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Viscosity Dependence of Some Protein and Enzyme Reaction Rates: Seventy-Five Years after Kramers

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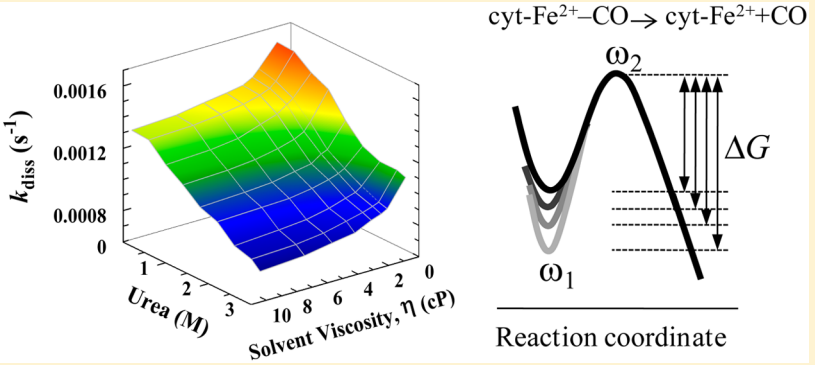

ABSTRACT: Kramers rate theory is a milestone in chemical reaction research, but concerns regarding the basic understanding of condensed phase reaction rates of large molecules in viscous milieu persist. Experimental studies of Kramers theory rely on scaling reaction rates with inverse solvent viscosity, which is often equated with the bulk friction coefficient based on simple hydrodynamic relations. Apart from the difficulty of abstraction of the prefactor details from experimental data, it is not clear why the linearity of rate versus inverse viscosity, $k \propto \eta^{-1}$, deviates widely for many reactions studied. In most cases, the deviation simulates a power law $k \propto \eta^{-n}$, where the exponent n assumes fractional values. In rate−viscosity studies presented here, results for two reactions, unfolding of cytochrome *c* and cysteine protease activity of human ribosomal protein S4, show an exceedingly overdamped rate over a wide viscosity range, registering n values up to 2.4. Although the origin of this extraordinary reaction friction is not known at present, the results indicate that the viscosity exponent need not be bound by the 0−1 limit as generally suggested. For the third reaction studied here, thermal dissociation of CO from nativelike cytochrome *c*, the rate−viscosity behavior can be explained using Grote−Hynes theory of time-dependent friction in conjunction with correlated motions intrinsic to the protein. Analysis of the glycerol viscosity-dependent rate for the CO dissociation reaction in the presence of urea as the second variable shows that the protein stabilizing effect of subdenaturing amounts of urea is not affected by the bulk viscosity. It appears that a myriad of factors as diverse as parameter uncertainty due to the difficulty of knowing the exact reaction friction and both mode and consequences of protein−solvent interaction work in a complex manner to convey as though Kramers rate equation is not absolute.

Kramers reaction rate theory is based on phenomenological assumptions that the one-dimensional reaction coordinate interacts with the invisible coordinates representing the remaining degrees of freedom for both reactant and surrounding particles, and the degree of the interaction is determined entirely by the frictional damping rate. During the past 75 years since its foundation,[1] Kramers theory has been extended to include many spatial dimensions,[2] and to absolute zero temperature.[3] In their authoritative review 25 years ago, Hangii et al.[3] have already discussed the decisiveness and the impact of Kramers ideas in classical and quantum regimes. Many fundamental experiments involving subatomic systems and atom recombination reactions in inert solvents have accurately reproduced Kramers rate formula in both energy diffusion and activated diffusion-controlled regimes straddled by the turnover region.

There has, however, been a pesky problem in how closely condensed phase reaction rates for both small and large molecules should follow Kramers rate equations. To exemplify the small-molecule case, the dependence of binaphthyl isomerization rates on the shear viscosity of alcohols shows Kramers scaling, but the rates of the same reaction in linear alkanes indicate deviation.[4] Some other earlier investigations also found reduced overdamping of the isomerization rate of *trans*-stilbene versus alkane shear viscosity.[5,6] With regard to protein and enzyme reactions, numerous studies sought to examine the extent to which the Kramers basic result, set as a rate−friction inverse relationship ($k \propto 1/\eta$, where the hydrodynamic proportionality of solvent viscosity η and friction coefficient γ is used), can be observed. These studies have generally concluded that the rate−viscosity relationship holds as $k \propto \eta^{-n}$, where the exponent n lies in the 0−1 bound for a variety of reactions, including enzyme kinetics,[7−16] protein folding,[17−23]

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end-to-end intrachain looping in small peptides,[24] inter- and intramolecular electron transfer processes,[25,26] the photodissociation rate in oxygen complexes of myoglobin and hemerythrin,[27,28] ligand binding to myoglobin,[29] and amide hydrogen exchange in some proteins.[30−32] A recent single-molecule study also finds a fractional viscosity exponent.[33] Taking all literature data at face values under experimental conditions used, one might suppose that macromolecular reactions are as overdamped as the Kramers equation would, and underdamping in some cases could be due to a host of factors, including characteristic equilibrium thermal fluctuations,[34] low-amplitude motions required for certain protein functions,[15,35] colored Gaussian noise,[36] insufficient coupling of the reaction site with the bulk solvent,[37,38] and anomalous internal friction.

Studies so far have scarcely revealed overdamping in excess of what Kramers rate theory would allow, except for slowly exchanging hydrogens in lysozyme for which the exponent n approaches 1.4,[30] and the rate of electron transfer from cytochrome c to P840 in the photosynthetic reaction center where n is found to be 1.9 and 2.4 at 283 and 295 K, respectively.[39] Such extra overdamping is of interest, because it implies operation of additional degrees of freedom for both solute and solvent compared to the number produced by diffusive fluctuations.

This work reports on the effect of glycerol-generated solvent friction on the rates of three different protein reactions: guanidinium-induced unfolding of cytochrome c, human ribosomal protein S4-catalyzed cleavage of the peptide-Phe-Arg↓-fluorophore, and thermal dissociation of heme-bound CO from nativelike cytochrome c. Data were collected repeatedly over several years to provide us with sufficient confidence. Whereas the rates for unfolding and enzyme reactions are found to be exceedingly overdamped, the ligand dissociation reaction can be modeled using Grote−Hynes (GH) theory.[40]

MATERIALS AND METHODS

Unfolding Reactions. The influence of solvent viscosity on the conformational stability of cytochrome c (Sigma, type VI) was checked by GdnHCl-induced unfolding of the protein at 53% (v/v) glycerol and 15 mM sodium phosphate (pH 7), held at different temperatures. Two stock solutions, both identical in content of the protein (6 μM) and glycerol [53% (v/v)], but one containing 4.2 M GdnHCl and the other not, were mixed appropriately to obtain protein samples whose denaturant content varied from 0 to 4.2 M. The samples were equilibrated at a given temperature (−0.5, 7.5, and 19 °C) for 6 h before measuring fluorescence at the same temperature. The fluorescence cuvette was thermostated at the same temperature. Extreme care was taken to regulate the temperature from sample incubation to emission measurement. Sample viscosity due to both glycerol and GdnHCl at the temperature of measurement was determined from standard tables and empirical relations.[41−43]

Unfolding kinetics were studied by both manual and stopped-flow mixing protocols. For manual mixing experiments, the initial native protein solution (3 mg mL^{-1}) was prepared in 57.5% (v/v) glycerol and 15 mM phosphate (pH 7). The unfolding buffer solution contained the same volume of glycerol and 3.9 M GdnHCl and was buffered identically. The unfolding run consisted of incubating the native protein solution and the unfolding buffer at a temperature in the range of −11.5 to 13 °C for 2 h and diluting the former into the latter 61-fold to obtain ∼4 μM protein and ∼3.8 M GdnHCl in the final unfolding milieu. Stopped-flow experiments were conducted identically using 8-fold dilution of the native protein solution. The denaturant content in the unfolding buffer was adjusted to match the conditions already employed in the manual mixing experiments.

The experiments were performed repeatedly using Fluorolog and Fluoromax series of Horiba fluorometers and a SFM400 mixing module (BioLogic). Low-temperature experiments were conducted by constant nitrogen purging in the cuvette chamber. Typically, five to seven kinetic traces were averaged at each value of solvent viscosity.

Human S4 Enzyme Kinetics. Recombinant human S4 (hS4) was produced as described previously,[44] and the steady-state kinetic experiments involved measurement of the initial velocity of hS4-catalyzed cleavage of the cysteine protease synthetic substrate analogue Z-FR↓-AMC (N-CBZ-Phe-Arg-aminomethylcoumarin) by detecting the level of AMC released with time. A typical reaction was initiated by injecting a 5 μL stock solution of Z-FR↓-AMC (1 mg/mL) into 400 μL of a 3 μM hS4 solution prepared in 20 mM Tris-HCl and 100 mM NaCl (pH 7.6) containing glycerol (v/v). The enzyme:substrate ratio is thus fixed at 1:6.7, the substrate concentration being 20 μM. In another set of experiments, the substrate concentration was increased to 40 μM. Even though the hS4 solution was already equilibrated at 37 °C in the fluorometer cuvette and the addition of the substrate solution did not change the temperature appreciably, a half-minute period was introduced between enzyme−substrate mixing and measurement of the time-base fluorescence of AMC at 460 nm (360 nm excitation). Kinetics were monitored using a Fluoromax 4P (Jobin-Yvon) or JASCO FP-8300 fluorometer equipped with temperature controller to an accuracy of ±0.2 °C.

Measurement of Dissociation of CO from Nativelike Ferrocytochrome *c* (NCO). The details of this experiment have been described previously.[45] Briefly, cytochrome c is unfolded in 10 M urea and 0.1 M phosphate (pH 7), reduced under nitrogen by the addition of sodium dithionite to a final concentration of 2 μM, and liganded with CO by passing the gas gently into the solution for 1 min. The CO-bound unfolded cytochrome c is then diluted 101-fold into the same buffer containing a desired solvent additive, and the escape of CO with time is measured by monitoring the optical density at 550 nm. In the experiments presented here, CO dissociation was measured as a function of two simultaneous solvent additives, urea in the range of 0−3 M and glycerol in the range of 0−60% (v/v). All steps in the experiment were conducted in a strictly anaerobic atmosphere at 25 °C. Peltier-thermostated UV-3101 PC (Shimadzu) and Cary 100 (Varian) spectrophotometers were used to record time-base changes in optical density.

RESULTS

The Cytochrome *c* Unfolding Rate Is Exceedingly Overdamped. To determine how closely protein unfolding rate follows Kramers provision, the rate constant for the unfolding of cytochorme c was measured at 3.8 M GdnHCl in the presence of a variable level of glycerol. The choice of these experimental conditions relied upon no major change in the conformational stability of cytochrome c at a solvent viscosity of up to ∼29 cP (see below), complete unfolding of the protein even at a very high level of the viscogen, and a single kinetic phase for protein unfolding measured by stopped-flow or manual mixing (Figure 1a,b). The results show that unfolding rate does

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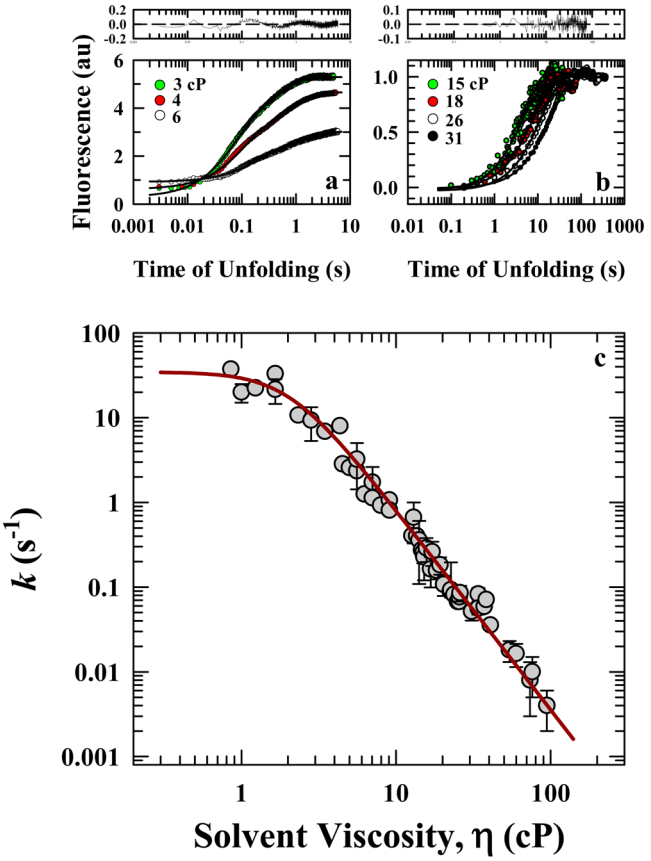

Figure 1. Unfolding of cytochrome *c* in 3.8 M GdnHCl and 15 mM phosphate (pH 7). Shown are some kinetic traces obtained by (a) stopped-flow and (b) manual mixing at the indicated values of glycerol viscosity. Solid lines through data represent two-exponential fits, where the minor phase that accounts for ∼10% of the total amplitude is included to improve the quality of fits. (c) Rate coefficient of the major unfolding phase as a function of solvent viscosity. The solid line is the fit to eq 2 with fit parameters $C = 1.3 \times 10^{10}$ cP s^{-1}, $n = 2.4$, $\sigma = 5.3 \pm 0.4$ cP, and ΔG = 10.4 kcal mol^{-1} (Table 1). Error bars were generated by multiple repeats of experiments.

decrease with solvent viscosity η (Figure 1c), but only by a deviation from that specified by the Kramers equation

$$k = \frac{C}{\eta} \exp\left(-\frac{\Delta G}{RT}\right) \quad (1)$$

where η is assumed to be proportional to friction coefficient γ (Stokes law) and parameter C contains the details of potential energy surfaces for the native protein and the transition barrier of height ΔG. The data in Figure 1c are simulated best by

$$k = \frac{C}{\eta^n + \sigma} \exp\left(-\frac{\Delta G}{RT}\right) \quad (2)$$

where n is the solvent viscosity exponent and σ represents the intrinsic viscosity for the protein. The fit yields $C = 1.3 \times 10^{10}$ cP s^{-1}, $n = 2.4$, $\sigma = 5.3 \pm 0.4$ cP, and ΔG = 10.4 kcal mol^{-1}, which are also listed in Table 1. The value of n here is significantly larger than fractional values ($\leq$1) reported earlier for diverse protein reactions, including the folding of a helix-forming peptide,[46] ligand association and dissociation, enzyme catalysis, and cellular secretion,[29,39,47,48] and electron transfer reactions,[25,26] suggesting substantially larger overdamping for the unfolding of cytochrome *c*. Interestingly, the value of the parameter σ, which could be identified with any nonsolvent frictional force, is fairly consistent with that obtained by Ansari et al.[49] in their study of the effect of viscosity on the rate of protein conformational changes. Although they did not include the exponent to the solvent viscosity, this set of data does not fit a Kramers-like equation should the exponent be excluded. It thus appears that the unfolding rate is strongly overdamped by the solvent viscosity when compared with results for the friction dependence of various macromolecular reactions reported to date. The higher damping here must reflect the effect of solvent viscosity on barrier crossing rather than error in the determination of unfolding rates, because the rate coefficients determined in the range of −11 to 19 °C at different glycerol percents were corrected for the Arrhenius activation energy to the reference temperature of 10 °C.

There remains a concern if the viscogen increases the conformational stability of the protein, in which case the retarded unfolding cannot be attributed entirely to the viscosity effect. GdnHCl-induced equilibrium unfolding experiments in different glycerol/water mixtures have not revealed a sweeping change in the value of ΔG at solvent viscosities of percent (Figure 2). Experiments at still higher viscosities are precluded by the limit of the denaturant solubility in a water/glycerol mixture, but the surmised stability increase of cytochrome *c* in going from 1 to 100 cP viscosity is roughly 4 kcal mol^{-1}. Although the significance of this change in the overdamped unfolding may appear uncertain at present, it is instructive to note that the unfolding rate in the range of 90−100 cP would have to increase by ∼2 orders of magnitude from what is reported here (Figure

Table 1. Fit Parameters to Kramers-like Equations for Different Systems in This Study

		C ($\times 10^{-10}$ cP s^{-1})	n	σ (cP)	ΔG (kcal mol^{-1})
unfolding of cytochrome *c*		1.30	2.4 ± 0.2	5.3 ± 0.4	10.4 ± 1
hS4 enzyme reaction		0.12	2.1 ± 0.2	2.1 ± 0.3	15.6 ± 1
	[urea] (M)	$\omega_1\omega_2$ ($\times 10^{-12}$ s^{-2})	λ_r ($\times 10^{-9}$ s^{-1})	B ($\times 10^{-9}$ cP s^{-1})	ΔG (kcal mol^{-1})
CO dissociation	0.0	12	9.4	1.8	12.6
	0.4	11	8.5	1.9	12.5
	0.8	9.2	13	2.9	12.7
	1.2	11	25	8.0	13.2
	1.6	11	31	8.5	13.4
	2.0	10	33	7.0	13.4
	2.4	19	20	5.3	13.6
	2.8	90	12	4.0	13.5
	3.0	8.9	22	9.9	13.2

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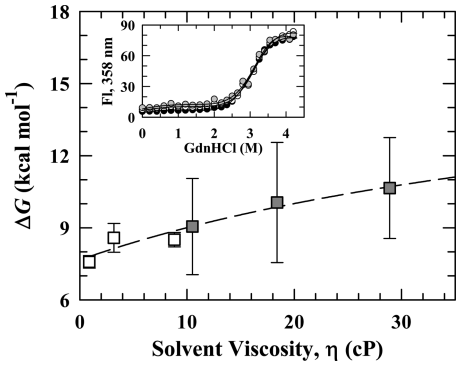

Figure 2. Stability of cytochrome *c* at different solvent viscosities produced by 53% glycerol at 19, 7.5, and −0.5 °C, pH 7, and 15 mM phosphate. Data shown as empty squares are taken from ref 38. The broken line through data points has been drawn by using the rational/empirical function $(1 + 0.0373x)/(0.13 + 0.0022x)$ and hence may not provide a physical meaning. The inset shows the equilibrium melting transitions monitored by tryptophan fluorescence at solvent viscosities of 10.5 (●), 18.4 (○), and 28.9 cP (gray circles). Solid lines represent fits according to a two-state transition with polynomial baselines.

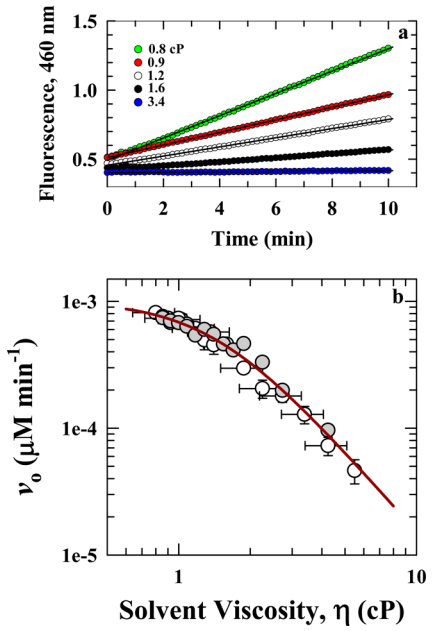

Figure 3. Catalysis by human ribosomal protein S4 (hS4) of the hydrolysis of Z-FR↓-AMC at 37 °C in 20 mM Tris-HCl and 100 mM NaCl (pH 7.6) or 40 mM sodium phosphate and 50 mM NaCl (pH 7.4). The enzyme:substrate ratio is 1:6.7 when the substrate concentration is 20 μM and 1:13.4 when 40 μM substrate is used. (a) Examples of time-base traces showing the cleavage of the substrate peptide Z-FR↓-AMC monitored by the fluorescence of the released AMC group at the indicated values of solvent viscosity. The slope obtained from the initial part of the trace provides the initial velocity (v_0) of catalytic cleavage. (b) Solvent viscosity dependence of v_0 for [S] = 20 μM (○) and [S] = 40 μM (gray circles). Data are fitted to eq 2 with parameters $C = 1.22 \times 10^9$ cP s^{-1}, $n = 2.13$, $\sigma = 2.09 \pm 0.2$ cP, and $\Delta G = 15.6$ kcal mol^{-1}.

1c) if the viscosity exponent *n* has to approach unity. Such a huge difference is highly unlikely, and hence, the *n* value is expected to remain much larger than 1. An earlier study at 25% glycerol and 283 K (3.4 cP) also did not find any considerable effect of viscogen-induced stability on the folding rate of cytochrome *c*.[21]

Higher Overdamping in the Cysteine Protease Activity of Ribosomal Protein S4. In this set of experiments, human ribosomal protein S4 (hS4) that has been recently described as a cysteine protease[44,50] was allowed to cleave the synthetic substrate Z-FR↓-AMC (N-CBZ-Phe-Arg-aminomethylcoumarin) at different glycerol contents in 20 mM Tris-HCl and 100 mM NaCl (pH 7.6) at 37 °C. The substrate concentration in these experiments was 20 μM, which is nearly equal to the reported K_m value of 19.8 μM.[44] Experiments at a later stage were also performed using 40 μM substrate. The appearance of the profile for the solvent viscosity dependence of the initial velocity (v_0) for the substrate cleavage by hS4 at a constant enzyme:substrate ratio (Figure 3) is similar to that for the viscosity dependence of cytochrome *c* unfolding (Figure 1), and the same Kramers-like empirical relation (eq 2) fits the data best. The catalysis reaction

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} [ES] \xrightarrow{k_2} E + P$$

where E, S, and [ES] are the enzyme, substrate, and enzyme–substrate complex, respectively, is consistent with the Michaelis–Menten mechanism.[50] Values of parameters fit to data in Figure 3 (Table 1) do not provide information about the shape and size of the transition barrier for the rate-limiting step ($k_2 \approx k_{cat}$) in a straightforward manner. The magnitude of the *C* value, which is small here, is independent of the solvent friction and is related to detail of the potential energy surface. More importantly, the exponent for the bulk viscosity ($n = 2.13$) is considerably larger than the 0–1 bound reported by many viscosity dependence studies of enzyme catalysis,[7,8,10−15] suggesting stronger overdamping of k_2. The $n > 2$ result is consistently obtained in all experiments performed under the condition of $[S] \geq K_m$ (Figure 3).

The CO Dissociation Rate in Nativelike Cytochrome *c* Appears To Reach Saturation at Higher Viscosity. This experiment is different from the two described above and involves direct measurement of the thermal dissociation of CO bound to the ferrous heme of cytochrome *c*. Briefly, CO is not a natural ligand for cytochrome *c* heme, but when CO-bound nativelike cytochrome *c* (cyt-Fe^{2+}-CO) is prepared by allowing the CO-liganded unfolded protein to refold, the trapped CO escapes due to thermally activated dissociation of the Fe^{2+}–CO bond (cyt-Fe^{2+}–CO → cyt-Fe^{2+} + CO). The CO dissociation rate, k_{diss}, can be determined from time-base absorbance measurement at 550 nm, which is the λ_{max} for cyt-Fe^{2+}–CO (Figure 4a). The experiment presented here measures the solvent viscosity dependence of k_{diss} for differently native states of CO-bound cytochrome *c* prepared by including low levels of urea in the refolding buffer. The urea variable was introduced to learn more about dynamics and the conformation of subdenatured states.

Figure 4b shows a decrease in k_{diss} with increasing values of both variables: solvent viscosity and urea concentration. The logarithmic plot of k_{diss} and solvent viscosity at all concentrations of urea shows an initial decrease that tends to level off at higher viscosities (Figure 4c). This behavior, which is quite different from the two sets of results presented above (Figures 1 and 3), has been described previously for the folding of nativelike cyt-Fe^{2+}–CO by laser photolysis of the CO ligand,[38] and the analysis is based on the idea of frequency-dependent friction due to Grote and Hynes in conjunction with defect diffusion dynamics.[37,40] Briefly, the Langevin equation is generalized by replacing the constant friction in the Stokes law with time-dependent friction, and the random force autocorrelation function is factored into

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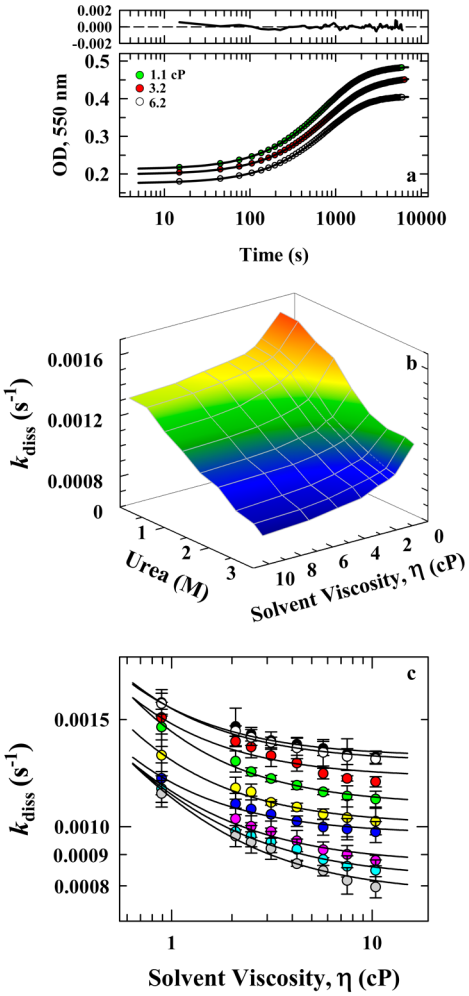

Figure 4. Thermal dissociation of CO according to cyt-Fe^{2+}–CO → cyt-Fe^{2+} + CO in 0.1 M phosphate buffer (pH 7) at 25 °C, as described in the text. (a) The reaction is monitored by time-base 550 nm absorbance due to cyt-Fe^{2+}. The traces at indicated values of solvent viscosity are fitted to a single-exponential function to extract the rate of CO dissociation, k_{diss}. (b) Variation of k_{diss} as a function of two simultaneous variables, solvent viscosity and urea. (c) The behavior of k_{diss} as a function of solvent viscosity is shown for several subdenaturing concentrations of urea (0, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, and 3.0 M) in order of decreasing k_{diss}. Solid lines through data are calculated according to eq 3 derived using GH theory in conjunction with internal dynamics of the protein.

solvent-dependent global fluctuations and solvent-decoupled local modes relevant for the CO dissociation process.[37,38] The global motions are still assumed to follow Kramers-like behavior. The final expression for the observed dissociation rate works out to[38]

$$k_{diss} \approx 3.28 \times 10^{-17}\omega_1\omega_2\left\{\frac{\lambda_r + B/\eta}{1 - \exp\left[-\left(\frac{\lambda_r + B/\eta}{\gamma_D}\right)\right]^{0.5}}\right\}\exp\left(-\frac{\Delta G}{RT}\right) \quad (3)$$

where ω_1 and ω_2 are frequencies of the reactant well and the barrier, respectively, λ_r is the unstable reactive motion at the barrier, B ($\sim k_{KRAMERS}\eta$) represents the product of the over-damped Kramers rate and solvent viscosity η, and γ_D is related to the diffusion coefficient for the solvent-independent local modes involved in the reaction. The γ_D dependency of the fit is conditional, because it is connected with global fluctuation and reactive frequency. A lower limit of 10 ns has been assumed for $1/\gamma_D$.[38] Fits to the η dependence of k_{diss} at all urea concentrations are shown in Figure 4c, and parameter values are listed in Table 1.

While a general deviation of η dependence of k_{diss} from Kramers rate theory can be concluded clearly from results up to this stage, urea-dependent variations of the model-related parameters provide further information. Figure 5a presents k_{diss} versus urea under several isoviscous conditions, where the gradient $(\partial k_{diss}/\partial[\text{urea}])_\eta$ appears to be constant within error, suggesting that the structure of the transition state for CO dissociation $[\text{cyt-Fe}^{2+}\text{–CO}]^{\ddagger}$ is not altered by glycerol viscosity. The frequency parameter $\omega_1\omega_2$ is insensitive to the presence of the denaturant urea in the reaction (Figure 5b), but model

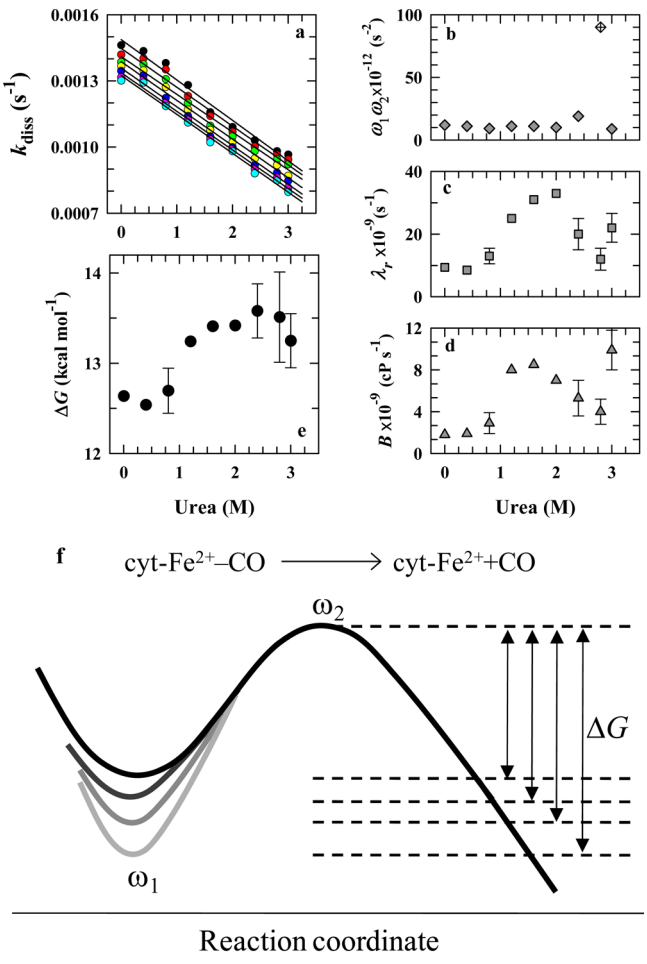

Figure 5. Protein stabilization by subdenaturing levels of urea and 0.1 M phosphate buffer (pH 7) at 25 °C. (a) Urea dependence of k_{diss} under isoviscous conditions. Lines with decreasing ordinate values correspond to 2.1, 2.5, 3.1, 4.2, 5.7, 7.5, and 10.4 cP, in order. (b–e) Urea dependence of fit parameters $\omega_1\omega_2$, λ_r, B, and ΔG for thermally activated dissociation of CO in the reaction cyt-Fe^{2+}–CO → cyt-Fe^{2+} + CO. The value of $\omega_1\omega_2$ is nearly constant (the crossed diamond is an outlier), but the trend of urea-dependent variation of λ_r, B, and ΔG is similar. The variation of ΔG indicates initial energetic stabilization of the protein up to ~2 M urea. At still higher urea concentrations, the denaturing effect of urea sets in, causing a decrease in barrier height ΔG. (f) Possible explanation for the urea-dependent change in ΔG. Although solvent viscosity leaves the barrier height and frequency unchanged, urea-mediated intraprotein cross-linking lowers the free energy of the reactant (black to gray), so as to increase ΔG.

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parameters λ_r, B, and ΔG follow the characteristic trend of a broad peak near 2 ± 0.5 M urea (Figure 5c−e). Of particular interest is ΔG versus urea (Figure 5e), because it pertains to energetic stabilization of the protein by the denaturant. Because glycerol presumably does not perturb the transition state (Figure 5a), the observed urea-dependent changes in ΔG must be due to vertical shifts of the reactant surface (Figure 5f). Binding of urea to the protein mediates intraprotein cross-links that stabilize the protein within the subdenaturing limit, but at still higher levels of the denaturant, its effect on protein structure unfolding sets in. This inference is consistent with the earlier report of protein stabilization by subdenaturing levels of urea.[45,51,52] We have refrained from interpreting the urea dependence of barrier parameters $\omega_1\omega_2$, B ($\sim k_{KRAMERS}\eta$), and λ_r, because they cannot be interpreted in a straightforward manner, but trends of their variation with urea indicate the robustness of the analysis.

DISCUSSION

Higher Overdamping. The version of the empirical equation used here to describe the dependence of protein unfolding and enzyme reaction rates on effective viscosity includes both the exponent n of the solvent's dynamic viscosity[47] and nonsolvent viscosity contribution σ.[49] The n values determined for unfolding and enzyme reactions are at least 2-fold larger than the 0−1 range suggested earlier for a variety of reactions, including enzyme catalysis,[7−16] which means the degree of overdamping can be much higher than what has been perceived so far. This is not a trivial result, because neither phenomenological explanations of data[48] nor GH theory[40] allows the n value to exceed unity. Incomplete coupling of the protein interior with the solvent viscosity is generally thought to give rise to fractional viscosity exponents observed empirically,[53,54] and the maximal value for the exponent has been projected to be ∼0.8.[34,48] The GH theory at the high-friction limit also finds a weaker dependence of the reaction rate on viscosity than what the Kramers equation predicts,[40] even though the theory does not directly invoke a fractional exponent to viscosity in the rate expression.

There is no theory at present to account for overdamping in excess of that permitted by the Kramers equation. On a qualitative note, the conditions of the stable-state picture (SSP) of chemical reactions[55] can be examined for some clues. SSP holds that the internal rate constant for equilibration of the stable reactant is larger than the barrier rate constant, and because the latter is rate-limiting, it is identified as the true reaction rate constant. In cases in which the rate constant for internal equilibration becomes smaller than the barrier rate constant, the observed rate constant for an irreversible reaction like the protein unfolding or the enzyme reaction is given by[40]

$$k_{obs} = \left(\frac{1}{1 + \frac{k_f}{k_R}}\right) k_f \qquad (4)$$

where k_f is the rate constant for over-the-barrier loss of the activated state and k_R is the relaxation rate constant that determines internal equilibration of the reactant. If the protein in the stable reactant side of the column becomes dynamically sluggish because of interaction with glycerol, then the condition of $k_R < k_f$ will hold. The value of k_R will continue to diminish with larger increments of the viscogen. The observed reaction rate is now determined not only by the passage of the activated state over the barrier alone but also by the internal relaxation rate within the reactant state. This in fact may be the case for many protein reactions in the presence of a large amount of glycerol, which can turn the protein exceedingly sluggish. A version of this argument is also found in the study of the viscosity dependence of myoglobin conformational change, where the conformational substates are thought to be frozen.[49]

With regard to higher overdamping in the case of hS4-catalyzed hydrolysis of Z-FR↓-AMC (Figure 3), the structural features of the protein may also play a role. Enzyme hS4 itself is intrinsically disordered, the N-terminal domain in particular. Conformational disorder is recognized from our current difficulty of obtaining the NMR structure of full-length hS4, consistent with the earlier report of the solution structure of a truncated eubacterial S4.[56] Dynamic disorder and large amplitude fluctuations, which are characteristics of intrinsically disordered proteins,[57−59] are suspected to produce larger chain solvation and dynamic constraints on the reactant [ES] so as to reduce the rate constant for internal relaxation (k_R). The possibility of this factor contributing to extra overdamping is consistent with the general thought that the response of enzyme reaction rates to solvent viscosity is dependent on internal dynamics and viscosity-dependent structural fluctuations of enzymes.[7,34,47,60−62]

In the context of overdamping results, two factors vis-à-vis properties of glycerol warrant a discussion. One, glycerol is a tremendous viscogen whose presence at high concentrations and low temperatures can affect the folding and conformational stability of the protein in a rather complex manner. While glycerol stabilizes by preferential hydration of proteins,[63] a low temperature destabilizes by cold denaturation, and manifestations of the two counteracting effects individually or in combination vary in a protein-dependent manner. The protein may not be largely cold-denatured at temperatures near 263 K as is the case here, but the destabilization at low temperatures may be compensated partly or wholly by stabilization due to glycerol. Existing evidence does not suggest overwhelming stabilization of cytochrome c at moderately high glycerol concentrations (Figure 2).[21,38] Two, the high value of viscosity exponent n and hence overdamping might also seem to imply that the experimental results are due to nonequilibrium effects if the relaxation time of glycerol is longer than the measurement time. High concentrations of glycerol, much higher than those used in experiments described here, supercooled to temperatures approaching the glass transition (∼200 K) do show extremely slow heterogeneous relaxation,[64] but the pattern of temperature dependence of the average rotation time reported by Zondervan et al.[64] suggests that glycerol under temperature conditions of the present study would relax faster than the experimental measurement time. Even if granted, these apparent caveats due to high glycerol and low temperature do not negate the general overdamping result presented in this work. To justify, the hS4 enzyme reaction has been studied in a glycerol/water mixture merely up to 50% and at the physiological temperature of 37 °C (Figure 3). These are simple and benign conditions under which difficulties arising from high glycerol and low temperature are encountered little, yet the dependence of the initial velocity (v_0) in the solvent viscosity range of 0.8−5.5 cP is highly overdamped (n = 2.13).

Rate Saturation at Higher Viscosity. Regardless of whether reactions are exceedingly overdamped, the general inverse rate−viscosity dependence means the reaction ceases to occur at infinite viscosity. On the contrary, the rate of the thermally activated dissociation of CO from nativelike cytochrome c is saturated at higher viscosity (Figure 4). This

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behavior has been observed also in past studies of photoisomerization of *trans*-stilbene,[5,6] reactions of CO and O_2 from within the myoglobin matrix,[27,37] and the folding of a frustrated nativelike ferrocytochrome *c*.[38]

This kind of deviation from the Kramers equation that seems to arise when the time between successive solute−solvent collisions in the barrier region is comparable with the barrier crossing time of the activated protein has been analyzed using the GH model. The model invokes a frequency-dependent friction to describe the correlation between random forces of the solvent and provides a reactive frequency λ_r that represents the unstable reactive motion in the barrier region. Consistent with this idea, the viscosity dependence of ligand dissociation kinetics of myoglobin[37] and folding of nativelike cytochrome *c*[38] have been analyzed to show that the rate saturation occurs at high viscosity, because λ_r exceeds in magnitude the global fluctuation of the protein. The same model (eq 3) has been used to analyze the present set of data that reproduces the rate saturation at high solvent viscosity. Calculations have also shown that the hindrance to barrier passage in the low-viscosity regime where global fluctuations are fast is due to the usual friction, but stronger memory effects in the higher-viscosity region hinder the escape over the barrier.[65] The model thus associates the high-viscosity rate saturation with stronger non-Markovian diffusion effects, which is a basic assumption in GH theory.[40] It follows that the barrier top is relatively sharp in this case, contrasting the impression of flat barriers for the strongly overdamped cases of cytochrome *c* unfolding and hS4-catalyzed reaction. This conjecture is based entirely on the observation of the weak dependence of the rate on viscosity, which means a shorter transit time over the barrier. It is acknowledged that understanding the details of barrier potentials in diffusion-dependent intramolecular activated processes is a long-standing problem, and analyses often lead to physically unreasonable barrier pictures.[3,6]

Solvent Viscosity Does Not Change the Barrier Properties. In the barrier region, the solvent friction influences only the motion of the activated state so that barrier recrossings are committed. The barrier parameters, including shape and height, are not altered. Therefore, if the rate coefficient is a continuous function of both viscosity and another variable (Figure 4b) where the latter affects the rate independently, the rate−variable gradient will not change under different isoviscous conditions. In CO dissociation experiments presented here (Figures 4 and 5a), the viscogen does not influence the mechanism by which urea exerts a slowing effect on the rate of CO dissociation.

Urea Influences the Rate of CO Dissociation by Modulating the Internal Dynamics of the Protein. Subdenaturing amounts of chemical denaturants stabilize proteins mainly through polyfunctional binding interactions,[45,51,66−69] which serve to cross-link different parts of the backbone and side chains leading to dynamic stiffness and greater internal friction.[51] The overall effect is fewer intramolecular thermal collisions, and hence a decrease in the rate of thermally activated dissociation of CO observed in rate−urea profiles under isoviscous conditions (Figure 5a). However, the Kramers-like dependence of the rate coefficient on viscosity (Figure 4c) reflects the influence of friction on both global fluctuations and the coupling of the local−global fluctuations in the barrier region. This is indeed true; however, to appreciate the magnitude of the observed rate constant, one needs to consider the process of reactant activation, as well. To do so, we again invoke SSP under the condition $k_R \ll k_f$ for the irreversible process cyt-Fe^{2+}−CO → cyt-Fe^{2+} + CO (eq 4). Because internal dynamics and hence internal equilibration rates are scaled by the degree of urea-mediated cross-linking within the reactant cytochrome *c* molecule, the $k_f \gg k_R$ condition prevails, and hence, an increase in the urea level within the subdenaturing limit decreases the rate of CO dissociation.

Denaturant-Altered Barrier Heights Can Be Determined by Kramers-like Analysis. Whereas solvent viscosity does not affect the transition barrier, chemical denaturants can independently alter the barrier height along the reaction coordinate. Kramers-like rate analysis under such two-variable conditions can provide some information about the effect of denaturant on barrier height even though the barrier properties are left unchanged by the bulk friction variable (Figure 5a). To illustrate, the barrier height (ΔG) for the reaction Fe^{2+}−CO → cyt-Fe^{2+} + CO increases with the denaturant up to ∼2 M urea and declines at higher levels (Figure 5e,f). The initial rise is within subdenaturing amounts of urea and is due to the protein stabilizing effect of the denaturant that shifts the reactant well vertically. The decrease in ΔG at higher urea concentrations (Figure 5e) is due to protein destabilization and unfolding.[45,51]

Deviation of Results from Kramers Rate Theory. One often comes across expressions referring to whether Kramers theory can be applied to condensed phase reactions. This need not be a question, because most of the theoretical framework has already been laid out for understanding thermally activated reactions,[3] and no modification to Kramers equations appears necessary.[16] Here, we provide evidence of critical overdamping in the cases of protein unfolding and enzyme reactions and have invoked the GH idea of frequency-dependent friction to deal with a special case of ligand dissociation. The basic problem in experimental work of condensed phase macromolecular reactions is the difficulty of knowing the actual reaction friction, which will have contributions from solvent, cosolvent, and the reactant itself. Concerns regarding replacement of friction with viscosity in experimental studies of Kramers theory have been raised by many from time to time.[23,47,70] The friction intrinsic to the reactant also surfaces in a different guise.[23,49,71] There could also be a concern for the modulation of the activation barrier by solvent density. The degrees of freedom representing the heat bath for energy dissipation can vary largely from system to system, depending on solute−solvent, solute−cosolvent, and solute−solute interactions. These difficulties are not confined to experimental studies alone; they are faced in theoretical and numerical analyses, as well.[3] Experiments to this end are performed with many-body interactive systems, and rate−viscosity results are weighed with the theory of an activated particle subjected to dissipative escape as envisaged by Kramers. It is this practical chasm between theory and experiments that produces the apparent inconsistency between the two yet makes the world of macromolecules a wonderful place in which to dwell.

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Notes

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■ ABBREVIATIONS

cyt *c*, cytochrome *c*; hS4, human ribosomal protein S4; Z-FR-AMC, N-CBZ-Phe-Arg aminomethylcoumarin; cyt-Fe^{2+}−CO, CO-bound nativelike cytochrome *c*; SSP, stable-state picture.

■ REFERENCES

(1) Kramers, H. A. (1940) Brownian motion in a field of force and the diffusion model of chemical reactions. *Physica 7*, 284−304.

(2) Portman, J. J., Takada, S., and Wolynes, P. G. (2001) Microscopic theory of protein folding rates. II: local reaction coordinates and chain dynamics. *J. Chem. Phys. 114*, 5082−5096.

(3) Hanggi, P., Talkner, P., and Borkovec, M. (1990) Reaction rate theory: fifty years after Kramers. *Rev. Mod. Phys. 62*, 251−341.

(4) Long, F. H., and Eisenthel, K. B. (1995) Ultrafast photochemistry and salvation in liquids and at liquid interfaces. In *Ultrafast Processes in Chemistry and Photobiology* (El-Sayed, M. A., Tanaka, I., and Molin, Y., Eds.) IUPAC.

(5) Brey, K. A., Gary, B., Schuster, G. B., and Drickamer, H. G. (1979) High-pressure study of the effect of viscosity on fluorescence and photoisomerization of trans-stilbene. *J. Am. Chem. Soc. 101*, 129−134.

(6) Rothenberger, G., Negus, D. K., and Hochstrasser, R. M. (1983) Solvent influence on photoisomerization dynamics. *J. Chem. Phys. 79*, 5360−5367.

(7) Gavish, B., and Werber, M. M. (1979) Viscosity-dependent structural fluctuations in enzyme catalysis. *Biochemistry 18*, 1269−1275.

(8) Gavish, B. (1986) in *The Fluctuating Enzyme* (Welch, G. R., Ed.) pp 263−339, John Wiley, New York.

(9) Ng, K., and Rosenberg, A. (1991) Possible coupling of chemical to structural dynamics in subtilisin BPN′ catalyzed hydrolysis. *Biophys. Chem. 39*, 57−68.

(10) Demchenko, A. P., Rusyn, O. I., and Saburova, E. A. (1989) Kinetics of the lactate dehydrogenase reaction in high-viscosity media. *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol. 998*, 196−203.

(11) Ricci, G., Caccuri, A. M., Lo Bello, M. L., Rosato, N., Mei, G., Nicotra, M., Chiessi, E., Mazzetti, A. P., and Federici, G. (1996) Structural Flexibility modulates the activity of human glutathione transferase P1−1. *J. Biol. Chem. 271*, 16187−16192.

(12) Frauenfelder, H., Wolynes, P. G., and Austin, R. H. (1999) Biological physics. *Rev. Mod. Phys. 71*, S419−S430.

(13) Uribe, S., and Sampedro, J. G. (2003) Measuring solution viscosity and its effect on enzyme activity. *Biol. Proced. Online 5*, 108−115.

(14) Frauenfelder, H., Fenimore, P. W., Chen, G., and McMahon, B. H. (2006) Protein folding is slaved to solvent motions. *Proc. Natl. Acad. Sci. U. S. A. 103*, 15469−15472.

(15) Loveridge, E. J., Tey, L.-H., and Allemann, R. K. (2010) Solvent effects on catalysis by *Escherichia coli* dihydrofolate reductase. *J. Am. Chem. Soc. 132*, 1137−1143.

(16) Sitnitsky, A. E. (2010) Model for solvent viscosity effect on enzymatic reactions. *Chem. Phys. 369*, 37−42.

(17) Chrunyk, B. A., and Matthews, C. R. (1990) Role of diffusion in the folding of the α subunit of tryptophan synthase from *Escherichia coli*. *Biochemistry 29*, 2149−2154.

(18) Klimov, D. K., and Thirumalai, D. (1997) Viscosity dependence of the folding rates of proteins. *Phys. Rev. Lett. 79*, 317−320.

(19) Zagrovic, B., and Pande, V. J. (2003) Solvent viscosity dependence of the folding rate of a small protein: distributed computing study. *J. Comput. Chem. 24*, 1432−1436.

(20) Plaxco, K. W., and Baker, D. (1998) Limited internal friction in the rate-limiting step of a two-state protein folding reaction. *Proc. Natl. Acad. Sci. U. S. A. 95*, 13591−13596.

(21) Bhattacharyya, R. P., and Sosnick, T. R. (1999) Viscosity dependence of the folding kinetics of a dimeric and monomeric coiled coil. *Biochemistry 38*, 2601−2609.

(22) Ramos, C. H. I., Weisbuch, S., and Jamin, M. (2007) Diffusive motions control the folding and unfolding kinetics of the apomyoglobin pH 4 molten globule intermediate. *Biochemistry 46*, 4379−4389.

(23) Hagen, S. J. (2010) Solvent viscosity and friction in protein folding dynamics. *Curr. Protein Pept. Sci. 11*, 385−395.

(24) Bieri, O., Wirz, J., Hellrung, B., Schutkowski, M., Drewello, M., and Kiefhaber, T. (1999) The speed limit for protein folding measured by triplet-triplet energy transfer. *Proc. Natl. Acad. Sci. U. S. A. 96*, 9597−9601.

(25) Pletneva, E. V., Fulton, D. B., Kohzuma, T., and Kostic, N. (2000) Protein docking and gated electron-transfer reactions between zinc cytochrome *c* and the new plastocyanin from the fern *Dryopteris crassirhizoma*. Direct kinetic evidence for multiple binary complexes. *J. Am. Chem. Soc. 122*, 1034−1046.

(26) Feng, C., Kedia, R. V., Hazzard, J. T., Hurley, J. K., Tollin, G., and Enemark, J. H. (2002) The effect of solvent viscosity on intramolecular electron transfer in sulfite oxidase. *Biochemistry 41*, 5816−5821.

(27) Beece, D., Eisenstein, L., Frauenfelder, H., Good, D., Marden, M. C., Reinisch, L., Reynolds, A. H., Sorensen, L. B., and Yue, K. T. (1980) Solvent viscosity and protein dynamics. *Biochemistry 19*, 5147−5157.

(28) Lavalette, D., and Tetreau, C. (1988) Viscosity-dependent energy barriers and equilibrium conformational fluctuations in oxygen recombination with hemerythrin. *Eur. J. Biochem. 177*, 97−108.

(29) Kleinert, T., Doster, W., Leyser, H., Petry, W., Schwarz, V., and Settles, M. (1998) Solvent composition and viscosity effects on the kinetics of CO binding to horse myoglobin. *Biochemistry 37*, 717−733.

(30) Gregory, R. G., Rosenberg, A., Knox, D., and Percy, A. J. (1990) The thermodynamics of hydrogen isotope exchange in lysozyme. *Biopolymers 29*, 1175−1183.

(31) Somogyi, B., Norman, J. A., Zempel, L., and Rosenberg, A. (1988) Viscosity and transient solvent accessibility of Trp-63 in the native conformation of lysozyme. *Biophys. Chem. 32*, 1−13.

(32) Rosenberg, A., Ng, K., and Punyiczki, M. (1989) Activity and viscosity effects on the structural dynamics of globular proteins in mixed solvent systems. *J. Mol. Liq. 42*, 31−43.

(33) Chung, H. S., and Eaton, W. A. (2013) Single-molecule fluorescence probes dynamics of barrier crossing. *Nature 502*, 685−688.

(34) Sitnitsky, A. E. (2008) Solvent viscosity dependence for enzymatic reactions. *Phys. A 387*, 5483−5497.

(35) Loveridge, E. J., Evans, R. M., and Allemann, R. K. (2008) Solvent effects on environmentally coupled hydrogen tunnelling during catalysis by dihydrofolate reductase from *Thermotoga maritimas. Chem. - Eur. J. 14*, 10782−10788.

(36) Bag, B. C., Hu, C.-K., and Li, M. S. (2010) Colored noise, folding rates and departure from Kramers behavior. *Phys. Chem. Chem. Phys. 12*, 11753−11762.

(37) Doster, W. (1983) Viscosity scaling and protein dynamics. *Biophys. Chem. 17*, 97−103.

(38) Kumar, R., and Bhuyan, A. K. (2008) Viscosity scaling for the glassy phase of protein folding. *J. Phys. Chem. B 112*, 12549−12554.

(39) Oh-oka, H., Iwaki, M., and Itoh, S. (1997) Viscosity dependence of the electron transfer rate from bound cytochrome *c* to p840 in the photosynthetic reaction center of the green sulfur bacterium *Chlorobium tepidum*. *Biochemistry 36*, 9267−9272.

(40) Grote, R. F., and Hynes, J. T. (1980) The stable state picture of chemical reactions. II. Rate constants for condensed and gas phase reaction models. *J. Chem. Phys. 73*, 2715−2732.

(41) Sheely, M. L. (1932) Glycerol viscosity tables. *Ind. Eng. Chem. 24*, 1060−1064.

(42) Hasinoff, B. B. (1977) The diffusion-controlled reaction kinetics of the binding of CO and O_2 to myoglobin in glycerol-water mixtures of high viscosity. *Arch. Biochem. Biophys. 183*, 176−188.

(43) Kawahara, K., and Tanford, C. (1966) Viscosity and density of aqueous solutions of urea and guanidine hydrochloride. *J. Biol. Chem. 241*, 3228−3232.

(44) Sudhamalla, B., Kumar, M., Kumar, R. S., Sashi, P., Yasin, U. M., Ramakrishna, D., Rao, P. N., and Bhuyan, A. K. (2013) Enzyme dimension of the ribosomal protein S4 across plant and animal kingdoms. *Biochim. Biophys. Acta, Gen. Subj. 1830*, 5335−5341.

DOI: 10.1021/acs.biochem.5b00315
Biochemistry 2015, 54, 4453−4461

(45) Bhuyan, A. K. (2002) (200) Protein stabilization by urea and guanidine hydrochloride. *Biochemistry 41*, 13386−13394.
(46) Jas, G. S., Eaton, W. A., and Hofrichter, J. (2001) Effect of viscosity on the kinetics of α-helix and β-hairpin formation. *J. Phys. Chem. B 105*, 261−272.
(47) Gavish, B., and Yedgar, S. (1995) Solvent viscosity effect on protein dynamics: Updating the concepts. In *Protein-Solvent Interactions* (Gregory, R. B., Ed.) Marcel Dekker, New York.
(48) Yedgar, S., Tetreau, C., Gavish, B., and Lavalette, D. (1995) Viscosity dependence of O_2 escape from respiratory proteins as a function of cosolvent molecular weight. *Biophys. J. 68*, 665−670.
(49) Ansari, A., Jones, C. M., Henry, E. R., Hofrichter, J., and Eaton, W. A. (1992) The role of solvent viscosity in the dynamics of protein conformational changes. *Science 256*, 1796−1798.
(50) Sudhamalla, B., Kumar, M., Roy, K. R., Kumar, R. S., and Bhuyan, A. K. (2013) Cysteine endoprotease activity of human ribosomal protein S4 is entirely due to the C-terminal domain, and is consistent with Michaelis-Menten mechanism. *Biochim. Biophys. Acta, Gen. Subj. 1830*, 5342−5349.
(51) Kumar, R., Prabhu, N. P., Yadaiah, M., and Bhuyan, A. K. (2004) Protein stiffening and entropic stabilization in the subdenaturing limit of guanidine hydrochloride. *Biophys. J. 87*, 2656−2662.
(52) Yasin, U. M., Sashi, P., and Bhuyan, A. K. (2014) Free energy landscape of lysozyme: multiple near-native conformational states and rollover in the urea dependence of folding energy. *J. Phys. Chem. B 118*, 6662−6669.
(53) Gavish, B. (1980) Position-dependent viscosity effects on rate coefficients. *Phys. Rev. Lett. 44*, 1160−1163.
(54) Barshtein, G., Almagor, A., Yedgar, S., and Gavish, B. (1995) Inhomogeneity of viscous aqueous solutions. *Phys. Rev. E: Stat. Phys., Plasmas, Fluids, Relat. Interdiscip. Top. 52*, 555−557.
(55) Northrup, S. H., and Hynes, J. T. (1980) The stable state picture of chemical reactions. I. Formulation for rate constants and initial condition effects. *J. Chem. Phys. 73*, 2700−2714.
(56) Markus, M. A., Gerstner, R. B., Draper, D. E., and Torchia, D. A. (1998) The solution structure of ribosomal protein S4 Δ41 reveals two subdomains and a positively charged surface that may interact with RNA. *EMBO J. 17*, 4559−4571.
(57) Dyson, H. J., and Wright, P. E. (2001) Nuclear magnetic resonance methods for elucidation of structure and dynamics in disordered states. *Methods Enzymol. 339*, 258−270.
(58) Mittag, T., and Forman-Kay, J. D. (2007) Atomic-level characterization of disordered protein ensembles. *Curr. Opin. Struct. Biol. 17*, 3−14.
(59) Mittag, T., Marsh, J., Grishaev, A., Orlicky, S., Lin, H., Sicheri, F., Tyers, M., and Forman-Kay, J. D. (2010) Structure/function implications in a dynamic complex of the intrinsically disordered Sic1 with the Cdc4 subunit of an SCF ubiquitin ligase. *Structure 18*, 494−506.
(60) Agarwal, P. K. (2005) Role of protein dynamics in reaction rate enhancement by enzymes. *J. Am. Chem. Soc. 127*, 15248−15256.
(61) Lodola, A., Sirirak, J., Fey, N., Rivara, S., Mor, M., and Mulholland, A. S. (2010) Structural fluctuations in enzyme catalyzed reactions: determinants of reactivity in fatty acid amide hydrolase from multivariate statistical analysis of quantum mechanics/molecular mechanics paths. *J. Chem. Theory Comput. 6*, 2948−2960.
(62) Musiani, F., Ippoliti, E., Micheletti, C., Carloni, P., and Ciurli, S. (2013) Conformationla fluctuations of UreG, an intrinsically disordered enzyme. *Biochemistry 52*, 2949−2954.
(63) Gekko, K., and Timasheff, S. N. (1981) Mechanism of protein stabilization by glycerol: preferential hydration in glycerol-water mixtures. *Biochemistry 20*, 4667−4676.
(64) Zondervan, R., Kulzer, F., Berkhout, G. C. G., and Orrit, M. (2007) Local viscosity of supercooled glycerol near T_g probed by rotational diffusion of ensembles and single dye molecules. *Proc. Natl. Acad. Sci. U. S. A. 104*, 12628−12633.
(65) Kolomietz, V. M., and Radionov, S. V. (2011) Non-Markovian diffusion over potential barrier in the presence of periodic time modulation. *Phys. Rev. E: Stat. Phys., Plasmas, Fluids, Relat. Interdiscip. Top. 84*, 051123.
(66) Pace, C. N., Laurents, D. V., and Thomson, J. A. (1990) pH dependence of the urea and guanidine hydrochloride denaturation of ribonuclease A and ribonuclease T1. *Biochemistry 29*, 2564−2572.
(67) Mayr, L. M., and Schmid, F. X. (1993) Stabilization of a protein by guanidine hydrochloride. *Biochemistry 32*, 7994−7998.
(68) Zarrine-Afsar, A., Mittermaier, A., Kay, L. E., and Davidson, A. R. (2006) Protein stabilization by specific binding of guanidinium to a functional arginine bidning surface on an SH3 domain. *Protein Sci. 15*, 162−170.
(69) Kumar, S., Sharma, D., and Kumar, R. (2014) Effect of urea and alkylureas on the stability and structural fluctuation of the M-80 containing Ω-loop of horse cytochrome *c*. *Biochim. Biophys. Acta, Proteins Proteomics 1844*, 641−655.
(70) Sekhar, A., Latham, M. P., Vallurupalli, P., and Kay, L. E. (2014) Viscosity-dependent kinetics of protein conformational exchange: microviscosity effects and the need for a small viscogen. *J. Phys. Chem. B 118*, 4546−4551.
(71) Pradeep, L., and Udgaonkar, J. B. (2007) Diffusional barrier in the unfolding of a small protein. *J. Mol. Biol. 366*, 1016−1028.

DOI: 10.1021/acs.biochem.5b00315
Biochemistry 2015, 54, 4453−4461