Protein Stabilization by Urea and Guanidine Hydrochloride†

Abani K. Bhuyan*

School of Chemistry, University of Hyderabad, Hyderabad 500046, India

Received May 20, 2002

ABSTRACT: The urea, guanidine hydrochloride, salt, and temperature dependence of the rate of dissociation of CO from a nonequilibrium state of CO-bound native ferrocytochrome *c* has been studied at pH 7. The heme iron of ferrocytochrome *c* in the presence of denaturing concentrations of guanidine hydrochloride (GdnHCl) and urea prepared in 0.1 M phosphate, pH 7, binds CO. When the unfolded protein solution is diluted 101-fold into CO-free folding buffer, the protein chain refolds completely, leaving the CO molecule bonded to the heme iron. Subsequently, slow thermal dissociation of the CO molecule yields to the heme coordination of the native M80 ligand. Thus, the reaction monitors the rate of thermal conversion of the CO-liganded native ferrocytochrome *c* to the M80-liganded native protein. The rate of this reaction, k_{diss}, shows a characteristic dependence on the presence of nondenaturing concentrations of the denaturants in the reaction medium. The rate decreases by ∼1.9−3-fold as the concentration of GdnHCl in the refolding medium increases from nearly 0 to ∼2.1 M. Similarly, the rate decreases by 1.8-fold as the urea concentration is raised from 0.l to ∼5 M. At still higher concentrations of the denaturants the denaturing effect sets in, the protein is destabilized, and hence the CO dissociation rate increases sharply. The activation energy of the reaction, E_a, increases when the denaturant concentration in the reaction medium is raised: from 24.1 to 28.3 kcal mol^{-1} for a 0.05−2.1 M rise in GdnHCl and from 25.2 to 26.9 kcal mol^{-1} for a 0.1−26.9 M increase in urea. Corresponding to these increases in denaturant concentrations are also increases in the activation entropy, S_{diss}/R, where *R* is the gas constant of the reaction. The denaturant dependence of these kinetic and thermodynamic parameters of the CO dissociation reaction suggests that binding interactions with GdnHCl and urea can increase the structural and energetic stability of ferrocytochrome *c* up to the limit of the subdenaturing concentrations of the additives. NaCl and Na_2SO_4, which stabilize proteins through their salting-in effect, also decrease the rate with a corresponding increase in activation entropy of CO dissociation from CO-bound native ferrocytochrome *c*, lending support to the view that low concentrations of GdnHCl and urea stabilize proteins. These results have direct relevance to the understanding and interpretation of the free energy−denaturant relationship and protein folding chevrons.

Despite their everyday use in protein folding studies the exact mechanism of action of GdnHCl and urea on proteins is not understood. Available information, including the results of isothermal calorimetry (*1*) and X-ray crystallography in the presence of these two denaturants (*2*−*4*), indicates binding or interaction of GdnHCl and urea to folded and unfolded states of proteins. It is held that the number of denaturant binding sites is more in the unfolded state than in the native state (*1*) and unfolding results from exposure of extra binding sites concomitant with additional interaction between the protein and the denaturant as the concentration of the latter is increased (*5*).

But how the structural and energetic properties of proteins change with varying concentrations of the denaturants is not clear. Analyses of X-ray data for certain protein crystals soaked in low concentrations of GdnHCl and urea have shown a reduced average isotropic MSD (mean square deviations), suggesting a reduction in mobility of native proteins (*3*, *4*). Constraints on intramolecular dynamics are expected to reduce the amplitudes of thermal fluctuations. While the former has been determined directly from X-ray diffraction (*3*, *4*), and indirectly from isothermal calorimetric data (*1*), biochemical data providing evidence for reduced thermal fluctuations in the presence of low concentrations of denaturants do not exist.

In studies with ferrocytochrome *c* presented in this paper low concentrations of GdnHCl and urea have been shown to reduce the spatial displacements of thermal fluctuations. It is found that the protein stability increases as the solvent composition is altered from strongly native to moderately native conditions by the addition of GdnHCl and urea. When the concentrations of the denaturants are increased further, their stabilizing effect is overwhelmed by their own protein unfolding effect. The stabilizing effect has been deduced from thermodynamic and kinetic properties of thermal dissociation of CO from CO-bound ferrocytochrome *c*. The heme of native ferrocytochrome *c* does not bind CO. High-affinity binding, however, occurs when the protein is

† This work was supported by a grant (BRB/15/227/2001) from the Department of Biotechnology, Government of India, and by the School of Chemistry, University of Hyderabad. A.K.B. is the recipient of a Swarnajayanti Fellowship from the Department of Science and Technology, Government of India.

* Address for correspondence: e-mail, akbsc@uohyd.ernet.in; fax, 91-40-3012460.

10.1021/bi020371n CCC: $22.00 © 2002 American Chemical Society
Published on Web 10/17/2002

unfolded in the presence of GdnHCl or is destabilized strongly by addition of higher concentrations of urea. The native state of ferrocytochrome *c* prepared by allowing the CO-bound unfolded protein to refold carries the CO molecule still bonded to the heme iron. The trapped CO, however, escapes as thermal motions facilitate the dissociation of the $Fe^{2+}-CO$ bond. In the present study the properties of this dissociation reaction in the 0−3.7 M range of GdnHCl and 0−6.9 M range of urea have been analyzed to show that subdenaturing concentrations of both denaturants stabilize the protein.

MATERIALS AND METHODS

Horse heart cytochrome *c* (type VI from Sigma) was used without further purification. Urea and GdnHCl were obtained from Gibco BRL. Sodium dithionite was purchased from Merck. Sodium chloride and sodium sulfate were obtained from Sigma. Protein concentration was determined by the use of molar extinction coefficients. All experiments were done in 0.1 M sodium phosphate buffer in an inert atmosphere. Extreme care was taken to minimize air exposure of solutions at all stages of the experiments. During low-temperature measurements vapor fogging and condensation on the outer surfaces of cuvettes and flow cells were prevented by blowing a constant stream of nitrogen.

Preparation of CO-Liganded Native Ferrocytochrome c and Measurement and Analysis of CO Dissociation Kinetics. Cytochrome *c* (1.8 mM), initially dissolved in 6.35 M GdnHCl or 10 M urea, 0.1 M phosphate, pH 7, was deaerated and reduced by adding sodium dithionite to a final concentration of 1.8 μM. Ferrocytochrome *c* thus obtained is completely unfolded when the protein solution contains 6.35 M GdnHCl but is only denatured in the presence of 10 M urea. The heme iron of ferrocytochrome *c* was then liganded with CO by bubbling the gas gently into the solution for ~30 s. Because of binding of CO preferentially to the unfolded state of the protein, the mass action principle applies, and hence the urea-denatured ferrocytochrome *c* unfolds completely. The CO-bound unfolded ferrocytochrome *c* (UCO) was then diluted 101-fold by transferring 20 μL of the protein solution into a cuvette containing 2 mL of the degassed and dithionite-reduced CO-free refolding buffer (0.1 M phosphate, pH 7, 22 °C, containing the desired solvent additive). This procedure allows complete refolding of ferrocytochrome *c* with the CO molecule still bonded to the heme iron of the native protein (NCO).

The kinetics of dissociation of CO from NCO was recorded by time-resolved spectra in the visible region (500−600 nm) or by time dependence of the absorbance change at 549 nm. The dead time of measurement (i.e., the time elapsed between refolding the CO-liganded unfolded protein and recording the first time point of kinetics of CO dissociation) was ~15 s. The CO dissociation kinetics were slow, and the generation of a single kinetic trace required data collection for several hours; the lower the temperature, the longer the time required. Measurements were done using a UV-3101 PC (Shimadzu) UV−vis−NIR spectrophotometer interfaced to a TCC controller for temperature regulation.

Rates of CO dissociation were extracted from single-exponential fits of the kinetic data. Use of a double-exponential function did not improve the fit.

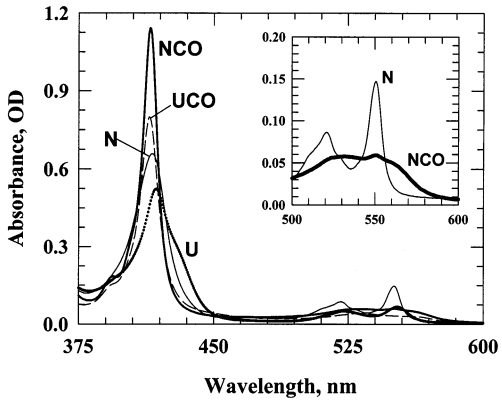

FIGURE 1: Soret−visible optical absorption spectra of U, UCO, NCO, and N, all at pH 7, 0.1 M sodium phosphate, 22 °C. The U species is ferrocytochrome *c*, unfolded in 6.35 M GdnHCl and reduced by adding sodium dithionite to a final concentration of 1.8 μM. UCO is obtained by adding 1 atm of CO to U. The NCO state is refolded CO-bound ferrocytochrome *c* and was obtained by diluting the UCO solution into the refolding buffer (100 mM phosphate, pH 7) to a final denaturant concentration of 1.1 M GdnHCl. N is native ferrocytochrome *c*, where M80 is the iron ligand. The N spectrum was generated by dissolving the native protein directly in the buffer containing 1.1 M GdnHCl. The inset shows the spectral region and the spectra used to monitor the CO dissociation process in the reaction NCO → N + CO.

Stopped-Flow Measurement of Unfolding Kinetics of Ferrocytochrome c in the Absence of CO. Kinetics of GdnHCl-induced unfolding of native ferrocytochrome *c* were monitored at 10 °C by both Soret heme absorbance and tryptophan fluorescence probes. In stopped-flow experiments, native cytochrome *c* (0.09 and 0.4 mM for Soret heme absorbance and fluorescence measurements, respectively), prepared in 0.1 M phosphate, pH 7, and 1 M GdnHCl, was reduced by the addition of <5 mM sodium dithionite in an inert atmosphere. Two buffers, one containing ~8 M GdnHCl and another with no denaturant, both at neutral pH, were reduced by the addition of ~0.5−1 mM dithionite shortly before the experiment. These two buffers and the reduced protein solution, all filled in gastight syringes, were loaded in the stopped-flow syringes cooled to 10 °C and equilibrated for 15 min. Unfolding to different final concentrations of GdnHCl was initiated by mixing 25 μL of the native protein solution with 275 μL of the two buffers mixed variably. The final concentrations of the unfolded protein were 7.5 and 33 μM in Soret absorbance and fluorescence-monitored kinetics, respectively. These measurements were done using a SFM4 stopped-flow instrument (Biologic). A 0.8 mm square flow cell was used. The dead time of the instrument, determined by using the procedure prescribed by the instrument manufacturer, was ~1.5−2 ms.

RESULTS

The U, UCO, NCO, and N Forms of Ferrocytochrome c. Figure 1 shows the changes in the heme absorption spectrum in the reaction sequence U → UCO → NCO. Ferrocytochrome *c*, unfolded in 6.35 M GdnHCl (U), was allowed to react with 1 atm of CO to obtain UCO. When the UCO solution is diluted into the refolding buffer such that the final concentration of the denaturant in the refolding milieu is ~1.1 M, the NCO spectrum is obtained. The N spectrum was generated by dissolving the protein directly in 0.1 M

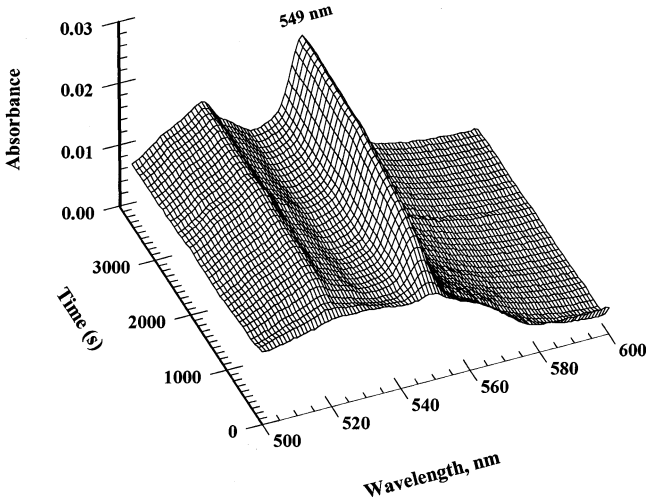

FIGURE 2: Time-resolved visible absorption spectrum showing thermal dissociation of CO according to the reaction NCO → N + CO, following refolding of UCO to NCO in the presence of 0.05 M GdnHCl, pH 7, 28 °C.

phosphate buffer containing 1.1 M GdnHCl. This N spectrum is of course not distinguishable from the N spectrum obtained after complete dissociation of CO molecules from the NCO sample. Band positions and associated extinction coefficients of the spectrum of individual species are distinct. The spectrum of NCO is, however, more similar to the spectrum of UCO than to that of N. This similarity in the appearance of spectra of UCO and NCO only indicates that the CO molecule is still liganded to the ferrous heme of the refolded protein. In terms of secondary and tertiary structural contents and overall molecular conformation, as assessed from fluorescence and CD spectra, and NMR chemical shift dispersions, the NCO state is very similar to native ferrocytochrome *c* (data not shown). The heme absorption spectrum reports faithfully that native ferrocytochrome *c* obtained by refolding the UCO molecule contains CO in the place of M80 as the axial heme ligand.

The inset to Figure 1 enlarges the 500−600 nm region of the spectra of NCO and N. The NCO spectrum, recorded within 15 s of diluting the UCO solution from 6.35 to 1.1 M GdnHCl (pH 7, 22 °C), shows featureless broad bands which develop within a few hours into distinct α and *β* bands (549.5 and 520 nm, respectively), indicating the occurrence of slow NCO → N + CO conversion due to thermally activated dissociation of CO. The conversion is essentially the Fe^{2+}−CO → Fe^{2+}−M80 replacement and does not involve any major conformational adjustment.

Thermal Dissociation of CO from NCO. Figure 2 presents the primary data showing the spectral evolution associated with the NCO → N process after allowing UCO to refold to ∼0.05 M GdnHCl at 28 °C, pH 7. Immediately after the refolding reaction (UCO → NCO) has occurred, the visible absorption spectrum of NCO appears similar to that of UCO, indicating that CO remains bonded to the ferrous heme. The absorbance at the heme $\pi \rightarrow \pi^*$ bands, 520 nm (*β*) and 549.5 nm (α), increases exponentially with time due to dissociation of CO (NCO → N + CO) yielding to the formation of the Fe^{2+}−M80 bond. The time dependence of changes in absorbance of α and *β* bands is best described by a single-exponential function. Use of a double-exponential function did not improve the fit. Because the kinetics of CO dissociation are slow, the rates were extracted more conveniently by recording the absorbance change at 549 nm alone as a function of time.

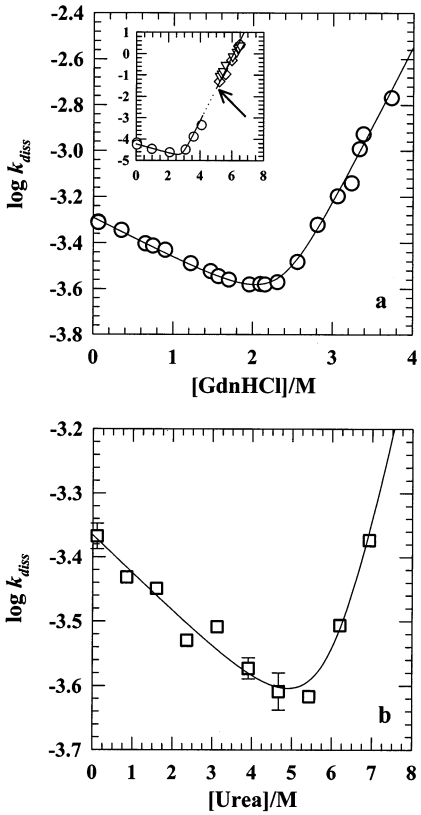

FIGURE 3: (a) GdnHCl-dependent changes in the rate of CO dissociation, k_{diss}, at 22 °C and at 10 °C (inset). In the inset the k_{diss}−GdnHCl gradient is shown to connect the slope of the stopped-flow unfolding rate of the protein, k_u, as a function of GdnHCl measured at 10 °C. k_u values were measured by changes in tryptophan fluorescence (◇) and 430 nm Soret heme absorbance (▽). The arrow points to the C_m of equilibrium unfolding of ferrocytochrome *c* at 10 °C, 0.1 M phosphate, pH 7. (b) Urea dependence of k_{diss} at 22 °C, 0.1 M phosphate, pH 7. The decrease in k_{diss} has been interpreted to originate from the protein stabilizing effect of the denaturants. In higher concentrations of denaturants k_{diss} values increase because of the protein destabilizing/unfolding effect. The lines through the data in both panels have been drawn by inspection only.

The refolding protocol employed involves 101-fold dilution of the UCO solution with CO-free folding buffer so that the concentration of CO in the refolding medium is substantially reduced. Therefore, the CO binding rate to N formed by dissociation of CO from NCO is negligible. Together with the fact that the Fe^{2+}−M80 bond in the native conformation of ferrocytochrome *c* is extra stable (*6*), the rate obtained from the spectra in Figure 2 can be equated to the CO dissociation rate, k_{diss}. The dissociation process described here, unlike photodissociation, is thermally driven and, hence, is slow. The value of k_{diss} varies with the intensity of the monitoring beam of the spectrophotometer and can be attributed to a small degree of photodissociation of NCO. To avoid any significant variation in the extent of photodissociation from one kinetic run to another, all experimental data were taken under identical setting of parameters for absorbance measurements.

Denaturant Dependence of k_{diss}. Figure 3a shows the GdnHCl concentration dependence of the logarithm of k_{diss},

the rate of CO dissociation, measured at 22 °C. As the final concentration of GdnHCl in the refolding medium is raised, k_{diss} initially decreases and then increases, displaying an inflection centered around 2.1 M GdnHCl. In going from 0.05 to 2 M GdnHCl the value of k_{diss} decreases 1.9-fold. This "chevron-like" feature of the log k_{diss}−GdnHCl space was seen consistently in several experiments both at 22 °C and at 10 °C (Figure 3 and the inset); k_{diss} decreases by ∼2−3-fold as the denaturant concentration in the folding medium is raised from 0 to ∼2.1 M and increases sharply thereafter. The decrease suggests that GdnHCl tends to block the dissociation of CO from NCO to form N, and the increase in k_{diss} can be interpreted to arise from protein destabilization and structural unfolding in higher concentrations of the denaturant facilitating the dissociation of CO. The GdnHCl dependence of the stopped-flow unfolding rate of ferrocytochrome *c*, k_u, at 10 °C (Figure 3, inset) illustrates the protein destabilization effect. The slope defined by the linear dependence of log k_u on GdnHCl appears similar to the positive slope of the GdnHCl dependence of log k_{diss}.

Figure 3b shows the log k_{diss}−urea space at 22 °C. k_{diss} decreases by ∼1.8-fold as the urea concentration increases from 0.1 to ∼5 M, suggesting that urea prolongs the lifetime of NCO by stabilizing the protein. At still higher concentrations of the denaturant the unfolding effect of the denaturant sets in, and hence k_{diss} increases sharply. Thus, the feature of dependence of the CO dissociation rate on denaturant concentration is similar for GdnHCl and urea. GdnHCl is a stronger protein denaturant than urea. Accordingly, the minima in the log k_{diss}−denaturant spaces are observed at ∼2.1 M GdnHCl and ∼5 M urea, respectively (Figure 3).

Effects of NaCl and Na_2SO_4 on the Rate of CO Dissociation. If the decrease in the value of k_{diss} with increasing concentration of GdnHCl and urea, as exhibited by left limbs of the log k_{diss}−denaturant chevrons (Figure 3), is indeed due to increase in protein stability in the presence of the denaturants, then known protein stabilizers are also expected to retard the rate of CO dissociation. To check for this expectation, the rate of the NCO → N + CO reaction was measured as a function NaCl and Na_2SO_4. In these experiments the UCO solution (6.35 M GdnHCl, 0.1 M phosphate, pH 7, 22 °C) was diluted 101-fold into the refolding buffer containing no denaturant but different concentrations of a salt additive. Thus, the final concentration of GdnHCl in the refolding medium was ∼0.05 M.

Figure 4 shows the logarithm of k_{diss} as a function of molar concentrations of NaCl and Na_2SO_4. Retardation in the CO dissociation rate is clearly seen for both NaCl and Na_2SO_4. The value of k_{diss} decreases 1.6-fold when the concentration of NaCl in the medium is raised to 2.5 M, and in the 0−2 M range of Na_2SO_4 concentration the rate decreases 1.8-fold. The exact functional dependence of k_{diss} on the concentration of the salt additives has not been evaluated, and the solid lines in the figure show apparent trends only. These observations strengthen the implication that the observed decrease in the rate of the NCO → N + CO reaction in the presence of GdnHCl and urea is due to NCO stabilization by the denaturants.

Additive Effect of NaCl and Urea. Since both NaCl and urea (<5 M) individually decrease the rate of the NCO → N + CO reaction, coexistence of the two in the reaction medium is expected to produce a cumulative effect on the rate of CO dissociation. Figure 5 shows the logarithm of k_{diss} as a function of urea in the absence and the presence of 1 M NaCl. In the presence of the salt the profile is shifted vertically down to lower k_{diss}. A slight horizontal shift toward higher concentration of urea is also apparent. The negative gradients within the stabilizing range of urea concentration (i.e., the slopes of the left limbs) are very similar for the two curves. Both vertical and horizontal shifts indicate the cumulative effect of urea and NaCl on stabilization of NCO. The added stability of NCO in the presence of 1 M NaCl results in deceleration of both the dissociation of CO from NCO (vertical shift) and the unfolding of NCO by urea (horizontal shift).

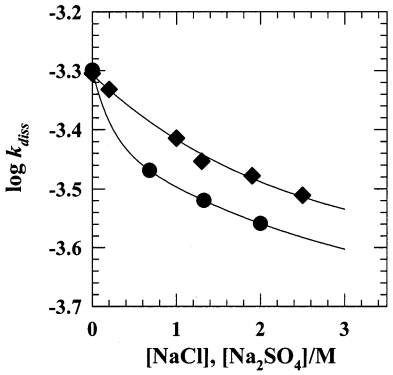

FIGURE 4: Effect of NaCl (◆) and Na_2SO_4 (●) on the rate of the reaction, NCO → N + CO, at 22 °C, pH 7. The reaction medium contained no denaturant. The lines through data have been drawn to guide the eye only and do not represent a functional dependence of k_{diss} on salt concentrations.

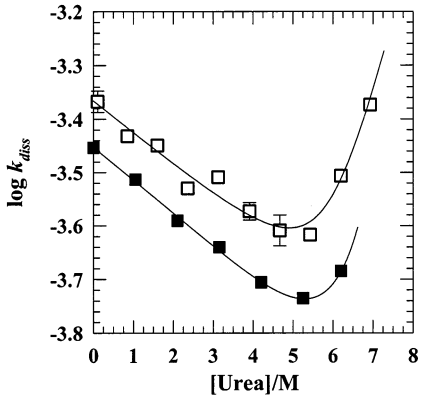

FIGURE 5: Dependence of the rate of the NCO → N + CO reaction on urea concentration in the absence (□) and in the presence (■) of 1 M NaCl, 22 °C, pH 7. The lines through the data have been drawn by inspection only.

Dependence of Activation Enthalpy and Entropy of CO Dissociation on the Concentration of GdnHCl, Urea, NaCl, and Na_2SO_4. A decrease in the rate of dissociation of CO in the presence of a stabilizing solvent additive would mean that the energy barrier separating the bound state of CO (NCO) from the unbound one (N + CO) is relatively higher. If the decrease in the magnitude of the rate coefficient is the result of stabilization of NCO by the stabilizing additives used (GdnHCl, urea, NaCl, and Na_2SO_4), the decrease in entropy in the presence of these agents must be compensated by an increase in activation enthalpy of the CO dissociation

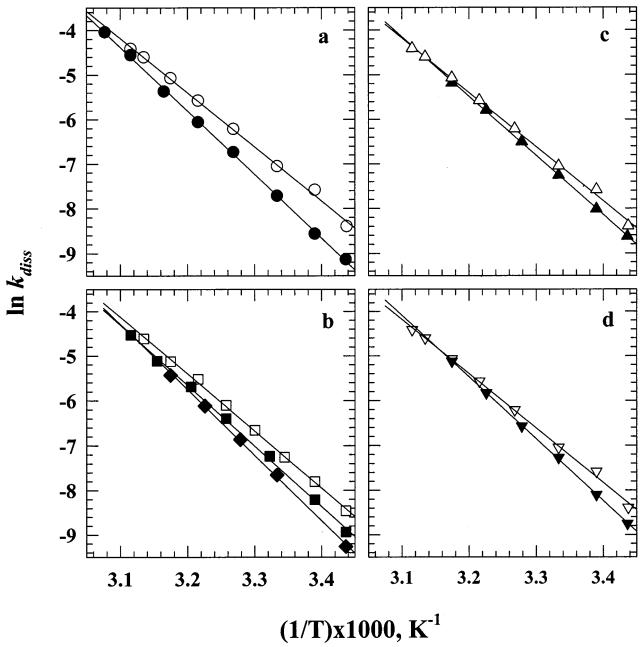

FIGURE 6: Arrhenius plots for the CO dissociation reaction, 0.1 M phosphate, pH 7, with (a) 0.05 M (○) and 2.1 M (●) GdnHCl, (b) 0.1 M (□) and 4.15 M urea without (■) and with (◆) 1 M NaCl, (c) no additives (△) and 2 M NaCl (▲), and (d) no additives (▽) and 1.33 M Na_2SO_4 (▼). Activation energies, frequency factors, and activation entropies are listed in Table 1.

reaction. This is understood from the relation

$$H_{diss}/RT = \ln(\nu/k_{diss}) + S_{diss}/R \quad (1)$$

obtained by casting the transition state equation

$$k_{diss} = \nu \exp\left(-\frac{G_{diss}}{RT}\right) \quad (2)$$

in terms of $G_{diss} = H_{diss} - TS_{diss}$, where the vibrational frequency, $\nu = kT/h \approx 10^{13}$ s^{-1} (k and h are Boltzmann's and Planck's constants), and G_{diss}, H_{diss}, and S_{diss} are changes in free energy, enthalpy, and entropy, respectively, between the initial CO-bound state and the transition state. The enthalpy and entropy changes associated with the CO dissociation reaction can be determined conveniently by using the Arrhenius equation

$$k_{diss} = A \exp(-E_a/RT) \quad (3)$$

where A is the frequency factor (s^{-1}) and E_a is the activation energy (kcal mol^{-1}). Comparison of eqs 1 and 3 gives

$$E_a = H_{diss}$$
$$\ln(A/\nu) = S_{diss}/R \quad (4)$$

Figure 6 shows the Arrhenius plots for the CO dissociation reaction in the presence of 0.05 and 2.1 M GdnHCl (Figure 6a), 0.1 M urea without salt and 4.15 M urea in the presence and the absence of 1 M NaCl (Figure 6b), 0.05 M GdnHCl in the presence and the absence of 2 M NaCl (Figure 6c), and 0.05 M GdnHCl in the presence and the absence of 1.33 M Na_2SO_4 (Figure 6d). The activation energies, frequency factors, and entropy changes are listed in Table 1. The data show clearly that in the presence of the stabilizing additives

Table 1: Activation Energies (E_a), Frequency Factors (A), and Entropy Changes (S_{diss}/R) for CO Dissociation from Ferrocytochrome *c* in 0.1 M Phosphate, pH 7[a]

stabilizing additive	additive concn, M	E_a (=H_{diss}), kcal mol^{-1}	log A, s^{-1}	S_{diss}/R
GdnHCl	0.05	24.1	14.6	+3.6
	2.10	28.3	17.3	+9.9
urea	0.10[b]	25.2	15.4	+5.4
	4.15 (−1 M NaCl)	26.9	16.3	+7.7
	4.15 (+1 M NaCl)	29.1	17.9	+11.2
NaCl	2.0	26.4	16.1	+7.1
Na_2SO_4	1.33	27.5	16.8	+8.8

[a] Errors in values of E_a and log A are <1 kcal mol^{-1} and 1.1 s^{-1}, respectively, and the corresponding error in S_{diss}/R is ∼2. The error values were determined from three or more independent measurements. Note that S_{diss}/R, the entropy change, is expressed here as a dimensionless quantity. In the text entropy values are given in the unit of k_B (eqs 6 and 7). [b] Values of E_a, log A, and S_{diss}/R shown under this condition represent upper bounds (see text for details).

the activation energy for CO dissociation increases. For measurements of k_{diss} in the presence of 0.1 M urea the UCO species (i.e., the CO-liganded unfolded ferrocytochrome *c*) was prepared in the presence of 10 M urea. Some crystals of urea were seen due to the cooling effect of nitrogen gas that was used for reducing the solution of unfolded cytochrome *c* prior to the addition of CO gas. This undesirable situation may have introduced relatively large errors for this set of measurements. The values reported for 0.1 M urea (Table 1) represent upper bounds. For all other measurements of k_{diss} involving urea, including those shown in Figures 3b and 5, the initial UCO state was prepared in 6.35 M GdnHCl.

DISCUSSION

In this study a nativelike conformation of ferrocytochrome *c*, in which the Fe^{2+}−M80 bonding is replaced by Fe^{2+}−CO, has been prepared. Since native ferrocytochrome *c* does not bind CO, the CO-liganded engineered conformation, NCO, is unstable and is converted to native ferrocytochrome *c*, N, by slow thermal dissociation of CO. Effects of low concentrations of the protein denaturants, GdnHCl and urea, and the protein stabilizing ionic additives, NaCl and Na_2SO_4, on the rate of dissociation of CO at 22 °C have been examined. The CO dissociation is decelerated by lower concentrations of the protein denaturants but is accelerated at higher concentrations. Temperature dependence of the CO dissociation rate, k_{diss}, both in the presence and in the absence of the solvent additives indicates that in the presence of lower concentrations of the denaturants, where CO dissociation is decelerated, the activation energy is relatively higher. The discussion in the following endeavors to relate these results to the protein stabilizing effect of lower concentrations of GdnHCl and urea.

The Identity of NCO and the Nature of the CO Dissociation Reaction. It is pertinent to assert at the onset of the discussion that the NCO conformation is just the native conformation of ferrocytochrome *c*, except that the Fe^{2+}−M80 bonding has been replaced by Fe^{2+}−CO, a natural consequence of allowing the UCO molecules to refold. The NCO conformation is not in equilibrium with any other conformation. It is unstable and breaks down to the native state, N, because the affinity of M80 for the heme in the native state of ferrocytochrome *c* is very high. NCO is not

a folding intermediate. It is rather the native conformation of ferrocytochrome *c* trapped by binding of CO (*7*).

The dissociation of CO dealt with here is a unimolecular thermally activated reaction, where the NCO molecules accumulate energy necessary for the dissociation reaction by fluctuation. Collisions between different groups of atoms or structural elements afforded by internal dynamics of the protein act as an intramolecular source of energy. Since NCO is an artificially prepared nonequilibrium state, the observed rate of disappearance of NCO, limited by the dissociation of CO, is actually the rate of activation of NCO:

$$d[N]/dt = k_{diss} = ck_1 \quad (5)$$

where c is a parameter related to thermal fluctuations and k_1 is the rate of activation of NCO.

Stabilization of NCO by GdnHCl and Urea. Dependence of k_{diss} on GdnHCl and Urea. The linear decrease in the rate coefficient of the NCO → N + CO reaction at lower concentrations of GdnHCl and urea (Figures 3 and 5) provides the primary indication that the denaturants stabilize the protein conformations. Similar observations are made for the dependence of log k_{diss} as a function of NaCl and Na_2SO_4 (Figure 4). These ionic additives are known to stabilize proteins when used within the range of their salting-in concentrations (*8*, *9*), suggesting that the deceleration of the dissociation of CO in subdenaturing concentrations of the denaturants is due to protein stabilization. As the concentrations of GdnHCl and urea are increased in the medium, the denaturing action sets in, and the destabilizing effect dominates. The rate then is limited by the motional modes associated with protein unfolding (Figures 3 and 5). Indeed, the positive slope of the log k_{diss}−GdnHCl chevron appears to merge with the positive slope of the unfolding limb of the folding chevron of ferrocytochrome *c* at 10 °C (inset to Figure 3a). As GdnHCl is a stronger denaturant than urea, the respective minimum in the log k_{diss}−denaturant chevrons appears at ∼2.1 and ∼5.0 M (Figure 3).

Both NCO and N molecules are stabilized, most likely to the same extent, since the conformations of the two molecules are much alike and both can interact with denaturants to the same extent. In general, the kinetics of a reaction depend on the properties of the reactants and products and also on the properties of the activated state. However, since the NCO → N + CO reaction is irreversible, for N does not bind CO readily, only the relative properties of the reactant and the activated state are relevant. The analysis is achieved by thermodynamic formulation of the conventional transition state theory (*10*).

Thermodynamic Interpretation of the Rate of CO Dissociation. In this formulation, appropriate for unimolecular reactions, the frequency factor, A, and the activation energy, E_a, of the reaction are related to the entropy and the enthalpy of activation (refs *10* and *11*, for example), respectively. Equations 1−4 show the pertinent relations, where both A and E_a are taken to be temperature independent.

The activation entropy of the CO dissociation reaction in the presence of a denaturant is positive, suggesting that the structure of NCO in the transition state, NCO*, is less ordered relative to that in its ground state (Table 1). The activation entropy increases from 3.6 units in the presence of 0.05 M GdnHCl to 9.9 units in the presence of 2.1 M GdnHCl, an ∼2.8-fold rise. Clearly, in the presence of increasing subdenaturing concentrations of GdnHCl relatively large-scale conformational constraint must be negotiated to make to the transition state. Similar observation is made in the case of urea, implying that the extent of disorder in the transition state with reference to the "ground state" increases in the presence of increasing subdenaturing concentrations of the denaturant. This picture is fully consistent with the reorganization energies required for the CO dissociation reaction, as shown by higher values of activation enthalpy, H_{diss}, corresponding to increases in the concentration of the denaturants (Figure 6, Table 1). These interpretations of rate coefficients for CO dissociation strongly suggest a protein-stabilizing role of subdenaturing concentrations of GdnHCl and urea. The thermodynamic parameters derived from the rate data in the presence of NaCl and Na_2SO_4 also show increase in activation enthalpy and entropy (Table 1). The parallelism in the changes in activation parameters observed for these protein-stabilizing ionic additives and the two denaturants lends support to the conclusion of denaturant stabilization of ferrocytochrome *c*. However, global stabilization of ferrocytochrome *c* by low concentrations of the two denaturants is not proved by this observation alone. It is rather more likely that local motions or the particular thermal fluctuations needed for CO loss are decreased in the presence of the denaturants.

Interaction of GdnHCl and Urea with Proteins. Both stabilizing and destabilizing effects of denaturants must arise from their influence on protein conformations. Unfortunately, a clear understanding of their mode of action is lacking. While denaturants can interact directly with proteins (*1*−*4*, *12*, *13*), they also exert a pronounced effect on the structure of water and, hence, on the surface hydration of proteins (*5*, *14*). The assessment of the relative importance of these effects in protein unfolding has been difficult. To make progress in the discussion of protein stabilization in the presence of subdenaturing concentrations of the denaturants, we consider the nature of direct protein−denaturant interactions. Polyfunctional interactions between protein groups and GdnHCl and urea have been known for a long time (*1*, *15*). An earlier crystallographic study of Hibbard and Tulinsky using α-chymotrypsin crystals soaked in 3.0 M urea and 2.0 M GdnHCl has indicated interactions of $GdnH^+$ ions and urea with protein (*2*). Recently reported crystal structures of proteins with urea (*3*, *4*) and GdnHCl (*4*), both present within the protein-stabilizing range of concentrations reported in this study, provide direct evidence that the denaturants interact with proteins by multiple hydrogen-bonding interactions. The illustrated binding of denaturants, particularly for RNase A (*4*), involves both the protein side-chain and backbone atoms, and the interaction modes are not only variable-length hydrogen bonding but also van der Waals interactions. No particular type of amino acid appears to be a preferential target for denaturant binding. Makhatadze and Privalov (*1*) have estimated the number of binding sites for GdnHCl and urea in the native and unfolded states of cytochrome *c*. Using their numbers for native state binding sites, it can be roughly estimated that in the range of subdenaturing concentrations of these denaturants (i.e., corresponding to the left limb of the log k_{diss}−denaturant

chevrons shown in Figures 3 and 5), under the experimental conditions employed in this study, the number of GdnHCl and urea molecules bound to cytochrome *c* will increase up to 12 and 26, respectively.

Structural, Dynamic, and Thermodynamic Consequences of Protein−Denaturant Interactions. The interactions of the denaturant molecules with different groups of the protein through noncovalent bonding can establish nonspecific networks of intramolecular interactions. Such network patterns have actually been observed in crystal structures of proteins with low concentrations of denaturants (*4*). The denaturant-mediated cross-linking interactions, however nonspecific they are, might serve to stabilize resident parts of the protein structure. The side chains of abundant surface lysines of horse cytochrome *c* are attractive groups to be bonded into such interactions. Structural ordering of some of the surface side chains seen in the crystal structure of hen lysozyme soaked in urea (*3*) provides a basis for this conjecture.

An immediate consequence of denaturant-mediated cross-linking of different parts of the protein molecule is a decrease in the motional freedom. If two to three amino acid residues are engaged in the binding of one denaturant molecule, assuming a minimum of two hydrogen bonds per molecule of the denaturant (*1*, *3*, *4*), the internal motion at the sites of some 25 residues of cytochrome *c* will be constrained when 2 M GdnHCl is present. Similarly, in the presence of ∼4 M urea, roughly 50 residues would experience constrained dynamics. Existing X-ray data on lysozyme, ribonuclease A, and dihydrofolate reductase do show a significant decrease in the *B*-factor of side-chain and backbone atoms in the presence of low concentrations of GdnHCl and urea compared to the native conformations in the absence of denaturants (*3*, *4*). The substantial reduction in motional amplitudes in the presence of subdenaturing concentrations of GdnHCl and urea conceivably reduces the magnitude of subglobal unfolding motions. Cross-linking of different groups increases barriers to motions in the more compact conformers of the protein. In this scenario, denaturant stabilization of the protein, akin to low-temperature stabilization, originates from a reduction in fluctuations in the positions of individual or clusters of atoms around their average. We call this effect denaturant-induced protein stiffening.

Restricted dynamics in the presence of low concentrations of denaturants must alter the thermodynamic properties of the protein molecule, most obviously, the conformational entropy of the system. In the presence of a subdenaturing concentration of the denaturant, the average conformation of the cytochrome *c* molecule, relative to that in an aqueous solution, undergoes a "disorder-to-order" structural transition. This entropy-expensive process could support local folding reactions that are expected to contribute to the structural stability of the protein.

The magnitude of the entropy loss of ferrocytochrome *c* in the presence of subdenaturing concentrations of a denaturant, with reference to the entropy of the native protein in the aqueous solution, can be estimated easily. Consider the Arrhenius frequency factors, A, and activation entropies, S_{diss}, for the CO dissociation reaction in the presence of 0.05 and 2.1 M GdnHCl and 1.33 M Na_2SO_4 (Table 1). The rate of dissociation of CO from NCO by the reaction NCO → N + CO, k_{diss}, is inversely proportional to the number of NCO conformers, n_{NCO}. The Boltzmann relation

$$S = k_B \ln n \tag{6}$$

where S is the entropy and n, the number of states, can be used to express the ratio of the frequency factors in the presence of 2.1 and 0.05 M GdnHCl.

$$\frac{A_{(2.1)}}{A_{(0.05)}} = \frac{n_{NCO(2.1)}}{n_{NCO(0.05)}} = \exp\left(\frac{S_{NCO(2.1)} - S_{NCO(0.05)}}{k_B}\right) \tag{7}$$

From the values of A listed in Table 1 the ratio $A_{(2.1)}$:$A_{(0.05)}$ ≈ 500, so that $S_{NCO(2.1)} - S_{NCO(0.05)} = 6.2k_B$. This is an appreciable loss of entropy. In the absence and the presence of urea (+1 M NaCl) the entropy difference is ≈$5.8k_B$, and for the reaction in the absence and the presence of 1.33 M Na_2SO_4, it is ≈$5.1k_B$. These estimates, also obtained from thermodynamic interpretation of the rate data (eqs 1−4; Table 1), clearly show the protein stiffening effect of the denaturants.

Protein Stiffening and Entropic Stabilization. Reduction in local entropy as a result of stiffening of one or more chain segments facilitated by denaturant-mediated cross-linking leads to an increase in protein stability (see ref *16*). Oxidation state-dependent structures of cytochrome *c* themselves provide a good example of entropic stabilization of protein structures. Ferrocytochrome *c*, the oxidation state used in this study, is more stable than ferricytochrome *c* (*17*), and the protein interior of the former, compared to the latter, is more tightly packed. The relative compactness is also indicated by X-ray scattering data (*18*). For *Saccharomyces cerevisiae* iso-1-cytochrome *c* the stabilization of the oxidized protein upon reduction has been shown to be entirely entropic (*19*).

Segregation of Entropic and Electrostatic Contributions. Subdenaturing concentrations of both GdnHCl and urea stabilize cytochrome *c*; the former is ionic and the latter is not. What difference does it make? In solution, GdnHCl exists as a $GdnH^+$ cation and Cl^- anion. Protein stabilization by cation and anion binding is known (ref *8*, for example). We have suggested that $GdnH^+$ ions commit intramolecular cross-links by forming hydrogen bonds and van der Waals interactions with different nonspecific parts of the protein and, thus, stabilize the protein through entropic effect. But then $GdnH^+$ ions can also interact electrostatically with a specific cation binding site to stabilize a protein, as suggested by Mayr and Schmid (*20*). This is electrostatic stabilization that is distinct from entropic stabilization. Similarly, Cl^- anions dissociated from GdnHCl can also stabilize proteins by electrostatic effects, through Debye−Hückel charge screening, for example. The stabilization of the acid molten globule state of cytochrome *c* by low concentrations of GdnHCl, due to binding of Cl^- to the cationic sites (*21*, *22*), provides a classic example. Such interactions could exist at neutral pH also. It is possible that the Cl^- anions of GdnHCl find target sites on cytochrome *c* because of predominance of cationic lysyl side chains on the protein surface. The resulting electrostatic interactions, be they ion-pair type or charge-screening type, can stabilize cytochrome *c*. Thus, GdnHCl stabilization originates from (1) protein stiffening or entropic effect due to the cross-linking action of $GdnH^+$ cations and (2) electrostatic effect due to the interaction of

Cl^-, and also possibly of $GdnH^+$, with charged groups of the protein. While the entropic effect operates invariably, the contribution of the electrostatic effect would depend on the formation of ionic interaction(s). In the case of GdnHCl stabilization of cytochrome *c* both effects are in action. The dependence of k_{diss}, the rate of dissociation of CO in the reaction NCO → N + CO, on NaCl and Na_2SO_4 (Figure 4) shows electrostatic stabilization due to the presence of anions. What matters is the availability of anions irrespective of the source.

Urea is nonionic, and protein stabilization in its presence is entirely entropic. The extent of stabilization of cytochrome *c* by urea as such appears to be less compared to that by GdnHCl (Table 1). Addition of 1 M NaCl to the urea solution augments the stabilizing effect (see also Figure 5), and the activation energy of CO dissociation increases by ≈3 kcal mol^{-1} (Table 1). Use of eq 7 yields a value of $3.7k_B$ for the difference in the entropy of the protein in the presence and the absence of 1 M NaCl.

To summarize this section, both GdnHCl and urea stabilize proteins through protein stiffening or entropic effect, but in the case of GdnHCl electrostatic effect also can contribute to the stability depending on the extent of ionic interactions between the denaturant ions and the charged sites on the protein.

Denaturants Reduce the Amplitudes of Thermal Fluctuations. How do restricted internal dynamics resulting from denaturant binding to the protein influence the rate of the reaction? In the present study increasing denaturant concentrations within the subdenaturing limit decreases the rate of the NCO → N + CO reaction (Figures 3 and 5). This is an intramolecular reaction where thermal fluctuations break the Fe^{2+}−CO bond. If internal dynamics of cytochrome *c* are constrained because of denaturant binding, the amplitudes of thermal fluctuations responsible for the loss of CO will also be reduced. Both entropy and fluctuations have the same origin, and the entropy of the system is a measure of atomic fluctuations. Thus, a reduction in the vibrational entropy in the presence of denaturants correlates with decreased spatial displacements of thermal fluctuations required of the CO-bound heme and the surrounding atoms. Consequently, the rate of dissociation of CO decreases (Figures 3 and 5). The reduction in amplitudes of thermal fluctuations is, of course, detected as a decrease in the crystallographic *B*-factor (*23*, *24*). Consistent with this fact is the observed decrease in *B*-factor of conformations of proteins, including lysozyme, dihydrofolate reductase, and RNase A, soaked in low concentrations of denaturant solutions (*3*, *4*). The *B*-factor, of course, increases in denaturant concentrations beyond the subdenaturing limit, as has been shown for RNase S (*25*).

Denaturant-Promoted Structural Rigidity and Protein Unfolding. The rigidity of the protein caused by binding of denaturants within the limit of subdenaturing concentrations may actually promote tight interactions. Binding site flexibility allows for broadened specificity (*26*). This may come at the price of reduced affinity due to the loss of conformational entropy on binding. Conversely, a rigid binding site should result in narrow specificity and tight binding for well-matched surfaces (*26*, *27*). If this is true, the rigidity developed initially as a result of binding of low concentrations of denaturants should lead to progressively tighter binding, as their concentrations become increasingly more destabilizing to the protein. Binding of GdnHCl and urea to proteins is unfavorable thermodynamically, as suggested by their equilibrium binding constants of 0.6 and 0.061 M^{-1}, respectively (*1*). It would seem possible that their binding affinities are promoted by their initial action of making the binding surfaces rigid. The tighter binding, and the resultant destabilizing effect, eventually overruns their own protein-stabilizing effect.

Other Examples of GdnHCl Stabilization of Proteins. Stabilizing interactions between GdnHCl and at least two proteins have been reported previously. Pace et al. (*28*) have reported stabilization of RNase T1, at both acidic and neutral pH, by ≈2 kcal mol^{-1}. This effect has also been reported by Mayr and Schmid (*20*), who have shown in addition that low concentrations of GdnHCl increase thermal stability and decrease the rate of unfolding of RNase T1. Low concentrations of GdnHCl (0.5−1.4 M) have also been found to stabilize an equilibrium folding intermediate of protein disulfide isomerase by 2.3 kcal mol^{-1} (*29*). But the existence of the effect remains to be reported for a sizable set of proteins. Because the stabilization energy involved is small, ∼2 kcal mol^{-1} or less, the effect may escape detection in equilibrium unfolding experiments.

Implications in Analysis and Interpretation of Protein Folding Data. The observed denaturant stabilization of the protein under nativelike conditions, or in the pretransition region, invalidates, at least for this protein, the general procedure of estimating the free energy of unfolding in water, $\Delta G°$, within the formalism of the linear free energy model (*30*, *31*). In the present case, less stability of the protein in lower concentrations of the denaturant relative to that in, say, 2.1 M GdnHCl, would imply an increase in the equilibrium *m* value in the linear free energy relation, $\Delta G = \Delta G° - m$[GdnHCl], as strongly nativelike conditions are approached. As a consequence, the ΔG−denaturant plot in <2 M GdnHCl will deviate upward from linearity, and hence, a linear extrapolation of the ΔG−denaturant gradient backward to the ordinate would suggest an underestimated value of $\Delta G°$. Thus, for ferrocytochrome *c* the linearly extrapolated value of 18.8 kcal mol^{-1} for $\Delta G°$ (*17*) may not reflect the actual stability of the protein. Such nonlinearity, predicted from isothermal calorimetric study of protein interactions with GdnHCl and urea (*1*), has been experimentally observed for barnase (*32*).

The observed effect of GdnHCl and urea on protein stability under subdenaturing conditions will obviously affect the dependence of folding and unfolding rates on the denaturant in a rather involved manner. The rollover in the protein folding rate−denaturant space, seen for some proteins, is interpreted classically as an indication of transient accumulation of kinetic intermediate(s) (*33*). However, even two-state proteins with no apparent accumulation of kinetic intermediates can show deviation from linearity (*34*). According to the suggestion made in this study, under moderate to strongly nativelike conditions the extent of structural and energetic stability of kinetic intermediates, when present, would be convoluted by the stabilizing effect of denaturants.

Finally, while the present results are convincing to demonstrate the GdnHCl- or urea-induced stabilization of ferrocytochrome *c* at subdenaturing concentrations of the denaturants, there appears an inconsistency with results obtained from hydrogen-exchange studies. GdnHCl-induced

gradual denaturation of ferrocytochrome *c* has been observed previously in hydrogen−deuterium exchange probed by real-time NMR (*35*) and equilibrium NMR (*36*) methods. More comprehensive understanding of the present results and the nature of protein−denaturant interactions will be required to resolve this apparent conflict.

SUMMARY AND CONCLUSION

Low concentrations of GdnHCl and urea stabilize proteins. The stability increases as the denaturant concentration is raised to the limit of its subdenaturing effect. With further increase in the concentration the classical protein unfolding effect of the denaturant beats its own protein stabilizing effect. The initial stabilization is entropic due to constraints on thermal fluctuations in one or more parts of the protein brought about by noncovalent interactions of the denaturant molecules with the protein.

ACKNOWLEDGMENT

Thanks are due to Raghavan Varadarajan and Jayant Udgaonkar for discussions.

REFERENCES

1. Makhatadze, G. I., and Privalov, P. L. (1992) *J. Mol. Biol. 226*, 491−505.
2. Hibbard, L. S., and Tulinsky, A. (1978) *Biochemistry 17*, 5460−5468.
3. Pike, A. C. W., and Acharya, R. (1994) *Protein Sci. 3*, 706−710.
4. Dunbar, J., Yennawar, H. P., Banerjee, S., Luo, J., and Farber, G. K. (1997) *Protein Sci. 6*, 1272−1733.
5. Timasheff, S. N. (1992) *Biochemistry 31*, 9857−9864.
6. Schejter, A., and Plotkin, B. (1988) *Biochem. J. 255*, 353−356.
7. Bhuyan, A. K., and Kumar, R. (2002) *Biochemistry* (in press).
8. Pace, C. N., and Grimsley, G. R. (1988) *Biochemistry 27*, 3242−3246.
9. Timasheff, S. N. (1993) *Annu. Rev. Biophys. Biomol. Struct. 22*, 67−97.
10. Laidler, K. J. (1987) *Chemical Kinetics*, 3rd ed., pp 89−115, Harper & Row, New York.
11. Forst, W. (1973) *Theory of Unimolecular Reactions*, p 181, Academic Press, New York.
12. Tanford, C. (1970) *Adv. Protein Chem. 24*, 1−95.
13. Simpson, R. B., and Kauzmann, W. (1953) *J. Am. Chem. Soc. 75*, 5139−5152.
14. Breslow, R., and Guo, T. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 167−169.
15. Robinson, D. R., and Jencks, W. P (1965) *J. Am. Chem. Soc. 87*, 2462−2470.
16. Dill, K. A. (1990) *Biochemistry 29*, 7133−7155.
17. Bhuyan, A. K., and Udgaonkar, J. B. (2001) *J. Mol. Biol. 312*, 1135−1160.
18. Trewhella, J., Carlson, V. A. P., Curtis, E. H., and Heidorn, D. B. (1988) *Biochemistry 27*, 1121−1125.
19. Cohen, D. S., and Pielak, G. J. (1995) *J. Am. Chem. Soc. 117*, 1675−1677.
20. Mayr, L. M., and Schmid, F. X. (1993) *Biochemistry 32*, 7994−7998.
21. Goto, Y., Takahashi, N., and Fink, A. L. (1990) *Biochemistry 29*, 3480−3488.
22. Hagihara, Y., Aimoto, S., Fink, A. L., and Goto, Y. J. (1993) *J. Mol. Biol. 231*, 180−184.
23. Petsko, G. A., and Ringe, D. (1984) *Annu. Rev. Biophys. Bioeng. 13*, 331−371.
24. Frauenfelder, H., Parak, F., and Young, R. D. (1988) *Annu. Rev. Biophys. Biophys. Chem. 17*, 451−479.
25. Ratnaparkhi, G. S., and Varadarajan, R. (1999) *Proteins: Struct., Funct., Genet. 36*, 282−294.
26. Rader, S. D., and Agard, D. A. (1997) *Protein Sci. 6*, 1375−1386.
27. Sahu, S. C., Bhuyan, A. K., Majumdar, A., and Udgaonkar, J. B. (2000) *Proteins: Struct., Funct., Genet. 41*, 460−474.
28. Pace, C. N., Laurents, D. V., and Thomson, J. A. (1990) *Biochemistry 29*, 2564−2572.
29. Morjana, N. A., McKeone, B. J., and Gilbert, H. F. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 2107−2111.
30. Schellman, J. A. (1987) *Annu. Rev. Biophys. Biophys. Chem. 16*, 115−137.
31. Agashe, V. R., and Udgaonkar, J. B. (1995) *Biochemistry 34*, 3286−3299.
32. Johnson, C. M., and Fersht, A. R. (1995) *Biochemistry 34*, 6795−6804.
33. Matouschek, A., Kellis, J. T., Jr., Serrano, L., Bycroft, M., and Fersht, A. R. (1990) *Nature 346*, 440−445.
34. Otzen, D. E., Kristensen, O., Proctor, M., and Oliveberg, M. (1999) *Biochemistry 38*, 6499−6511.
35. Bhuyan, A. K., and Udgaonkar, J. B. (1998) *Proteins: Struct., Funct., Genet. 32*, 241−247.
36. Xu, Y., Mayne, L. C., and Englander, S. W. (1998) *Nat. Struct. Biol. 5*, 774−778.

BI020371N