Specific Ammonium Ion Requirement for Functional Ribosomal RNA Tertiary Structure†

Yun-Xing Wang, Ming Lu, and David E. Draper*

Department of Chemistry, The Johns Hopkins University, Baltimore, Maryland 21218

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ABSTRACT: In compactly folded RNAs, coordination or hydrogen bonding of cations in specific sites is a potentially important aspect of the tertiary structure. NH_4^+ specifically stabilizes the tertiary structure of a conserved, 58-nt fragment of the large subunit ribosomal RNA, as judged in two ways: a melting transition associated with tertiary interactions is sharpened and stabilized more effectively by NH_4^+ than by any alkali metal cation, and the affinity of the RNA fragment for ribosomal protein L11 or the antibiotic thiostrepton is ~10-fold stronger when measured in NH_4^+ than in Na^+. The dependence of the melting temperature on NH_4^+ concentration shows that a single bound ion is responsible for these effects. The requirement of different ribosome functions for NH_4^+ suggests that other such sites exist in ribosomal RNAs.

RNA folding must overcome a large electrostatic energy barrier, since each nucleotide phosphate carries a full negative charge. The problem is particularly severe in RNAs which require a highly compact conformation for their function. Screening of phosphate charges by nonspecifically bound counterions helps stabilize folded conformations; in addition, one might expect to find the three-dimensional structure of an RNA forming "pockets" in which cations with the correct coordination or hydrogen bond geometry bind. If specific ion binding promotes the correct conformation of an RNA, then the RNA function will be sensitive to the identities of the counterions as well as their concentrations. Only a few instances of this specificity have been observed. Some ribozymes are much more active in Mg^{2+} than other divalent ions, and recent studies suggest specific coordination of the ion to base amino, sugar hydroxyl, or backbone phosphate groups (Perreault *et al.*, 1991; Fu & McLaughlin, 1992; Piccirilli *et al.*, 1993). A second kind of ion-specific coordination takes place in the "G-quartet" structure, in which guanine carbonyl groups line a pocket specifically binding K^+ (Guschlbauer *et al.*, 1990); HIV RNA dimers, which occur in the mature virion, are thought to depend on such K^+-stabilized structures (Sundquist & Heaphy, 1993). Here we report a third variety of specific ion–RNA binding, in which NH_4^+ stabilizes a ribosomal RNA tertiary structure recognized by protein and antibiotics.

MATERIALS AND METHODS

RNA Preparation and Melting Experiments. The *Escherichia coli* large-subunit ribosomal RNA sequence G1051–U1158 was synthesized from plasmid DNA by T7 RNA polymerase runoff transcription as described (Ryan & Draper, 1989; Laing & Draper, 1993), using a clone which could be cleaved with *Rsa*I to give the 3′ terminus shown in Figure 1. The RNA was purified by column gel electrophoresis, using 8 M urea and 8% acrylamide. Melting data were collected in a Perkin-Elmer Lambda 4 spectrophotometer interfaced to a computer and temperature probe (Laing & Draper, 1993). RNA samples were renatured at 65 °C for 15 min in the indicated buffers prior to the experiment. The heating rate was 0.5°/min. Methods used to take the first derivative of the temperature vs absorbance data and to fit sequential two-state transitions to the data have been described (Laing & Draper, 1993).

Ligand–RNA Binding Assays. For binding measurements a larger rRNA fragment, A1029–G1122, was transcribed using [α-^{35}S]ATPαS (Ryan & Draper, 1989). The L11 protein was prepared from *E. coli* MRE600 ribosomes and used to titrate the RNA in nitrocellulose filter binding assays as previously described (Ryan & Draper, 1989), with the exception that incubation of the protein and RNA was at room temperature. Thiostrepton was purchased from Calbiochem and used in filter binding assays in the same way as for the L11 protein, except that 5% DMSO (v/v) was included in the buffers to increase the antibiotic solubility (Ryan *et al.*, 1991).

RESULTS

NH_4^+ Stabilizes Tertiary Structure in a Conserved Ribosomal RNA Domain. Figure 1 shows the secondary structure of a highly conserved domain in the large-subunit ribosomal RNA. The RNA is a recognition site for protein L11 of *E. coli* and a class of thiazole-containing antibiotics (Ryan & Draper, 1989, 1991). Both L11 and the antibiotics affect the uncoupled GTPase activity of the ribosome (Cundliffe, 1986), and the RNA probably forms part of the binding site for elongation factor G (Moazed *et al.*, 1988). The pairing between U1082 and A1086 has been suggested by phylogenetic comparisons and confirmed by compensatory mutations (Egebjerg *et al.* 1990; Ryan & Draper, 1991). Melting experiments have identified an additional set of "tertiary" interactions which depend on the binding of a single Mg^{2+} ion and unfold at moderate temperatures (~40 °C) (Laing & Draper, 1993; Laing *et al.*, 1993). Although these tertiary interactions have not yet been identified in detail, they probably take place within the junction region and define the three-dimensional folding of the RNA needed for ligand recognition: mutations either disrupting the 1082–1086 pair or changing some of the universally conserved bases in the junction region destabilize the tertiary structure without

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* To whom correspondence should be addressed.
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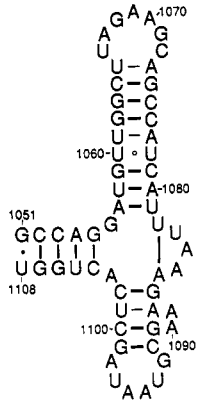

FIGURE 1: Highly conserved domain of the large subunit ribosomal RNA. The sequence and numbering are from *E. coli*, and the secondary structure is phylogenetically conserved (Gutell *et al.*, 1992). Thin lines connect pairs which are not conserved as Watson–Crick pairs but are likely to be paired in the *E. coli* sequence. A U·U pair is shown, based on NMR evidence (unpublished data). The sequence shown was used in all the melting experiments described here. [Figure adapted from Laing and Draper (1993).]

affecting other higher temperature transitions (Laing & Draper, 1993; M. Lu and D. E. Draper, unpublished observations), and the same mutations weaken both L11 and antibiotic binding (Ryan *et al.*, 1991).

The unfolding of the RNA fragment is shown in Figure 2A as the first derivative of the absorbance with respect to temperature. The tertiary structure unfolding is the broad transition occurring between ~20 and 50 °C in the presence of 100 mM K^+ and 3 mM Mg^{2+} (middle curve). When Na^+ is substituted for K^+ in the buffer, a distinct transition no longer appears in this temperature range, only a gradual unfolding which is under way by 10 °C. A similar melting profile is obtained if only K^+ (and not Mg^{2+}) is included in the buffer. Li^+, Rb^+, and Cs^+ also fail to induce a distinct low-temperature transition (data not shown). However, NH_4^+ ion is more effective than K^+ at stabilizing this tertiary structure (Figure 2A). None of the ions tested differ in their effects on the higher temperature unfolding transitions.

An apparent enthalpy and melting temperature for the low-temperature transition can be obtained by fitting the melting profile to a set of sequential unfolding transitions (Laing & Draper, 1993). The quantities obtained are only apparent, since the broad unfoldings seen in buffers with low Mg^{2+} concentrations or with monovalent ions other than K^+ or NH_4^+ are probably not two-state transitions (Laing *et al.*, 1993). The quantities are nevertheless useful for comparing the effects of different ions and are graphed in Figure 2B as a function of ionic radius. It is clear that NH_4^+ is more effective than any of the other ions tested at increasing both the apparent T_m and ΔH of the transition. Melting experiments done in the presence of K^+ consistently showed a more distinct transition than those done with the other alkali metal ions (as in Figure 2A), but the fitting of low-enthalpy transitions to these curves has substantial error, and a preference for K^+ is not evident in Figure 2B.

Increases in the apparent ΔH and T_m of the low-temperature transition have been observed with increasing Mg^{2+} concentration (at constant K^+ or NH_4^+ concentration) and attributed to the organization of a more extensive set of RNA tertiary interactions by a single Mg^{2+} ion bound to the folded RNA (Laing *et al.*, 1993). NH_4^+ (and to some extent K^+) apparently has a similar role and stabilizes the same (or closely related) tertiary structure as Mg^{2+}.

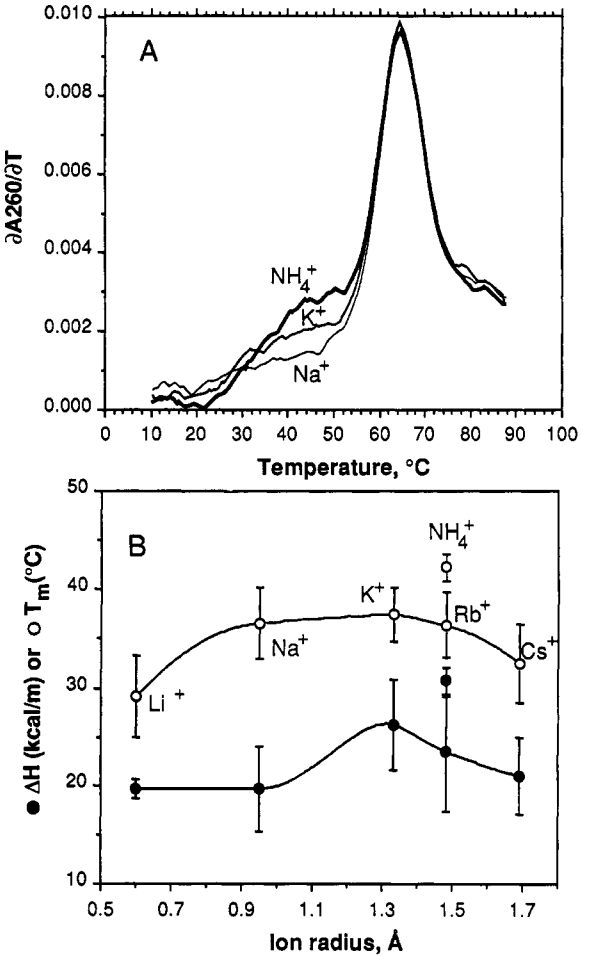

FIGURE 2: Monovalent ion effects on the 58-nt rRNA fragment unfolding. (A) Thermal denaturation of the rRNA fragment, detected by absorbance changes at 260 nm; the first derivative of the absorbance with respect to temperature is shown. Buffers contained 10 mM MOPS, pH 7.2, 3 mM $MgCl_2$, and 100 mM chloride salt of the indicated cation. (B) Apparent enthalpy and melting temperature of the first unfolding transition, derived from data sets as in panel A. The values were obtained by fitting four sequential transitions to the melting profile and fixing the enthalpy and hyperchromicity of the last three at the values found for melts in KCl-containing buffers (Laing & Draper, 1993). The melting temperatures of these three transitions did not vary significantly with the ion present. Data presented are averages of three to six separate melting experiments. Ionic radii are from Pauling (1960).

A Single NH_4^+ Ion Stabilizes the rRNA Tertiary Structure. The number of bound ligands released upon unfolding of an RNA structure can be estimated from the dependence of the transition melting temperature on ligand concentration, using the formula $d(1/T_m)/(d \ln [L]) = -nR/\Delta H$, where n is the number of ligands, [L] is the concentration of free ligand, ΔH is the enthalpy of the transition, and R is the gas constant (Laing *et al.*, 1993). The equation applies in the limit of saturating ligand concentrations and assumes that the enthalpy of ligand binding is negligible. To make this measurement, we melted the RNA fragment in buffers containing LiCl and NH_4Cl concentrations totalling 100 mM. Debye–Hückel screening contributions of the ions to the RNA stability should therefore remain approximately constant, while any binding of NH_4^+ to specific site(s) manifests itself as an increase in transition T_m. $1/T_m$ for the low-temperature transition is plotted as a function of the NH_4^+ concentration in Figure 3. The graph becomes linear at NH_4^+ concentrations above ~50 mM, and the slope at these concentrations yields a value for n of 1.1 ± 0.15. We conclude that there is one NH_4^+ binding

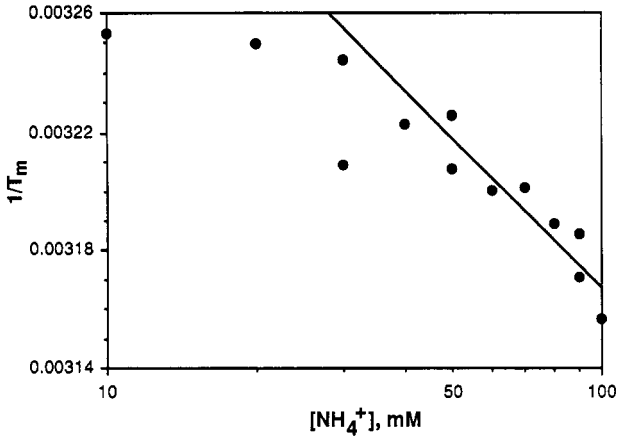

FIGURE 3: Dependence of the rRNA fragment tertiary structure stability on NH_4^+ ion concentration. Melting temperatures were determined as in Figure 2, using the same buffer but with the NH_4Cl and LiCl concentrations totaling 100 mM. The line is a least squares fit to the data obtained above 50 mM NH_4Cl, with $d(1/T_m)/(d \ln [NH_4^+]) = -7.3 \times 10^{-5}$ deg^{-1}. The reciprocal T_m in 100 mM LiCl was 0.003282 deg^{-1}.

site in this RNA. In principle, the curvature of Figure 3 at low NH_4^+ concentrations is related to the ion binding affinity, but because the transition may not be two-state at low ion concentrations, the affinity cannot be calculated easily.

Properties of the NH_4^+ Binding Site. The potential of NH_4^+ to donate four hydrogen bonds in a tetrahedral geometry may be responsible for its ability to stabilize the RNA fragment structure more effectively than the alkalai metal cations. To see if all four bonding positions are essential, methylamine was tested. The melting profiles obtained with this ion were identical to those seen with Na^+ (data not shown). Either all four hydrogen-bonding positions are essential or the bulk of the methyl group prevents the ion from fitting into some "pocket" in the RNA.

Mg^{2+} also stabilizes the RNA fragment tertiary structure by binding at a single site (Laing *et al.*, 1993). The site is distinct from the NH_4^+ binding site, since concentrations of NH_4^+ or K^+ up to 1 M do not induce the low-temperature transition in the absence of Mg^{2+}, and high Mg^{2+} concentrations do not stabilize the structure in the presence of Na^+ as the monovalent ion (Laing *et al.*, 1993, and data not shown). Some cooperative interaction between the two sites might be expected, and we found that Ca^{2+}, which only weakly stabilizes the tertiary structure in the presence of K^+ (Laing *et al.*, 1993), becomes more effective with NH_4^+ as the monovalent ion (data not shown).

NH_4^+ Stimulates L11 and Thiostrepton Binding to the rRNA Fragment. Since both Mg^{2+} and NH_4^+ stabilize the same RNA tertiary structure, and Mg^{2+} must be present to detect either L11 or antibiotic binding to the RNA (Ryan & Draper, 1989; Ryan *et al.*, 1991), we suspected that monovalent ions would affect the binding affinity of these ligands. A comparison of L11 binding in buffers containing 175 mM each of either NH_4^+, K^+, Cs^+, or Na^+ is shown in Figure 4A. There is a definite trend to the binding constants measured, with NH_4^+ strenghtening binding about 7-fold over Na^+, and K^+ and Cs^+ stabilizing the complex to an intermediate degree. The same trend is seen in the binding of thiostrepton antibiotic (Figure 4B), with more than an order of magnitude separating the largest and smallest affinity constants. Although ion effects on ligand binding and RNA structure stabilization show the same trends, there are quantitative differences; for instance, Na^+ is worse than Cs^+ at promoting ligand binding but is about the same at stabilizing RNA structure. This

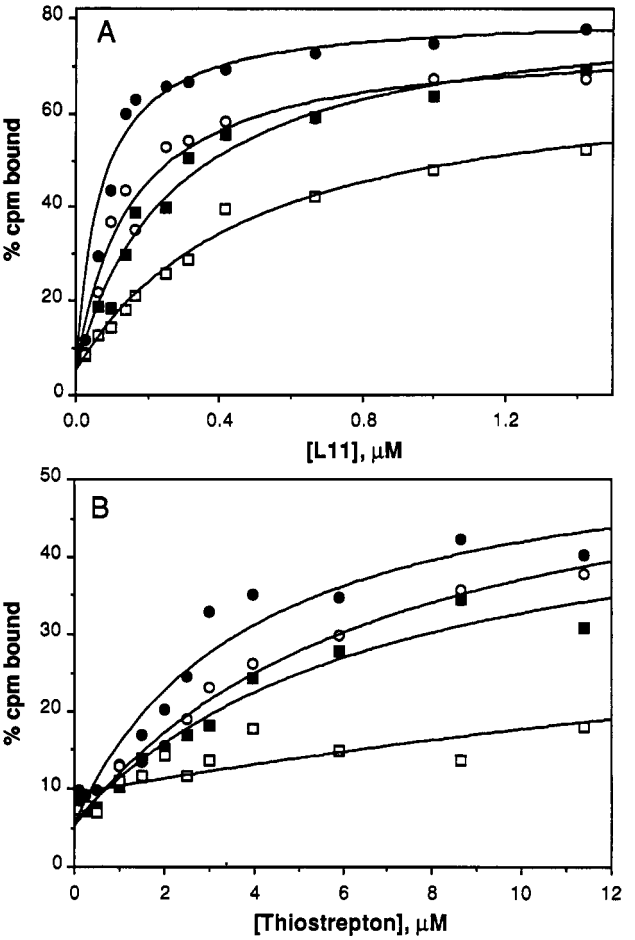

FIGURE 4: Titration of the rRNA fragment with either L11 (panel A) or thiostrepton (panel B) using a filter binding assay. Buffers contained 175 mM (□) NaCl, (■) CsCl, (○) KCl, or (●) NH_4Cl and contained in addition 30 mM Tris (pH 7.6) and 2 mM $MgSO_4$. Lines are single-site binding isotherms fit to the data by a nonlinear least squares program, allowing the equilibrium constant and maximum retention values to vary. Respective equilibrium association constants (μM^{-1}) for NH_4Cl-, KCl-, CsCl-, and NaCl-containing buffers are (panel A) 15, 6.6., 3.6, and 2.1 and (panel B) 0.26, 0.14, 0.14, and 0.022. For the thiostrepton titration in NaCl, the plateau value was fixed at 55.6%, the average obtained for titrations in the other three salts.

may be due to the difficulty of quantitating differences among the low-enthalpy (and non-two-state) RNA tertiary structure transitions with ions other than K^+ or NH_4^+. We conclude from these binding experiments that specific ion stabilization of the correct RNA structure is functionally relevant. The ion effects on L11 binding affinity may explain in part the complex dependence of the ribosome GTPase activity on monovalent cation identity (Sander *et al.*, 1976, 1978), since the protein is known to stimulate the GTPase activity and is loosely bound to the ribosome (Cundliffe, 1986).

DISCUSSION

How general should we expect to find the phenomenon of specific NH_4^+ ion interaction with RNA structures? The same ion requirements we find for the small rRNA fragment folding and ligand binding were observed more than 20 years ago for several activities of ribosomes, including the peptidyltransferase activity assayed with isolated 50S subunits and the mRNA-dependent binding of tRNA to 30S subunits (Miskin *et al.*, 1970; Zamir *et al.*, 1971). In both cases, prolonged dialysis against Na^+ resulted in inactive particles which could be reactivated by warming in NH_4^+; K^+, Rb^+,

and Cs^+ promoted intermediate levels of activity. Therefore, at least two sites in ribosomes (one on each subunit) interact specifically with NH_4^+. Chemical probing experiments localized structural differences between the small subunit rRNA active and inactive forms to sequences creating the decoding site (Moazed *et al.*, 1986), but similar experiments have not been tried with the inactive large subunit. To our knowledge, the only other RNA activity stimulated by NH_4^+ is tRNA cleavage by the RNase P ribozyme; the effect is more dramatic in the ribozyme from *Clostridium sporogenes* than from *E. coli* (Gardiner *et al.*, 1985; Roselli & Marsh, 1990). For many RNA reactions a comparison of NH_4^+ and other ions may not have been attempted.

NH_4^+-specific binding by a four-stranded poly(I) complex has been reported and postulated to involve hydrogen bonding of the ion to a tetrahedral arrangement of base carbonyl groups (Howard & Miles, 1982). Na^+ and K^+ formed complexes with poly(I) of slightly different structure, presumably because of the different bonding possible with these ions. We assume the rRNA fragment selectivity for NH_4^+ is also the result of a match between a tetrahedral array of hydrogen bond acceptors in the RNA and NH_4^+. Methylamine fails to stabilize the RNA tertiary structure, showing that substitution of even one NH_4^+ hydrogen by methyl cannot be accommodated in the binding site. The RNA binding pocket must surround the entire ion and probably hydrogen bonds at all four possible positions. Bases essential for the Mg^{2+}- and NH_4^+-dependent teritary structure of the rRNA fragment have been identified and are within or surrounding the junction loop (Laing & Draper, 1993, and unpublished data).

Although few RNA functions have been linked to specific ion binding sites so far, recent selection experiments suggest that ion binding sites of specific size and geometry are easily created in RNA structures. Pb^+, which coordinates ligands strongly, binds to a single specific site in tRNA (Brown *et al.*, 1985) and in an RNA internal loop selected for Pb^+-induced cleavage (Pan & Uhlenbeck, 1992). The guanidinium group of arginine binds in a bulge–loop structure of the HIV tat RNA (Puglisi *et al.*, 1992) and to other RNA structures selected from a random pool (Connell *et al.*, 1993). Although the alkali metal and alkali earth ions do not coordinate ligands strongly, partial neutralization of the high negative charge density developed in some RNA structure is a major impetus for binding. The columns of positive charges along the axis of either G-quartet or hemiprotonated oligo(dC) four-stranded helices are examples (Kang *et al.*, 1992; Gehring *et al.*, 1993). We suspect that sites specific for NH_4^+ and other ions are common in RNAs in which a compact structure is functionally required.

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REFERENCES

Brown, R. S., Dewan, J. C., & Klug, A. (1985) *Biochemistry 24*, 4785–4801.

Connell, G. J., Illangesekare, M., & Yarus, M. (1993) *Biochemistry 32*, 5497–5502.

Cundliffe, E. (1986) Involvement of Specific Portions of Ribosomal RNA in Defined Ribosomal Functions: A Study Utilizing Antibiotics, in *Structure, Function, and Genetics of Ribosomes* (Hardesty, B., & Kramer, G., Eds.) pp 586–604, Springer-Verlag, New York.

Egebjerg, J., Douthwaite, S. R., Liljas, A., & Garrett, R. A. (1990) *J. Mol. Biol. 213*, 275–288.

Fu, D.-J., & McLaughlin, L. W. (1992) *Biochemistry 31*, 10941–10949.

Gardiner, K. J., Marsh, T. L., & Pace, N. R. (1985) *J. Biol. Chem. 260*, 5415–5419.

Gehring, K., Leroy, J.-L., & Guéron, M. (1993) *Nature 363*, 561–565.

Guschlbauer, W., Chantot, J.-F., & Thiele, D. (1990) *J. Biomol. Struct. Dyn. 8*, 491–511.

Gutell, R. R., Schnare, M. N., & Gray, M. W. (1992) *Nucleic Acids Res. 20*, 2095–2109.

Howard, F. B., & Miles, H. T. (1982) *Biopolymers 21*, 147–157.

Kang, C., Zhang, X., Ratliff, R., Moyzis, R., & Rich, A. (1992) *Nature 356*, 126–131.

Laing, L. G., & Draper, D. E. (1993) *J. Mol. Biol.* (submitted for publication).

Laing, L. G., Gluick, T. C. & Draper, D. E. (1993) *J. Mol. Biol.* (submitted for publication).

Miskin, R., Zamir, A., & Elson, D. (1970) *J. Mol. Biol. 54*, 355–378.

Moazed, D., von Stold, B. J., Douthwaite, S., & Noller, H. F. (1986), *J. Mol. Biol. 191*, 483–493.

Moazed, D., Robertson, J. M., & Noller, H. F. (1988) *Nature 334*, 362–364.

Pan, T., & Uhlenbeck, O. C. (1992) *Biochemistry 31*, 3887–3895.

Pauling, L. (1960) *The Nature of the Chemical Bond*, Cornell University Press, Ithaca, NY.

Perreault, J.-P., Labuda, D., Usman, N., Yang, J.-H., & Cedergren, R. (1991) *Biochemistry 30*, 4030–4025.

Piccirilli, J. A., Vyle, J. S., Caruthers, M. H., & Cech, T. R. (1993) *Nature 361*, 85–88.

Puglisi, J. D., Tan, R., Calnan, B. J., Frankel, A. D., & Williamson, J. R. (1992) *Science 257*, 76–80.

Roselli, D. M., & Marsh, T. L. (1990) *Mol. Microbiol. 4*, 1393–1400.

Ryan, P. C., & Draper, D. E. (1989) *Biochemistry 28*, 9949–9956.

Ryan, P. C., & Draper, D. E. (1991) *Proc. Natl. Acad. Sci. U.S.A.* (in press).

Ryan, P. C., Lu, M., & Draper, D. E. (1991) *J. Mol. Biol. 221*, 1257–1268.

Sander, G., Marsh, R. C., & Parmeggiani, A. (1976) *Eur. J. Biochem. 61*, 317–323.

Sander, G., Parlato, G., Crechet, J.-B., Nagel, K., & Parmeggiani, A. (1978) *Eur. J. Biochem. 86*, 555–563.

Sundquist, W. I., & Heaphy, S. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 3393–3397.

Zamir, A., Miskin, R., & Elson, D. (1971) *J. Mol. Biol. 60*, 347–364.