulated by different parameters describing the effects of solvent interactions on the reaction, but in the case of protein folding, a quantification of these modulating factors has proven elusive (*3*).

Smoluchowski first dealt extensively with the viscosity dependence of the rates of chemical reactions (*23*). Then, in a seminal work expanding the scale of reaction rate theory for reactions in solution, Kramers discussed the proper means of expressing rates that accurately reflect the nature of barrier-crossing processes in solution. Kramers described three regimes of solvent friction, each with differing effects on reaction rates for low-barrier processes. In the high-friction limit, encompassing aqueous solvents, he described solvent collisions as impeding the diffusive path of reactant molecules along a reaction coordinate. The effect of such "overdamped oscillations" on reaction rates is to decrease reaction rates in solution, an effect that strengthens as solvent friction increases in this regime *(26)*. The resultant Kramers' Theory predicts a $1/\eta$ dependence for reaction rate constants in this limit of high η (*1*) [reviewed in (*2*, *3*, *5*, *26*)].

The application of this rule to intramolecular activated processes has raised complex issues about internal interactions within macromolecules (*3*). For example, if the rate-limiting step in a folding reaction involves internal rearrangements, then the internal friction of the protein, not solvent friction, will limit the reaction rate. In such a case, one would expect to see no dependence of folding rates upon solvent viscosity. In an intermediate case in which both solvent and internal friction limit the acquisition of the transition state, one would expect some dependence upon each type of friction. Eaton and co-workers modeled this dependence as $1/(\eta + \sigma)$, with σ representing an internal friction term in the neighborhood of 4 cP, compared with $\eta = 1$ cP for water at 20 °C (*27*, *28*). If folding rates depend in such a way on some convolution of these two friction terms, then one would not expect a $1/\eta$ dependence of folding rates upon solvent viscosity. Moreover, if two different folding processes were limited by different frictional factors, then one would expect differing effects upon rates from a change in solvent viscosity. Given ours and other workers observation that protein folding does exhibit a $1/\eta$ dependence, internal friction must be small compared to solvent friction.

Application of Kramers' Theory to protein folding studies will result in slightly different values for activation enthalpies and heat capacities determined from the temperature dependence of folding rates. If folding rates have a $1/\eta$ viscosity dependence, they should be scaled by the change in the viscosity of water [e.g., a factor of 3 from 5 to 70 °C (*15*)] for the proper determination of these activation parameters.

Implications for Models on Protein Folding. Of the models for protein folding currently postulated, a few actively predict a viscosity dependence for folding. We proposed a search−nucleation model where formation of a stable collapsed species is rate-limiting (*21*). Folding is limited by diffusive chain motions through solution with multiple transient collisions in a search for a topologically, but not necessarily structurally, nativelike transition state. The limiting step is the formation of a core with a sufficient number of long-range contacts that adequately define the native topology. At this point, the nucleation event, a considerable amount of configuration entropy is lost and folding can proceed rapidly downhill. On this downhill portion, the majority of the detailed, native structure is formed in an increasingly solvent-excluded environment. Exposed chain loops condense onto the core, forming the detailed nativelike elements such as secondary structures, possibly in a series of distinct intermediate steps (*29*). Since the internal friction-limited steps occur after the rate-limiting step, this model predicts that folding will be limited by the rate of chain diffusion through solution.

Other folding models also feature implicit predictions about the expected viscosity dependence of the folding process. The diffusion−collision model offers a picture of folding steps limited by the diffusion of structural elements such as helices through solution and thus would predict a viscosity dependence for portions of the folding reaction as well (*30*). The applicability of the diffusion−collision model to β-sheet proteins is less obvious.

In the limit that all the diffusive portions of the reaction occur in multiple fast preequilibrium steps on the uphill path to the transition state, the viscosity dependence of each of these steps cancels out. The overall reaction rate will exhibit the viscosity dependence of only the final step. Hence, if the entire reaction can be shown to have $1/\eta$ viscosity dependence, then even the final step to the top of the barrier must involve diffusion of the chain through solution. This is most striking for CspB, where over 90% of the surface area is buried in the transition state, but the folding reaction still appears to be diffusion-dependent (*11*).

A folding reaction exhibiting $1/\eta$ viscosity dependence is inconsistent with models that postulate a nativelike transition state where the major collapse has occurred in earlier steps and only internal rearrangements such as side chain packing dominate the rate-limiting step. This includes molten globule-like models where the rate-limiting step is the internal rearrangement of partially structured and collapsed folding intermediates (*31*−*33*). Instead, such models would predict rates that depend more upon internal friction terms, an eventuality excluded by these and other similar results.

This logic can be applied to proteins which are postulated to fold in a multi-state manner with a submillisecond "burst-phase" collapsed intermediate that only accumulates at low denaturant concentrations (U↔I↔N). This scenario has been proposed for ubiquitin (*34*) and cyt *c* (*35*), but alternative interpretations related to a response of the unfolded state to the newer, poorer solvent condition are possible (*18*, *21*, *36*, *37*). Even in the multistate scenario, this reaction is kinetically two-state (U↔N) at higher denaturant concentrations since the putative intermediate is unstable and is not significantly populated. If the net reaction can be shown to have a $1/\eta$ viscosity dependence when folding is effectively two-state, then the barrier between the unstable intermediate and the native state must also have a $1/\eta$ viscosity depen-

dence. Hence, a $1/\eta$ dependence for the reaction under any condition would argue against the existence of a highly collapsed, defined intermediate state which only needs to undergo internal rearrangements in the transition to the native state.

A feature of both the unlinked and linked coiled coil transition state is that nearly all of the TFE-sensitive surface area is buried (*14*). The apparently contradictory result is that this area, most likely representing the peptide backbone (*14*), is buried from TFE but still the chain is moving through solvent that has a viscosity identical to that of bulk solvent. Potentially, at the low level of TFE used in those studies (5%), a layer of bound water exists around the chain that sequesters the backbone, but the chain still undergoes large-scale motions through solution that involve displacement of bulk solvent.

Encounter-Limited versus Folding-Limited Reactions. Our investigation of the dimeric reaction raises an issue discussed by Baldwin and colleagues involving the question of "encounter-limited" versus "folding-limited" folding in a dimeric system, in their case the S-peptide and S-protein of RNase A (*38*). They define encounter-limited folding as a dimeric folding process in which the barriers to encounter, rather than any genuine folding steps, limit the folding rate. A number of factors argue against encounter-limited folding in the GCN4-p2 dimeric system. First, folding is 2 orders of magnitude slower than canonical diffusion-limited rates, which should be on the order of 10^9 M^{-1} s^{-1} as observed for the diffusion-limited folding of the arc repressor dimer (*20*). The fact that dimeric folding is slower than expected for a purely diffusion-limited process indicates that not all collisions are productive; i.e., the folding process is not purely limited by encounters in solution. Rather, following collision, another distinct event is required to overcome the rate-determining barrier for folding.

Further, the m_f values suggest that the amount of buried surface area in the transition state is near 50% (*17*) and is the same for both species. This extent of surface burial in the transition state of the dimeric species would seem unlikely in the absence of productive folding events, and its equivalency with the burial in the (folding-limited) monomeric transition state strongly suggests that some productive folding interactions have also been made in the dimeric reaction. Moreover, the changes in the m_f and m_u values following the addition of different amounts of glycerol are the same for the monomeric and dimeric species. Thus, the two transition states interact with both denaturant and viscosogenic cosolvents to the same extent and are largely equivalent in this respect. It is therefore unlikely that the folding of the monomeric species would be folding-limited while that of the dimeric version be encounter limited. Hence, dimeric folding is folding-limited rather than encounter-limited, and the encounter complex of the dimeric species is probably equivalent to the unfolded state of the monomeric species

Conclusions. The viscosity dependence of a folding reaction provides a stringent test for potential models for protein folding. A $1/\eta$ viscosity dependence is consistent with an early rate-limiting step dominated by motions through solution (*21*), but is inconsistent with molten-globule and other models where the rate-limiting step is dominated by internal rearrangements. We are able to account for the viscosogenic cosolvent's effect on the stability of the transition state by comparing the folding behavior of the dimeric to the monomeric version of coiled coil. The relative effect of viscosity on both folding and unfolding rates is the same for both versions of the coiled coil. Under the assumption that the relative interaction of the transition state with the viscosogenic solvent and denaturant is the same (chevron-shift analysis), folding rates of both versions of the coiled coil are seen to exhibit a $1/\eta$ viscosity dependence. Although results with TFE as a cosolvent and with cyt *c* suggest some need for concern in accepting the assumptions of the chevron-shift method, our results with the dimeric and monomeric coiled coil strengthen the claim of prior chevron-shift analyses (*10, 11*), namely, that protein folding reactions can be limited by the rates at which chains diffuse through solution.

NOTE ADDED IN PROOF

Plaxco and Baker (*39*) concluded that internal friction is negligible in the folding of the IgG binding domain of protein L having obtained an accurate $1/\eta$ dependence using the chevron-shift (isostability) analysis with a zero internal friction term.

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