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Ribonuclease T_1 Is Stabilized by Cation and Anion Binding†

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ABSTRACT: The stability of the folded conformation of ribonuclease T_1 is increased by 0.8, 1.8, and 3.3 kcal/mol in the presence of 0.1 M NaCl, $MgCl_2$, and Na_2HPO_4, respectively. This remarkable increase in the conformational stability results primarily from the preferential binding to the native protein of one Mg^{2+} or two Na^+ ions at cation-binding sites and by the binding of one HPO_4^{2-} ion at an anion-binding site. Only modest binding constants, 6.2 (Na^+), 155 (Mg^{2+}), and 282 M^{-1} (HPO_4^{2-}), are required to account for the enhanced stability. One important goal of the modification of proteins through genetic engineering is to increase their stability. Our results suggest that the creation of specific cation- and anion-binding sites on the surface of a protein through amino acid substitutions might be a generally useful way of achieving this goal. The design of these sites will be aided by the recent availability of detailed structural information on cation- and anion-binding sites.

Ribonuclease T_1 (RNase T_1) is a small (104 amino acids), well-characterized enzyme excreted from *Aspergillus oryzae* that cleaves single-stranded RNA molecules at guanine residues (Heinemann & Saenger, 1982; Takahashi et al., 1970). The three-dimensional structure has recently been refined to 1.9-Å resolution (Arni et al., 1987). RNase T_1 has many properties that make it an excellent model for studying various aspects of protein folding (Pace & Creighton, 1986). Oobatake et al. (1979) first showed that RNase T_1 is stabilized by NaCl. The melting temperature of RNase T_1 is increased 20 °C in the presence of 2 M NaCl as compared to an increase of about 1.5 °C for ribonuclease A (RNase A) (von Hippel & Wong, 1965). The aim of the experiments reported here was to reach a better understanding of how salts stabilize RNase T_1.

We show that the marked stabilization of RNase T_1 at moderate salt concentrations results mainly from the relatively weak binding of cations and anions by the native, folded conformation of the protein. We suggest that constructing ion-binding sites on the surface of globular proteins through genetic engineering may be an easier way to increase their conformational stability than adding disulfide bonds or other stabilizing interactions.

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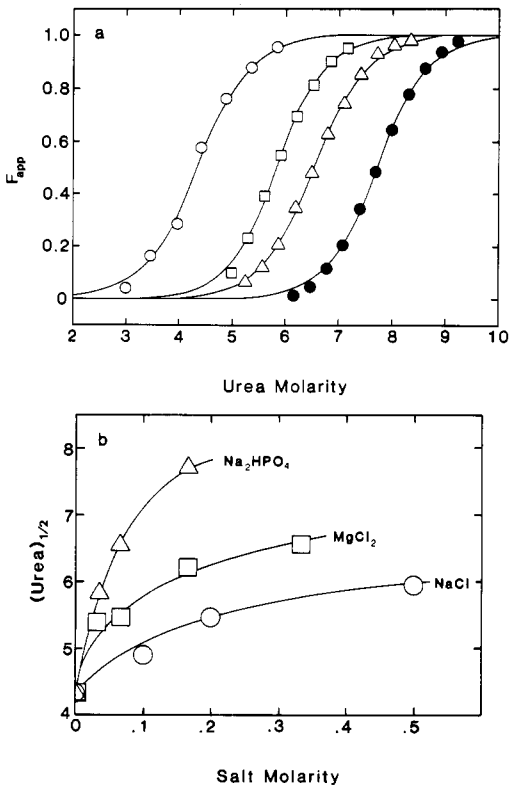

FIGURE 1: (a) Unfolding of RNase T_1 by urea at pH 7 [30 mM 4-morpholinepropanesulfonic acid (MOPS) buffer], 25 °C in the presence of 0 (○), 0.033 (□), 0.067 (△), and 0.167 M (●) Na_2HPO_4. (b) Midpoint of the urea denaturation curve, $[urea]_{1/2}$, plotted as a function of the concentration of added salt. The $[urea]_{1/2}$ values are from urea denaturation curves such as those shown in (a) determined in the presence of NaCl, $MgCl_2$, and Na_2HPO_4 at pH 7 (30 mM MOPS buffer), 25 °C.

Experimental Procedures

Ribonuclease T_1 was prepared by a new purification procedure (Pace et al., 1987). Urea denaturation curves and the results in Figure 4 were determined by measuring the intrinsic fluorescence (278-nm excitation and 320-nm emission) of 0.54 μM RNase T_1 solutions at 25 °C with a Perkin-Elmer MPF 44B spectrofluorometer (Pace, 1986). Solutions were equilibrated for at least 3 h to assure that unfolding reached equilibrium. The unfolding of RNase T_1 by urea is completely reversible under all conditions used here. Urea denaturation curves were analyzed to calculate the fraction of unfolded protein, F_{app} (Figure 1a), the equilibrium constant for unfolding, K_{app} (Figure 2), or the standard free energy change for unfolding, ΔG_{app} (Figure 3), by assuming a two-state unfolding mechanism, as described elsewhere (Pace, 1986).

Results

Urea denaturation curves were used to investigate the effect of salts on the conformational stability of RNase T_1. Figure 1a shows typical urea denaturation curves determined in the presence of Na_2HPO_4. The midpoints of these curves, $[urea]_{1/2}$, and others determined in the presence of NaCl and $MgCl_2$ are plotted as a function of salt concentration in Figure 1b. The results show clearly the marked stabilization of RNase T_1 by these salts. This is not a general effect of salts on proteins. For example, $[urea]_{1/2}$ increases from 4.38 to 7.88 M in the presence of 1.5 M NaCl for RNase T_1 but only from 7.05 to 7.80 M for RNase A under the same conditions (G. Grimsley, unpublished observations).

The fact that low concentrations of salt cause such large changes in the stability suggests that ion binding by RNase

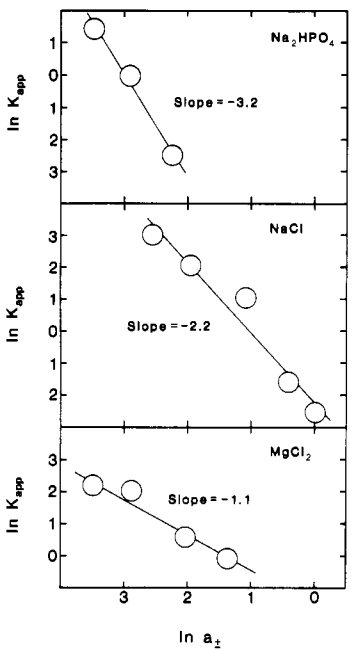

FIGURE 2: Plot of ln K_{app} as a function of ln $a_\pm$, according to equation 1. K_{app} is the equilibrium constant for unfolding at 6.5 M urea, and $a_\pm$ is the mean ion activity of the salt.

T_1 may make the major contribution to the observed increase in the conformational stability. When a ligand, L, binds preferentially to either the folded or unfolded conformations of a protein, folded ↔ unfolded + (Δn)L, then

$$d(\ln K_{app})/d(\ln a_L) = \Delta n = n_U - n_F \quad (1)$$

where K_{app} is the equilibrium constant for unfolding, a_L is the activity of L, and n_U and n_F are the number of ligand molecules bound by the unfolded and folded conformations of RNase T_1 (Record et al., 1978). Data from experiments such as those shown in Figure 1a have been analyzed in this way, and the results (Figure 2) indicate that about one, two, and three ions are released when RNase T_1 unfolds in the presence of $MgCl_2$, NaCl, and Na_2HPO_4, respectively. Ion release was expected since von Hippel et al. (1973) have shown that none of these ions bind appreciably to peptide groups, and none would be expected to bind tightly or specifically to unfolded RNase T_1. If the NaCl and $MgCl_2$ data are compared at the same Cl^- ion concentration, $MgCl_2$ has a greater effect. This argues that the major effect must be due to cation binding in the case of $MgCl_2$ and NaCl. The results with Na_2HPO_4 support this. It is known that HPO_4^{2-} binds specifically at the active site of RNase T_1 (Inagaki et al., 1985), as is observed with RNase A (Nelson et al., 1962). Thus, the Δn value of −3 suggests that one HPO_4^{2-} ion and two Na^+ ions are released when RNase T_1 unfolds in the presence of Na_2HPO_4.

Data such as those shown in Figure 1a can be analyzed to determine the free energy of unfolding, ΔG_{app}, as a function of urea concentration (Pace, 1986). The dependence of ΔG_{app} on urea concentration is linear, and a least-squares analysis was used to fit the data to the equation

$$\Delta G_{app} = \Delta G_{app}^{H_2O} - m[urea] \quad (2)$$

where $\Delta G_{app}^{H_2O}$ is the ΔG_{app} value in the absence of urea and m is a measure of the dependence of ΔG_{app} on urea concentration (Pace, 1986; Schellman, 1978). The m values did not vary significantly with the type of salt or the salt concentration. Average m values [kcal mol^{-1} (M urea)$^{-1}$] of 1.13 ± 0.04 (NaCl), 1.18 ± 0.06 ($MgCl_2$), and 1.22 ± 0.12 (Na_2HPO_4) were used with the $[urea]_{1/2}$ values in Figure 1b to calculate

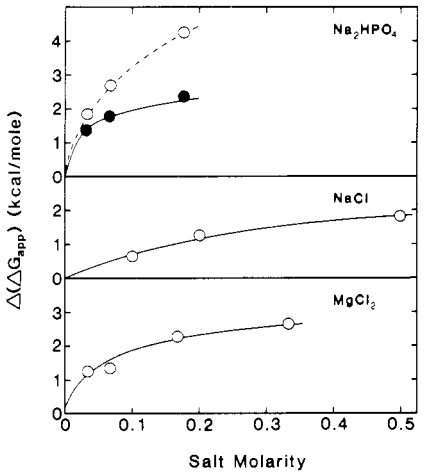

FIGURE 3: Change in ΔG_{app}, $\Delta(\Delta G_{app})$, as a function of salt concentration. $\Delta(\Delta G)$ values were calculated from the data in Figure 1 as described in the text and are shown by the experimental points (○). For Na_2HPO_4, the contribution of Na^+ ions, from the NaCl data, was subtracted from the $\Delta(\Delta G_{app})$ values for Na_2HPO_4 to give the contribution of HPO_4^{2-} to $\Delta(\Delta G_{app})$ (●). The solid lines were calculated with eq 3 by using binding constants of 200 for Mg^{2+} ions, 8.8 for Na^+ ions, and 343 for HPO_4^{2-} ions. If molar concentrations are used rather than mean ion activities, the binding constants that give the best fit to the experimental data are 155 M^{-1} for $MgCl_2$, 6.2 M^{-1} for NaCl, and 282 M^{-1} for Na_2HPO_4.

$\Delta(\Delta G_{app})$, the increase in the conformational stability of RNase T_1. The results are shown in Figure 3. Assuming that the entire stabilization results from the preferential binding of Δn ions at identical and independent binding sites by folded RNase T_1, the relationship among $\Delta(\Delta G)$, the mean ion activity of the ion, $a_\pm$, and the binding constant, K, is given by (Schellman, 1975)

$$\Delta(\Delta G) = \Delta G(\mathrm{L}) - \Delta G(\mathrm{L} = 0) = (\Delta n)\, RT \ln (1 + K[a_\pm]) \quad (3)$$

In Figure 3, the solid lines show that this equation fits the data quite well with binding constants based on mean ion activities of 8.8 for Na^+ ions, 200 for Mg^{2+} ions, and 342 for HPO_4^{2-} ions. The fit is equally good if molarities rather than mean ion activities are used in eq 3, and binding constants of 6.2 (NaCl), 155 ($MgCl_2$), and 282 M^{-1} (Na_2HPO_4) are obtained. The average deviation (in kcal/mol) of the experimental values of $\Delta(\Delta G)$ from the calculated curves in Figure 3 is ±0.10 for $MgCl_2$, ±0.08 for NaCl, and ±0.08 for Na_2HPO_4. The binding constant for phosphate is comparable to values of 235 (Anderson et al., 1968) and 400 M^{-1} (Nelson et al., 1962) reported for RNase A.

The effect of several other salts was tested at a single concentration. At 0.5 M, $\Delta(\Delta G_{app})$ values (kcal/mol) of 1.9 (NaCl), 1.9 (KCl), 2.2 (CsCl), and 2.4 (NaOAc) were observed; at 0.17 M, values of 1.9 ($CaCl_2$) and 2.2 ($MgCl_2$) were observed. The fact that K^+ and Cs^+ ions give an effect similar to that of Na^+ ions and that Ca^{2+} ions give an effect similar to that of Mg^{2+} ions suggests that the cation-binding sites are not very specific. For α-lactalbumin (Hiraoka & Sugai, 1985), calmodulin (Haiech et al., 1981), parvalbumin, and troponin C (Delville et al., 1980), it has been shown that Na^+ and K^+ ions bind at the Ca^{2+}-binding site. Sodium acetate may give a larger effect than NaCl because of the binding of acetate at the anion-binding site.

The intrinsic fluorescence of folded RNase T_1 increases in the presence of NaCl and $MgCl_2$ and decreases substantially in the presence of Na_2HPO_4 (Figure 4). In contrast, none of the salts affect the intrinsic fluorescence of unfolded RNase T_1. This provides direct evidence for ion binding to folded

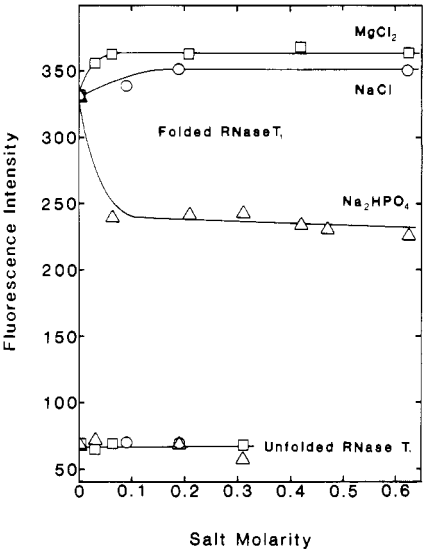

FIGURE 4: Intrinsic fluorescence intensity of RNase T_1 as a function of salt concentration at pH 7 (30 mM MOPS buffer), 25 °C. The fluorescence intensity of 0.54 μM solutions of RNase T_1 was measured at 320 nm after excitation at 278 nm. Measurements with unfolded RNase T_1 were made in 8 M urea.

RNase T_1 and suggests that different conformational changes are induced by the binding of cations and anions. The binding of nucleotides to the active site of RNase T_1 also leads to a large decrease in the fluorescence intensity (Pongs, 1970). The single tryptophan in RNase T_1 is buried, and several of the nine tyrosines transfer excitation energy to the tryptophan (Pongs, 1970). Consequently, the observed changes in intrinsic fluorescence may involve very small conformational changes in RNase T_1.

DISCUSSION

The native, folded conformation of RNase T_1 is about 5.5 kcal/mol more stable than unfolded conformations at pH 7, 25 °C, in the absence of salts. To increase the stability by 25% (1.4 kcal/mol) requires only 0.022 M Na_2HPO_4, 0.04 M $MgCl_2$, or 0.22 M NaCl. We think the evidence is good that this stabilization results mainly from the relatively weak binding of cations and anions to sites on the surface of RNase T_1. More direct evidence for a cation-binding site may soon be available. Crystallographic studies of RNase T_1 currently in progress suggest that there may be a cation-binding site near Asp-15 in RNase T_1 (Saenger, Heinemann, and Koepke, personal communication).

Salts can influence the stability of proteins in a variety of ways (von Hippel & Schleich, 1969). The solvent properties of salts have received considerable attention, perhaps because of widespread interest in the Hofmeister series (Collins & Washabaugh, 1985). The studies of Arakawa and Timasheff (1984) have shown that there is a wide range of interactions between salts and proteins ranging from preferential hydration to preferential salt binding depending on the kind of salt and the solvent conditions. Most salts decrease the solubility of nonpolar groups and increase the solubility of peptide groups (Nandi & Robinson, 1972). Schrier and Schrier (1967) have shown that these solvent effects can account for increases and decreases in the stability of RNase A caused by a variety of different salts. However, they can account for only a small part of the stabilizing effect of salts on RNase T_1.

For most proteins the charges are arranged on the surface so that there are more attractive than repulsive electrostatic interactions (Wada & Nakamura, 1981). For myoglobin (Friend & Gurd, 1979) and RNase A (Matthew & Richards,

1982) it has been suggested that these interactions contribute significantly to the conformational stability of the folded protein. In these cases, increasing ionic strength would be expected to weaken these interactions through Debye–Hückel screening and destabilize the protein. However, RNase T_1 has an unusual content of charged amino acids (see below) and has more repulsive than attractive electrostatic interactions at pH 7. Thus, some of the stabilizing effect of the salts is likely to be due to an ionic strength effect, but the few experimental studies suggest that this effect may be considerably smaller than the effects of ion binding (Hermans & Scheraga, 1961). Recent experimental (Russell et al., 1987) and theoretical (Sternberg et al., 1987; Gilson & Honig, 1987) studies of the effect of ionic strength on pK values may improve our understanding of ionic strength effects.

RNase T_1 is one of the most acidic proteins. It contains only 1 Lys and 1 Arg residue, as compared to a total of 14 of these residues for RNase A. This is surprising for an enzyme that must act on a negatively charged substrate. The binding of cations by RNase T_1 may help compensate for the shortage of basic residues. However, Pflugrath and Quiocho (1985) have shown that charged groups may be bound by proteins through hydrogen bonding with no formal counter charges present at the binding site.

Hiraoka et al. (1980) first showed that α-lactalbumin is dramatically stabilized by binding Ca^{2+} ions. The relationship between ion binding and conformational stability has since been investigated in detail (Mitani et al., 1986; Desmet et al., 1987), and a high-resolution structure of the protein has been reported (Stuart et al., 1986). The Ca^{2+}-binding site also binds Na^+ ions, and the binding constants are 2.9×10^9 M^{-1} for Ca^{2+} ions and 1240 M^{-1} for Na^+ ions. Thus, RNase T_1 and α-lactalbumin lie at opposite ends of the spectrum with regard to ion-binding affinity, and it is not surprising that α-lactalbumin is partially unfolded in the absence of ions. Acyl carrier protein (Schulz, 1977), alkaline phosphatase (Chlebowski & Mabrey, 1977), thermolysin (Dahlquist et al., 1976), and parvalbumin (Filimonov et al., 1978) are other proteins that have been shown to be much more stable in the presence of ions, probably also because of ion binding by the folded protein.

The marked stabilization of RNase T_1 by cation and anion binding reported here and the studies cited above suggest that constructing an ion-binding site on the surface of a folded protein provides a means of substantially increasing the conformational stability. It may be easier to construct an ion-binding site of modest affinity ($K \approx 200$ M^{-1}) than it is to engineer a new disulfide bond (Villafranca et al., 1987) or some other stabilizing interaction (Alber et al., 1987; Perry et al., 1987; Ward et al., 1987; Yutani et al., 1987; Matsumura et al., 1986). In fact, the results of Russell and Fersht (1987) suggest that they have created counterion-binding sites in some of their subtilisin mutants without even trying to do so. The design of ion-binding sites will be aided by the recent availability of detailed structural information on cation- (Stuart et al., 1986; Vyas et al., 1987; Herzberg & James, 1985) and anion- (Fujinaga et al., 1985; Pflugrath & Quiocho, 1985) binding sites. It has long been known (O'Sullivan & Tompson, 1890) that it is possible to stabilize enzymes by adding compounds that bind at the active site (Pace & McGrath, 1980). An obvious advantage of using ion binding for stabilization is that it need not interfere with the functioning of the active site of the enzyme.

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Registry No. RNase T_1, 9026-12-4; Mg, 7439-95-4; Na, 7440-23-5; HPO_4^{2-}, 14066-19-4.

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Occurrence of 2-5A and RNA Degradation in the Chick Oviduct during Rapid Estrogen Withdrawal†

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ABSTRACT: Rapid withdrawal of estrogen from immature chicks, previously stimulated with the hormone, results in the inhibition of transcription of mRNAs of egg white proteins, rapid degradation of existing estrogen-induced mRNAs of egg white proteins, and decline in ribosomes and weight of the oviduct. On rapid withdrawal of estrogen, ovalbumin mRNA decreased to 65% after 3 h and was not detected after 24 h. In contrast to ovalbumin mRNA, cellular RNA content remained unchanged at 3 h and subsequently decreased to 51% of the stimulated value by 48 h. To study the mechanism of rapid degradation of RNA during estrogen withdrawal, the role of 2-5A- $[p_x(A2'p)_nA; x = 2 \text{ or } 3, n \geq 2]$ dependent RNase was investigated. The effect of 2-5A-dependent RNase on the stability of RNA in vitro was determined by incubating oviduct polysomes with 2-5A-dependent RNase and exogenous 2-5A. Ovalbumin mRNA was degraded more rapidly than β-actin mRNA and rRNA, and the kinetics of RNA degradation were very similar to those observed in vivo. Levels of 2-5A in the chick oviduct increased shortly after estrogen withdrawal. Analysis of the oviduct RNA revealed that a distinct 18S rRNA derived fragment, 450 nucleotides in length, increased at 6 h after withdrawal and at subsequent time points when significant degradation of total cellular RNA was occurring. The 18S rRNA derived degradation product observed in vivo from the chick oviduct had the same mobility in denaturing agarose gels as the 18S rRNA cleavage product liberated on incubation of isolated oviduct ribosomes with purified 2-5A-dependent RNase and exogenous 2-5A. These results indicate that in the chick oviduct 2-5A-dependent RNase is activated and may be involved in the degradation of mRNA and rRNA during estrogen withdrawal.

Estrogen administration to immature chicks results in cytodifferentiation and proliferation of tubular gland cells in the magnum portion of the oviduct. In the tubular gland cells, egg white proteins, of which ovalbumin is the major constituent, are synthesized (Schimke et al., 1975; O'Malley et al., 1977; Palmiter et al., 1977). The oviduct increases steadily in weight from 20-30 mg to 0.5-0.75 g after 10 days of estrogen administration at which time ovalbumin synthesis constitutes 50-65% of protein synthesis in the oviduct. Continuous presence of estrogen is needed for sustained synthesis of egg white proteins. Discontinuation of estrogen administration (chronic withdrawal) leads to a gradual decline in

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