Stabilization of a Protein by Guanidinium Chloride†

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ABSTRACT: Guanidinium chloride is a commonly used denaturant to unfold native proteins and to determine their Gibbs free energy of stabilization, ΔG_{stab}. Here we show that this denaturant has a dual role for the stability and the folding of the model protein ribonuclease T1. When present at low concentration (0–0.3 M), guanidinium chloride stabilizes the folded protein toward thermal and urea-induced unfolding and decreases the rate of unfolding. At high concentration the function of guanidinium chloride as a denaturant dominates and ribonuclease T1 is cooperatively unfolded. Ribonuclease T1 is also strongly stabilized by other salts, such as NaCl, at low concentrations, and the dependence of the thermal stability on salt concentration is not linear. Such a complex behavior was not found in control experiments with pancreatic ribonuclease A. The stabilization in the presence of low concentrations of guanidinium chloride originates probably from the binding of guanidinium ions to one or a few cation binding sites that exist in native ribonuclease T1. It is not observed when an additional salt, NaCl, is present simultaneously. The favorable interaction of guanidinium chloride with the native protein leads to increased values for ΔG_{stab}, when unfolding transitions induced by guanidinium chloride are analyzed on the basis of the two-state model by the linear extrapolation procedure. The noncoincidence of these ΔG_{stab} values with stability data derived from urea-induced or thermal unfolding transitions does not imply that the two-state model is not appropriate but that the linear extrapolation to zero molar denaturant is incorrect. Such deviations from linearity and a stabilization of folded proteins by ionic denaturants could be fairly common. They can easily be detected by measuring thermal or urea-induced unfolding transitions in the presence of small concentrations of the denaturant of interest.

The stability of folded proteins in aqueous solution depends strongly on the solvent conditions. Salts influence protein stability either directly by preferential binding to the native or the unfolded state or indirectly by changing the properties of the solvent water [see Timasheff (1992) for a recent review]. These indirect stabilizing or destabilizing effects are similar for different proteins. They are usually additive and tend to follow the lyotropic or Hofmeister series of cations and anions (Hofmeister, 1888; von Hippel & Wong, 1962, 1965; Tanford, 1968). The modulation of protein stability by added salts was studied in detail for several proteins, such as bovine pancreatic ribonuclease A (RNase A)[1] (von Hippel & Wong, 1965; Ginsburg & Carrol, 1965). In these experiments the midpoints of the thermal unfolding transitions depended on the concentration of these salts in a monotonic, almost linear fashion.

Guanidinium chloride (GdmCl) is one of the most powerful destabilizing salts. It is widely used to denature proteins reversibly and to determine the difference in Gibbs free energy between the folded and the unfolded states, ΔG_{stab}. Usually, a simple two-state model, $U \rightleftharpoons N$, is used to analyze unfolding transitions and ΔG_{stab} is assumed to depend linearly on denaturant concentration (Pace, 1975; Schellman, 1978, 1987; Privalov, 1992). The value for ΔG°_{stab} in the absence of denaturant is now routinely obtained by a linear extrapolation from the transition region to zero molar denaturant (Pace, 1986; Santoro & Bolen, 1988). The Gdm^+ cation is the effective unfolding agent. Its unfolding potency can be weakened by combination with a strongly stabilizing anion, such as sulfate in $(Gdm)_2SO_4$, or increased by combination with a destabilizing anion, such as thiocyanate in GdmSCN (von Hippel & Wong, 1965).

Because of its ionic character, GdmCl might, however, not always act as a denaturant only. To detect and characterize potential stabilizing effects of GdmCl, we use ribonuclease T1 (RNase T1) as a model protein. RNase T1 is markedly stabilized by the addition of salts, such as NaCl, $MgCl_2$, or spermine (Oobatake et al., 1979a,b; Pace & Grimsley, 1988; Pace, 1990; Pace et al., 1990; Kiefhaber et al., 1990; Walz & Kitareewan, 1990; Hu et al., 1992). Part of this stabilization is probably caused by cation binding to folded RNase T1, and it was proposed that Gdm^+ ions might also interact with native RNase T1 (Pace et al., 1990). The possibility of stoichiometric denaturant binding and an interference with the determination of G°_{stab} values was discussed by Schellman (1987), but direct experimental evidence for this is still lacking.

We show here that the thermal stability of RNase T1 shows a complex dependence on salt concentration. Contrary to common experience, GdmCl stabilizes the folded conformation of RNase T1 when present at concentrations lower than 0.3 M. To directly demonstrate this stabilizing contribution, we compare the effects of low concentrations of GdmCl and of NaCl on thermal and urea-induced unfolding transitions and on the unfolding kinetics of RNase T1. As controls, analogous experiments are carried out with bovine pancreatic RNase A. The implications of the observed dual role of GdmCl as a stabilizing salt at low concentration and as a denaturant at high concentration for the thermodynamic analysis of unfolding transitions will be discussed.

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[1] Abbreviations: RNase T1, ribonuclease T1 from *Aspergillus oryzae*, cloned and expressed in *Escherichia coli*; RNase A, bovine pancreatic ribonuclease A; GdmCl, guanidinium chloride; T_m, midpoint of the thermal unfolding transition; ΔG_{stab}, Gibbs free energy of the folding reaction $U \rightleftharpoons N$ of a protein.

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MATERIALS AND METHODS

Materials. Lys25-RNase T1 was cloned and expressed in *Escherichia coli* (Quaas et al., 1988) and prepared as previously described (Mayr & Schmid, 1993). Bovine pancreatic RNase A (type XII-A) was from Sigma (St. Louis, MO); urea and GdmCl (grade "ultrapure") were from Schwarz/Mann (Orangeburg, NY). All other chemicals (analytical grade) were from Merck (Darmstadt, Germany). Protein concentrations were determined spectrophotometrically in a Kontron UVIKON 860 spectrophotometer by using an extinction coefficient of 21 020 M^{-1} cm^{-1} at 278 nm for RNase T1 (Takahashi et al., 1970) and 9700 M^{-1} cm^{-1} at 277 nm for RNase A (Sage & Singer, 1962). The concentrations of NaCl, GdmCl and urea were determined by measuring the refractive index (Pace, 1986).

Thermal Unfolding Transitions. The thermal denaturations of the proteins in the presence of various concentrations of NaCl or GdmCl (0–1.0 M) were measured in 0.01 M sodium acetate, pH 5.0, in a Gilford Response II spectrophotometer with an integrated temperature programmer. The protein concentrations were 9 μM for RNase T1 and 27 μM for RNase A. The thermal transitions were followed by the decrease in absorbance at 287 nm with a resolution of 10 data points per degree. The heating rate was 0.3 °C/min, and the path length was 10 mm. Unfolding was reversible to ≥98% as judged by the coincidence of heating and subsequent cooling transitions. The thermal unfolding transitions were analyzed on the basis of the two-state approximation by nonlinear regression as described previously (Mayr et al., 1993) to obtain values for the temperature at the midpoint of thermal denaturation (T_m). The standard errors determined for the T_m values from these fits were in the range of 0.1 °C and thus generally smaller than the size of the symbols used in Figures 2 and 3. Systematic errors would affect all T_m values in the same manner and are therefore not considered here. They are presumably small, however, since the T_m values at pH 5.0 determined here by absorbance and previously by calorimetry (Kiefhaber et al., 1990) agree within 0.2 °C.

Urea-Induced and GdmCl-Induced Unfolding Transitions of RNase T1. Native protein (0.67 μM) was incubated in 0.01 M sodium acetate, pH 5.0, 25 °C, in the presence of various concentrations of urea or GdmCl until the folding/unfolding equilibrium was reached (24–48 h). The extent of unfolding was determined for each solution by measuring the fluorescence at 320 nm (10-nm bandwidth) after excitation at 268 nm (3-nm bandwidth). The concentration of urea was varied between 0 and 9.8 M and the concentration of GdmCl between 0 and 6.25 M. The values for $\Delta G°_{stab}$ were derived by linear extrapolations to 0 M denaturant by a method that includes the pre- and posttransitional baselines for a nonlinear regression of the data (Santoro & Bolen, 1988). The concentrations of GdmCl and urea were determined after the experiments by measuring the refractions of the individual samples. The influence of 0, 0.1, and 0.5 M GdmCl on the urea-induced unfolding transition of RNase T1 was determined in analogous experiments.

Urea-Induced Unfolding Kinetics in the Presence of Salts. Unfolding of RNase T1 and RNase A was initiated by a 40-fold dilution of the proteins (in 0.01 M sodium acetate, pH 5.0) with urea to give final conditions of 8.75 M urea in 0.01 M sodium acetate, pH 5.0, at 25 °C. The reaction was followed by the decrease in tryptophan fluorescence at 320 nm (RNase T1) and the increase in tyrosine fluorescence at 303 nm (RNase A) (10-nm bandwidth) after excitation at 268 nm (3-nm bandwidth) in a Hitachi F-4010 spectrofluorometer. The protein concentrations were 0.5 μM for RNase T1 and 4 μM for RNase A. The kinetics of unfolding were analyzed by using the programs Kinfit (OLIS software) or Grafit (ERITHACUS software). The time constants τ of unfolding are the reciprocals of the measured rate constants k ($\tau = 1/k$).

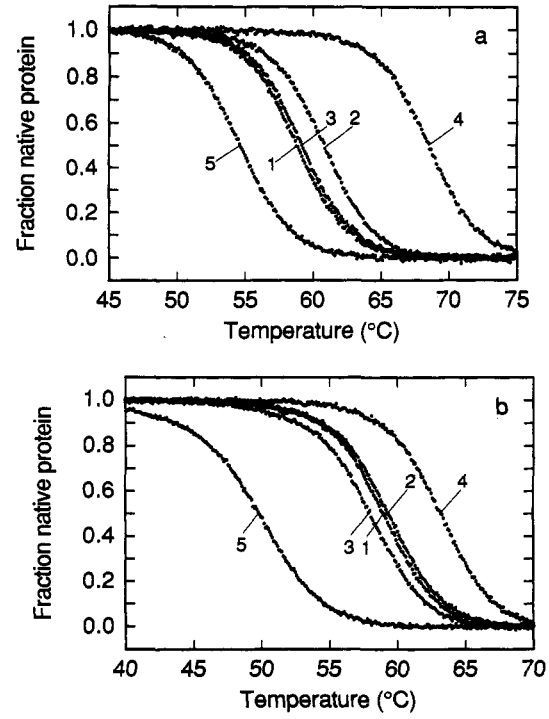

FIGURE 1: Thermal unfolding transitions of (a) RNase T1 and (b) RNase A in the presence of various concentrations of NaCl or GdmCl. The unfolding transitions in 0.01 M sodium acetate, pH 5.0, were monitored by the decrease in absorbance at 287 nm at a heating rate of 0.3 °C/min. A linear extrapolation of the baselines in the pre- and posttransitional regions was used to determine the fraction of unfolded protein within the transition region by assuming a two-state mechanism for unfolding. The protein concentrations were 9 μM for RNase T1 and 27 μM for RNase A in cells with path lengths of 10 mm. The reversibility of unfolding was tested by subsequent cooling. Curves 1, proteins in buffer only; curves 2, 0.1 M NaCl; curves 3, 0.1 M GdmCl; curves 4, 1.0 M NaCl; curves 5, 1.0 M GdmCl added.

RESULTS

Dependence on NaCl and GdmCl of the Thermal Stabilities of RNase A and RNase T1. To demonstrate directly the unusual effects of GdmCl on the stability of RNase T1, we compared thermal unfolding transitions in the presence of this denaturant with data in the presence of equivalent concentrations of NaCl. In addition, in control experiments the same measurements were carried out with pancreatic RNase A. This protein has been used as a model for investigating the effects of salts on protein stability and folding (von Hippel & Wong, 1962, 1965; Nelson & Hummel, 1962; Tsong & Baldwin, 1978; Ahmad & Bigelow, 1979).

The thermal stabilities of both RNase T1 and RNase A were measured by following the decrease in tyrosine absorbance at 287 nm upon unfolding as a function of temperature. The representative curves in Figure 1a reveal two interesting properties of RNase T1. As noted before, this protein is strongly stabilized by salt, and the addition of 0.1 M NaCl led to a marked increase in the temperature at the midpoint of the transition (T_m) by 2.1 °C. In the presence of 1.0 M NaCl T_m was increased by 9.8 °C. Contrary to expectation, RNase T1 did not lose stability when 0.1 M of the denaturant GdmCl was added, but was slightly stabilized, and the T_m value increased by 0.4 °C. This unusual stabilization was, however, abolished in the presence of 1.0 M GdmCl, and the T_m value was lowered from 59.2 (0.1 M GdmCl) to 54.7 °C

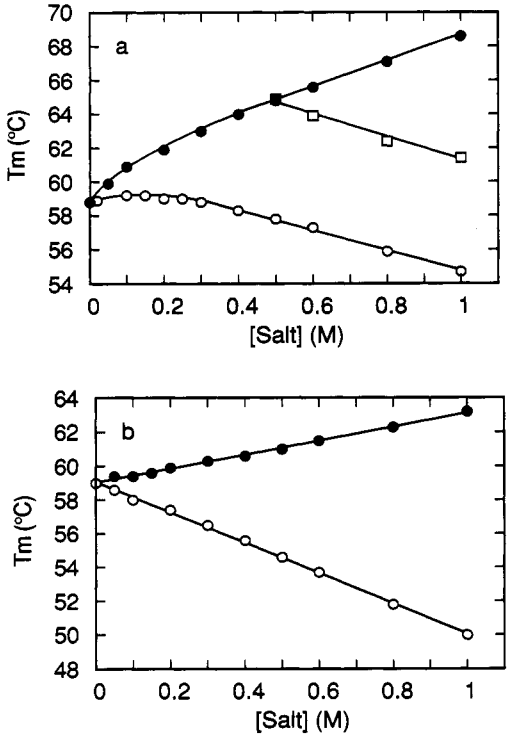

FIGURE 2: Dependence on GdmCl and NaCl of the thermal stabilities of (a) RNase T1 and (b) RNase A. The midpoints of the thermal unfolding transitions (T_m) are shown as a function of the concentration of added salts. The transitions in the presence of NaCl (●) and GdmCl (○) were determined in 0.01 M sodium acetate, pH 5.0, as shown in Figure 1. Concentrations of 9 μM RNase T1 and 27 μM RNase A were used. In the experiments denoted by (□), 0.1, 0.3, and 0.5 M GdmCl were added to a RNase T1 solution that contained already 0.5 M NaCl.

(1.0 M GdmCl). For RNase A, the addition of NaCl was slightly stabilizing, and GdmCl was destabilizing at 0.1 M as well as 1.0 M (Figure 1b). The effect of GdmCl on the thermal stability of RNase T1 was investigated further, and Figure 2a shows the T_m values of RNase T1 as a function of the NaCl and GdmCl concentrations. NaCl stabilizes this protein at all concentrations, and a particularly strong increase in T_m is found in the presence of 0–0.2 M NaCl. This was noted earlier and suggested to reflect ion binding to several sites on the native protein as well as a general stabilizing effect of NaCl on RNase T1 (Pace & Grimsley, 1988; Pace et al., 1990; Kiefhaber et al., 1990; Hu et al., 1992). GdmCl has a similar, though weaker, stabilizing influence between 0 and 0.2 M (Figure 2a). At higher concentration, however, the denaturing effect of GdmCl predominates, and the T_m value decreases with increasing denaturant concentration.

When analogous experiments were carried out with RNase A (Figure 2b), approximately linear profiles were obtained. Incidentally, RNase A and RNase T1 showed almost identical T_m values under the employed conditions in the absence of salts. NaCl slightly stabilizes RNase A, and the T_m value increased monotonically from 59 °C in the absence of NaCl to 63 °C in the presence of 1.0 M NaCl. The inverse was found for GdmCl. The T_m decreased linearly by 9 °C between 0 and 1.0 M GdmCl. It should be noted that, although the individual profiles in Figure 2 for the two RNases are significantly different, the differences in the ΔG_{stab} values at equal concentrations of NaCl and GdmCl are remarkably similar. At 60 °C, RNase T1 is destabilized by 20.7 kJ/mol and RNase A by 18.5 kJ/mol in the presence of 1.0 M GdmCl relative to 1.0 M NaCl.

The stabilization observed at low concentration of GdmCl could originate from preferential binding of Gdm^+ to a cation

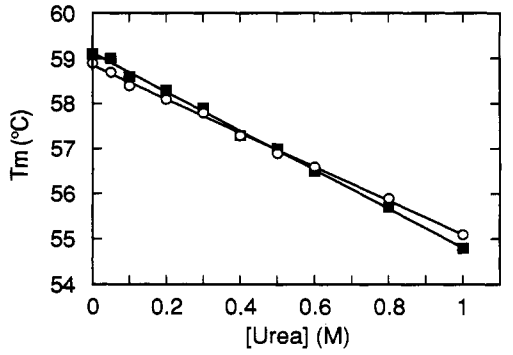

FIGURE 3: Dependence on urea of the thermal stabilities of RNase T1 (○) and RNase A (■). The T_m values were determined from transitions in 0.01 M sodium acetate, pH 5.0, as shown in Figure 1 in the presence of various concentrations of urea.

binding site, which was suggested to exist on native RNase T1 (Pace & Grimsley, 1988; Pace et al., 1990; Walz & Kitareewan, 1990). In this case the competitive binding of Na^+ ions should decrease or abolish the unusual stabilizing effect of GdmCl. This is indeed observed. When 0–0.5 M GdmCl was added to RNase T1 solutions that contained 0.5 M NaCl already, GdmCl acted as a destabilizing salt at all concentrations (cf. the open quadrangles in Figure 2a). This result provides strong support for the assumption that the stabilization of RNase T1 by low concentrations of NaCl and GdmCl occurs by the same mechanism, i. e., binding to a common cation binding site.

The stabilization of RNase T1 by GdmCl at low concentration and the striking difference from RNase A are clearly related with the ionic nature of GdmCl. The nonionic denaturant urea affects these two proteins in a very similar fashion. Almost identical and linear decreases in the T_m values were found for both RNase A and RNase T1, when thermal unfolding transitions were carried out in the presence of 0–1.0 M urea, instead of GdmCl (Figure 3).

Effect of GdmCl on the Urea-Induced Unfolding Transition of RNase T1. A stabilizing effect was also noted when urea-induced unfolding transitions of RNase T1 were determined in the presence of a constant low concentration of GdmCl. At 0.1 M GdmCl both the midpoint and the cooperativity of the urea transition are slightly increased (Figure 4a) and the $\Delta G°_{stab}$ value obtained after extrapolation to 0 M urea increased from −34.0 ± 0.4 to −38.0 ± 1.2 kJ/mol (Figure 4b). This is similar to the stabilization by 0.1 M NaCl observed by Pace and Grimsley (1988) at pH 7. The presence of 0.5 M GdmCl leads to a small destabilization of the protein toward unfolding by urea (Figure 4) as in the thermal unfolding transitions (cf. Figure 2a).

Unfolding Kinetics of RNase T1 and RNase A as a Function of Salt Concentration. In addition to their effect on protein stability, solvent additives have also marked effects on the kinetics of protein unfolding. Stabilizing salts usually decelerate, destabilizing salts accelerate the unfolding reaction. To examine the influence of GdmCl and of NaCl on the unfolding kinetics of RNase T1 and RNase A, we used a constant concentration of 8.75 M of the non-ionic denaturant urea. GdmCl and NaCl were then added at increasing concentration to these strongly unfolding conditions. The unfolding reactions of both RNase A and RNase T1 are monophasic processes under these conditions. The effects of 0–0.6 M GdmCl or NaCl on the rates of unfolding of the two RNases are shown in Figure 5a,b. As in the equilibrium experiments (Figure 2a) NaCl was found to be strongly stabilizing for RNase T1. A 5-fold deceleration of unfolding was observed in the presence of 0.6 M NaCl. When GdmCl

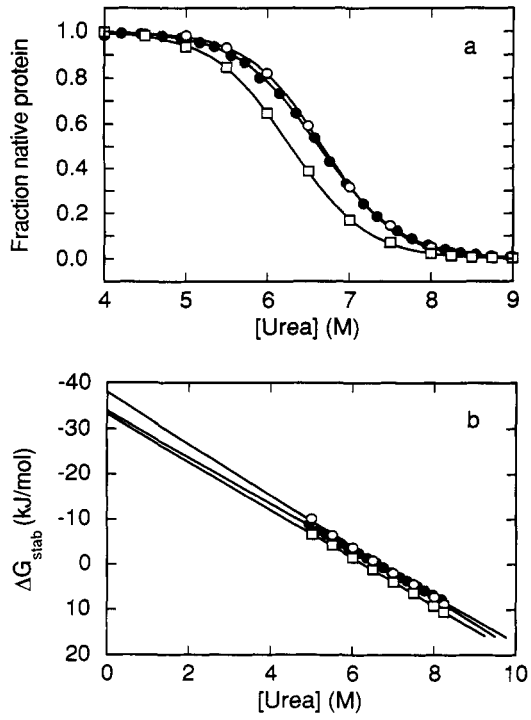

FIGURE 4: Urea-induced unfolding transitions of RNase T1 in the presence of (●) 0, (○) 0.1, and (□) 0.5 M GdmCl. (a) Normalized unfolding transitions; (b) ΔG_{stab} as a function of urea concentration, as calculated from the data in panel a by using the two-state approximation and the method of Santoro and Bolen (1988). The resulting fits are represented by the solid lines in both panels. The unfolding transitions were measured by fluorescence at 320 nm in 0.01 M sodium acetate, pH 5.0. RNase T1 concentration was 0.5 μM.

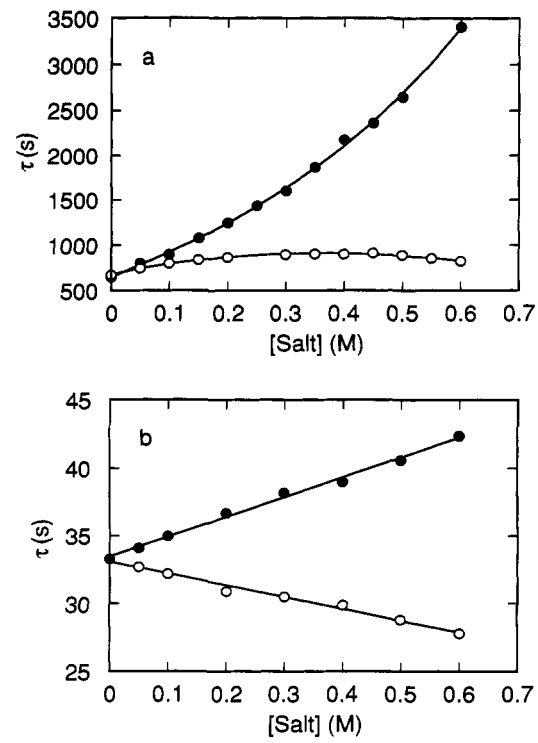

FIGURE 5: Kinetics of urea-induced unfolding of (a) RNase T1 and (b) RNase A in the presence of GdmCl (○) and of NaCl (●). The time constants of unfolding are shown as a function of salt concentration. The kinetics of unfolding were followed by the decrease in fluorescence at 320 nm for RNase T1 and by the increase in fluorescence at 303 nm for RNase A. The unfolding conditions were 8.75 M urea, 0.01 M sodium acetate, pH 5.0, at 25 °C. The protein concentrations were 4 μM for RNase A and 0.5 μM for RNase T1.

was added in increasing concentrations, a nonlinear behavior was noted. As in the experiments with NaCl, a stabilizing effect dominated in the presence of 0–0.3 M GdmCl, and the time constant of unfolding was increased, although GdmCl is thought to be a strong denaturant. At concentrations higher than 0.3 M, the denaturing power of GdmCl dominated and the time constant of unfolding decreased with GdmCl concentration. Again, a much simpler pattern was found in the control experiments carried out with RNase A (Figure 5b). The time constants of unfolding depended linearly on NaCl as well as GdmCl concentration. NaCl is weakly stabilizing, and the addition of 0.6 M NaCl led to a small, 1.3-fold increase in the time constant of unfolding. GdmCl is destabilizing at all concentrations and the time constant of unfolding decreased almost linearly with GdmCl concentration. A further analysis of the kinetic data in Figure 5 is not warranted, since all measurements were carried out in the presence of a very high concentration of urea, and the addition of the cosolutes NaCl or GdmCl affects not only the properties of the protein molecules but also the activities of urea and water.

DISCUSSION

The denaturant GdmCl displays two opposing functions for the folding and the stability of RNase T1. At low concentration (0–0.3 M), GdmCl acts in an unusual manner as a structure stabilizing additive. It increases the stability toward thermal and urea-induced denaturation and decreases the rate of unfolding. At higher concentration, the strongly destabilizing character of GdmCl dominates, and it behaves as a classical denaturant. Indeed, GdmCl-induced unfolding transitions of RNase A and RNase T1 show the same cooperativity, as indicated by almost identical m values (m = $d\Delta G_{stab}/d[GdmCl]$) at pH 7, when the slight difference in molecular weight is taken into account (Pace et al., 1990). A complex behavior as for GdmCl is not observed when the thermal stability of RNase T1 is investigated in the presence of a nonionic denaturant, such as urea. The mechanisms of stabilization of RNase T1 by low concentrations of GdmCl and NaCl are closely related and mutually exclusive. The suppression of the stabilizing effect of GdmCl by the addition of 0.5 M NaCl provides strong support for this suggestion. Mg^{2+} and spermine also compete for a common binding site on RNase T1 (Walz & Kitareewan, 1990).

The comparison with NaCl helps to clarify the stabilizing effect of GdmCl. NaCl stabilizes RNase T1 presumably in a twofold manner. Binding to one or two sites with fairly high affinity leads to the strong increase in stability between 0 and 0.2 M salt. Additional weak interactions and/or indirect, solvent-mediated effects lead to a continuing but smaller increase in stability at higher salt concentration. Calorimetric analyses indicate that RNase T1 is indeed stabilized by the binding of several Na^+ ions (Kiefhaber et al., 1990; Hu et al., 1992), and the fluorescence emission of the protein increases slightly when 0–1 M of NaCl or GdmCl, but not of urea, is added (Pace & Grimsley, 1988; Pace et al., 1990). This increase was also thought to reflect cation binding. On the other hand, the denaturing effect of GdmCl on folded proteins increases linearly with concentration, as in the case of RNase A (cf. Figure 2b). These different stabilizing and destabilizing effects of GdmCl on RNase T1 are apparently additive and lead to the observed complex dependences on GdmCl concentration of the stability and the unfolding kinetics (cf. Figures 2a and 5a). At low GdmCl concentration, the strong stabilization by salt binding to the high-affinity site(s) dominates and compensates the general, destabilizing function of GdmCl.

In contrast, the effects of NaCl and of GdmCl on the T_m of RNase A are deceptively simple. RNase A has a high positive net charge at pH 5 and several anion binding sites, which are sensitive to both pH and ionic strength. In their analysis of anion binding, Matthew and Richards (1982) suggested that about half of the free energy of stabilization of RNase A originates from electrostatic contributions. Clearly, several processes, such as weak interactions with defined binding sites and general as well as local Coulombic shielding, occur in a similar range of salt concentrations and lead to the observed small and linear increase of T_m with NaCl concentration (Figure 2b).

The selective stabilization of RNase T1 by an ionic denaturant is not related with the strong activation of several enzymes, in particular from thermophilic organisms, in the presence of small concentrations of a denaturant. In these cases the increase in enzymatic activity is probably brought about not by an increase in stability but by an increase in protein flexibility (Gerschitz et al., 1977; Rehaber & Jaenicke, 1992).

The preferential interaction of Gdm^+ ions with RNase T1 and the concomitant stabilization of the folded state interfere with the determination of the Gibbs free energy of stabilization. A two-state analysis by the linear extrapolation method of urea-induced and GdmCl-induced equilibrium transitions of RNase T1 under identical conditions of 0.01 M sodium actetate, pH 5.0, 25 °C, results in $\Delta G°_{stab}$ values of -34.2 ± 0.4 and -41.1 ± 0.3 kJ/mol, respectively (data not shown). A similar difference was noted by Pace et al. (1990) under different solvent conditions. The observed increase in stability by 7 kJ/mol reflects probably the free energy of the preferential interaction between folded RNase T1 and GdmCl.

These results lead to a conclusion that is important for the evaluation of the two-state model for the unfolding of proteins in equilibrium. A noncoincidence of $\Delta G°_{stab}$ values derived from experiments with different denaturants does not necessarily imply that the use of the two-state approximation is not appropriate. In fact, thermal and denaturant-induced unfolding of RNase T1 is well approximated by this model (Pace, 1990; Pace & Laurents, 1989; Pace et al., 1990; Kiefhaber et al., 1990; Hu et al., 1992). The noncoincidence in the presence of GdmCl results from the inadequate use of the linear extrapolation procedure to analyze the GdmCl-induced transitions. This method yields increased values for $\Delta G°_{stab}$ when binding sites for the denaturant exist on the native protein that are occupied before the unfolding transition occurs. A stabilization by ionic denaturants could be fairly common, because proteins frequently contain ion binding sites of varying affinity and specificity. Such additional stabilization is not easily detected in single, e. g., GdmCl-induced, unfolding experiments, since the denaturing effect clearly dominates in the region of the cooperative transition. It can, however, be detected by a simple and direct experimental approach, where thermal transitions of a protein are measured in the presence of small concentrations of the respective denaturants.

These limited data on two unrelated RNases certainly do not warrant a general assessment of the two-state approximation and the linear extrapolation procedure to 0 M denaturant. These issues have been discussed recently (Pace, 1986; Schellman, 1987; Santoro & Bolen, 1992). It is important, however, to recognize that the extrapolation to 0 M denaturant gives the stability of the protein conformation that exists in the transition region. In the case of RNase T1, this is the folded protein complexed with several Gdm^+ ions. More generally, it is the protein in a high-salt conformation. This was shown recently also for thioredoxin. In this case a linear relationship between the free energy of denaturation and GdmCl concentration was only observed when the high-salt form of this protein was maintained by adding NaCl in thermal unfolding experiments (Santoro & Bolen, 1992).

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