Kinetic and Thermodynamic Analysis of RNA–Protein Interactions in the RNase P Holoenzyme from *Escherichia coli*†

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ABSTRACT: A gel retardation assay has been used to examine the kinetic and equilibrium properties of the interaction between C5 protein and M1 RNA in the formation of the ribonuclease P holoenzyme from *Escherichia coli*. The interaction is relatively insensitive to the identity of the monovalent anions present and to pH in the range 7.0–9.0, but it has a more critical requirement for specific monovalent and divalent cations: NH_4^+, K^+, Mg^{2+}, Ca^{2+}, and Mn^{2+} all promote efficient formation of the complex. A positive ΔS (+6.4 cal mol^{-1} deg^{-1}) and a negative ΔH (−11.3 kcal mol^{-1}) combine to give a ΔG equal to −13.3 kcal mol^{-1} at 37 °C in 0.42 M salt. The binding reaction is sensitive to the concentration of monovalent and divalent cations, with the affinity increasing with increasing ionic strength ($\delta \log K_a/\delta \log [NH_4^+] = +2.7 \pm 0.1$). The dependence of K_d on the ionic strength and the positive ΔS suggests that hydrophobic and stacking interactions contribute significantly to the formation of the RNase P holoenzyme.

Many important cellular events including splicing of pre-mRNA, 3′-end formation, translation of mRNA, and processing of pre-tRNA are mediated by the specific interaction of proteins with RNA. Whereas, in general, DNA-binding proteins primarily recognize sequence, RNA-binding proteins recognize both sequence and structure at their target sites. It is of interest to characterize the nature and relative importance of the elements that confer specificity on such interactions. An approach taken by many groups has been to reproduce the binding reaction using purified components and to observe the effects of changes in solution conditions on the dissociation constant (K_d). Application of this approach to DNA–protein binding systems (Riggs et al., 1970; Lohman et al., 1980) and RNA–protein interactions (Carey & Uhlenbeck, 1983; Vartikar & Draper, 1989; Ryan & Draper, 1989; Weeks & Crothers, 1992) has led to inferences about the mechanism and molecular basis for these interactions. In our preceding paper (Talbot & Altman, 1994) we described an RNA-binding assay that we used to analyze RNA–protein interactions in the ribonuclease P (RNase P)[1] holoenzyme from *Escherichia coli*. RNase P, the endoribonuclease responsible for the biosynthesis of the 5′ termini of mature tRNA, is a ribonucleoprotein. In *E. coli*, the enzyme is made up of an RNA subunit of 377 nucleotides (M1 RNA) and a basic protein (C5) with a molecular mass of 13 800 Da. (Altman et al., 1993; Darr et al., 1992). The RNA subunit can catalyze the cleavage of appropriate substrates by itself *in vitro* (Guerrier-Takada et al., 1983), but both subunits are essential for the activity of the enzyme *in vivo* (Kole et al., 1980). We report here the dissociation constant for the C5 protein–M1 RNA interaction as determined under a variety of solution conditions. The kinetics of the binding reaction are also presented. The results are interpreted in terms of contacts made between the protein and the nucleic acid, and a working model for the interaction is proposed.

MATERIALS AND METHODS

Gel Retardation Assays. The preparation of C5 protein and internally ^{32}P-labeled M1 RNA and the details of the gel retardation assay have been described by Talbot & Altman (1994). The pH of the various buffers used in this study was adjusted at the temperature used in the incubation. The standard binding buffer contained 20 mM K-Hepes pH 8.0, 400 mM NH_4OAc, 10 mM $Mg(OAc)_2$, 0.01% (v/v) Nonidet P-40, 5% (v/v) glycerol.

Dissociation Kinetics. C5 protein–M1 RNA complexes were performed under standard binding conditions [20 mM K-Hepes pH 8.0, 400 mM NH_4OAc, 10 mM $Mg(OAc)_2$, 0.01% (v/v) Nonidet P-40, 5% (v/v) glycerol] at 37 °C for 10 min. Typical concentrations of C5 protein and radiolabeled M1 RNA were 0.4–0.8 nM and 0.1 nM, respectively; protein concentrations were sufficient for complex formation by 50–80% of input M1 RNA. Unlabeled M1 RNA was added to a final concentration of 5–100 nM ($t = 0$) and mixing was achieved by gentle pipetting. Aliquots (10 μL) were withdrawn at appropriate times and loaded immediately onto a running 4% (w/v) native polyacrylamide gel (Talbot & Altman, 1994). Dried gels were analyzed using a Betascope blot analyzer (Betagen Co., Waltham, MA).

The concentration of C5 protein referred to throughout this paper is the total concentration of protein in any preparation (see Talbot & Altman (1994)).

RESULTS

Kinetics. The interaction between C5 protein and M1 RNA was investigated with a gel retardation assay (Talbot & Altman, 1994). As seen in Figure 1, C5 protein–M1 RNA complexes can be separated from free M1 RNA by electrophoresis because of their higher molecular mass and altered net charge. C5 is a highly basic protein with an estimated pI of 12.2, so it has a net positive charge at the pH of the binding

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[1] Abbreviations: RNase, ribonuclease; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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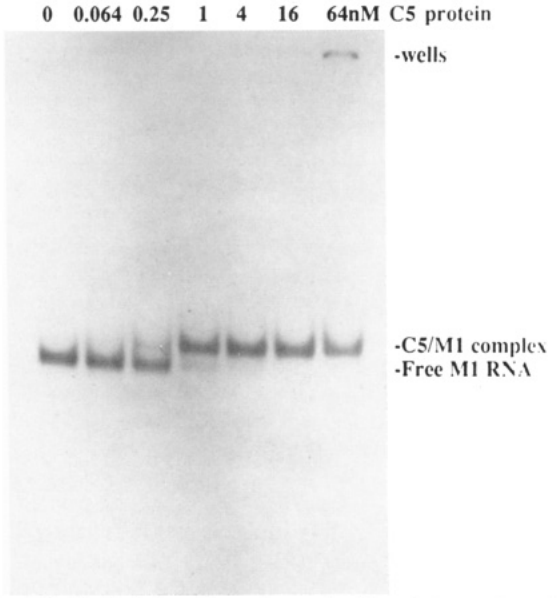

FIGURE 1: Analysis by native polyacrylamide gel electrophoresis of complexes formed between C5 protein and M1 RNA. Approximately 0.1 nM ^{32}P-labeled M1 RNA was incubated for 10 min at 37 °C with C5 protein at concentrations (in nanomoles of active C5 protein) indicated at the top of each lane. The more rapidly moving band on the autoradiogram corresponds to free M1 RNA and the slower band to the holoenzyme complex.

Table 1: Summary of Kinetic and Thermodynamic Parameters for Formation of the RNase P Holoenzyme[a]

dissociation constant (K_d)	4.0×10^{-10} M
dissociation rate constant (k_{off})	$6.0 \pm 0.1 \times 10^{-3}$ min^{-1}
association rate constant (k_{on})	1.5×10^{7} M^{-1} min^{-1}
$t_{1/2}$	115 ± 10 min
ΔG	−13.3 kcal mol^{-1} (−55.6 kJ mol^{-1})
ΔH	−11.3 kcal mol^{-1} (−47.4 kJ mol^{-1})
ΔS	+6.4 cal mol^{-1} deg^{-1} (+26.8 J mol^{-1} deg^{-1})

[a] All measurements were made at 37 °C.

assay (pH 8.0). Neutralization of the negative charge on the RNA results in slower migration of the complex. The K_d for the C5 protein–M1 RNA interaction was calculated as 0.4 nM (Talbot & Altman, 1994) which corresponds to a ΔG of −13.3 kcal mol^{-1} (−55.6 kJ mol^{-1}) at 37 °C ($\Delta G = RT \ln K_d$; Table 1).

The rate of dissociation of the C5 protein–M1 RNA complex was measured after formation of complexes between approximately 0.1 nM ^{32}P-labeled M1 RNA and 0.4–0.8 nM C5 protein. Sufficient protein was used to give 50–80% complex formation with M1 RNA. After formation of the complex, unlabeled M1 RNA was added in molar excess (50–1000-fold molar excess) and the rate of dissociation was monitored by the gel retardation assay (Figure 2). It is unlikely that complexes dissociate any further once they have entered the gel due to the "caging" effect of the gel matrix (Fried & Crothers, 1981). Since the unlabeled M1 RNA was added in molar excess, dissociated labeled M1 RNA must compete with the unlabeled RNA for rebinding to the protein, with resultant reduction in the amount of labeled M1 RNA in the complex with time until a new equilibrium is established. A control experiment (Figure 3) in which antisense M1 RNA was added to the C5 protein–M1 RNA complex at zero time showed no decrease in the amount of bound M1 RNA with time. Figure 3 shows the early time points from experiments with several different concentrations of protein and unlabeled competitor RNA. The initial rate of dissociation was

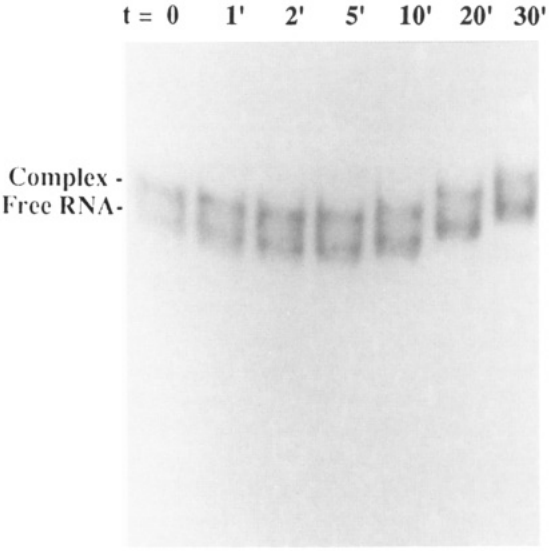

FIGURE 2: Kinetic stability of the RNase P holoenzyme complex. The autoradiogram is shown from which the dissociation rate for the C5 protein–M1 RNA complex was calculated. Time 0 indicates the equilibrium state at the start of the experiment. After addition of an excess of unlabeled M1 RNA (50 nM) as a competitor at $t = 0$, aliquots were loaded onto a running native gel at the times indicated.

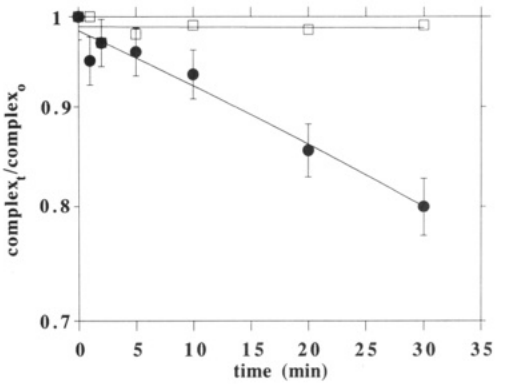

FIGURE 3: Dissociation kinetics of the RNase P holoenzyme complex. The data from Figure 2 are plotted as a fraction of the remaining complex. The half-life of the complex is 115 ± 10 min with the slope of the line (●) corresponding to a dissociation constant, k_{off}, of $6.0 \pm 0.1 \times 10^{-3}$ min^{-1}. The open boxes (□) show the rate of dissociation of the C5–M1 complex when antisense M1 is used as a competitor.

independent of the concentration of C5 protein, as expected for a unimolecular decomposition. The slope of the line in Figure 3 gives a value of the dissociation constant $k_{off} = 6.0 \times 10^{-3}$ min^{-1}, which corresponds to a half-life of 115 min for the complex.

An attempt was made to measure the rate constant (k_{on}) of the association between C5 protein and M1 RNA. Labeled M1 RNA (~0.1 nM) was mixed with sufficient C5 protein (0.4–0.8 nM) to give 50–80% complex of labeled M1 RNA, and aliquots were withdrawn and loaded onto a running native gel (Talbot & Altman, 1994) after very short intervals of time. Figure 4 shows that formation of the complex was complete after the first time point (10 s). Therefore, calculations of association rate constants are impractical. However, from the relationship $K_d = k_{off}/k_{on}$, the value of k_{on} was calculated to be 1.5×10^{7} M^{-1} min^{-1}.

Temperature Dependence of K_d. Protein-binding parameters were determined as a function of temperature, in binding buffers that had been adjusted to pH 8.0 at various temperatures. After incubation at various temperatures, binding reaction mixtures were fractionated on native gels (Talbot &

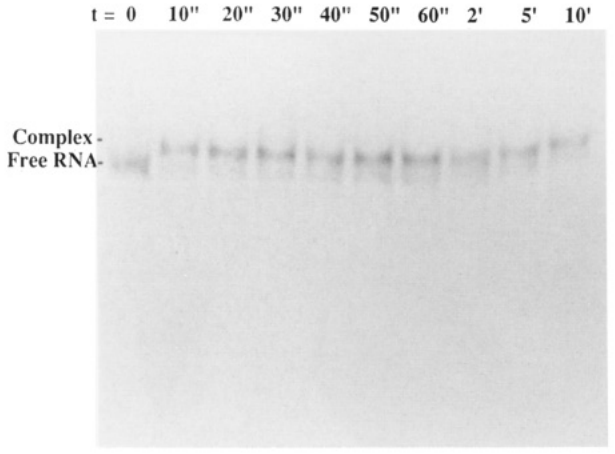

FIGURE 4: Kinetics of formation of the RNase P holoenzyme complex. [The autoradiogram shows the rate of formation of the C5 protein–M1 RNA complex.] At time 0, sufficient active C5 protein was mixed with radiolabeled M1 RNA to give 50–80% complexation. Aliquots were withdrawn from the reaction mixture and loaded onto a running native gel at the times indicated.

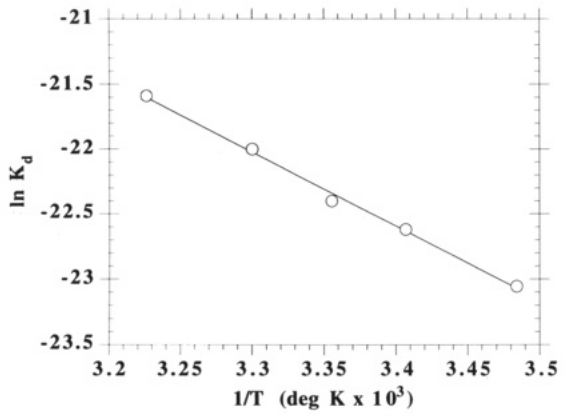

FIGURE 5: Temperature dependence of K_d. C5 protein excess binding curves were obtained in binding buffer adjusted to pH 8.0 at the indicated temperatures: 15, 20, 25, 30, and 37 °C. The slope gives a value for ΔH of −11.3 kcal mol^{-1}.

Altman, 1994). [Even though all gels were run at 4 °C, the values of K_d obtained are a reflection of the incubation temperature since, once the C5 protein–M1 RNA complex has entered the gel matrix, further dissociation or association is inhibited (Fried & Crothers, 1981)]. From the van't Hoff plot of these data (Figure 5), the ΔH for the binding reaction was calculated, from the slope of the line, to be −11.3 kcal mol^{-1} (−47.4 kJ mol^{-1}). The values calculated for ΔH and ΔG were used to obtain a value for ΔS of +6.4 cal mol^{-1} deg^{-1} at 37 °C ($\Delta G = \Delta H - T\Delta S$).

Effect of Solution Composition on K_d. The standard binding buffer used thoughout this study contained 20 mM K-Hepes, 400 mM NH_4OAc, 10 mM $Mg(OAc)_2$, 0.01% NP-40 and 5% glycerol. Table 2 shows the effects on K_d of the identity of monovalent cations and anions at 400 mM. The effects of anion were small with the affinity of C5 protein for M1 RNA decreasing 3-fold in the following order: $CH_3CO_2^- = NO_3^- > Cl^- = Br^- > I^-$. Changes in the monovalent cation, by contrast, had a relatively large effect, with the affinity of C5 protein for M1 RNA decreasing 14-fold as follows: $NH_4^+ \geq K^+ > Na^+ > Li^+$.

The effects of identity and concentration of divalent cations were also investigated. Protein-binding curves were determined with Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Cd^{2+} as their respective chlorides at a concentration of 5 mM, in the standard binding buffer prepared without $Mg(OAc)_2$

Table 2: The Effect of Ions on the Dissociation Constant (K_d) in the Formation of the RNase P Holoenzyme Complex

ion	K_d (nM)
CH_3COO^-	0.4
NO_3^-	0.4
Cl^-	0.6
Br^-	0.6
I^-	1.2
Li^+	5.4
Na^+	2.7
K^+	0.6
NH_4^+	0.4
Mg^{2+}	0.4
Ni^{2+}	>40
Co^{2+}	2.1
Mn^{2+}	0.4
Ca^{2+}	0.4
Cu^{2+}	>40
Cd^{2+}	0.6

(Table 2). The identity of the divalent cation had a significant effect on the affinity of C5 protein for M1 RNA. Affinity decreased 50-fold as follows: $Mg^{2+} = Ca^{2+} = Mn^{2+} > Co^{2+} > Cd^{2+}$. Ni^{2+} and Cu^{2+} ions completely inhibited formation of the complex. The optimum concentration of Mg^{2+} in the binding reaction was also investigated. The results in Figure 6 show that in the absence of any divalent metal ions the affinity of C5 protein for M1 RNA is reduced 25-fold. This suggests that Ni^{2+} or Cu^{2+} ions actively inhibit the interaction of C5 protein with M1 RNA since the K_ds are 2-fold lower than in the absence of any divalent cation. Optimal divalent metal ion concentrations for formation of the RNase P holoenzyme are in the range 1–10 mM, similar to the optimum concentration for the cleavage of substrates by RNase P (Guerrier-Takada & Altman, 1984). Concentrations of Mg^{2+} ion above 100 mM begin to inhibit formation of the complex (data not shown).

Ionic Strength and pH Dependence of K_d. C5 protein excess binding curves were obtained at various concentrations of various NH_4OAc in the standard binding buffer at 37 °C. In Figure 7 K_d is plotted against the concentration of NH_4OAc. The affinity of C5 protein for M1 RNA increased 500-fold with increasing ionic strength in the range 0.1–1.0 M ($\delta \log K_a/\delta \log [NH_4^+] = +2.7 \pm 0.1$). This result contrasts with the dependence on ionic strength observed with other RNA–protein interactions, such as the interaction between R17 coat protein and its translational operator (Carey & Uhlenbeck, 1983), HIV Tat derived peptides binding to the tar region of HIV-1 RNA (Weeks & Crothers, 1992), the *E. coli* ribosomal protein S4-16S ribosomal RNA complex (Vartikar & Draper, 1989), and binding of ribosomal protein of *E. coli* L11 to 23S rRNA (Ryan & Draper, 1989). In general, the affinity of a protein for its RNA ligand decreases with increasing ionic strength, reflecting the contribution of ionic contacts to the RNA–protein interaction. At high ionic strength, any anion-binding sites on the protein are occupied to a greater extent. If the sites occupied by anions are the same ones as those occupied by the RNA, then competition between these two ligands will increase the K_d (reduce the affinity). The ionic strength dependence of the C5 protein–M1 RNA interaction suggests that ionic contacts do not contribute significantly to the formation of the specific complex but, rather, hydrophobic interactions are the driving force for formation of the complex. The change in entropy (ΔS) for formation of the complex is +6.4 cal mol^{-1} deg^{-1} at 37 °C (Table 1). This change in entropy could result from configurational changes in M1 RNA or C5 protein and/or the freeing of water molecules opon binding. Hydrophobic interactions are also associated with

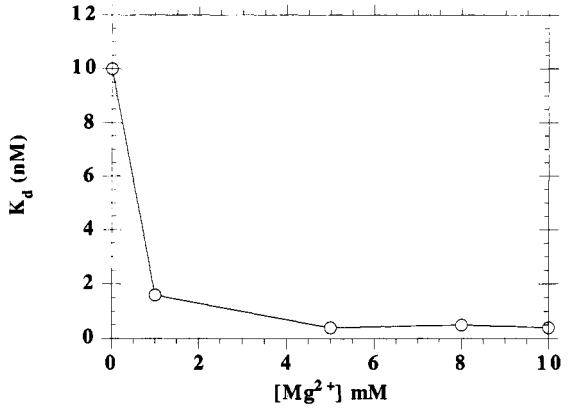

FIGURE 6: Effect of Mg^{2+} concentration on the dissociation constant (K_d) for the C5–M1 interaction.

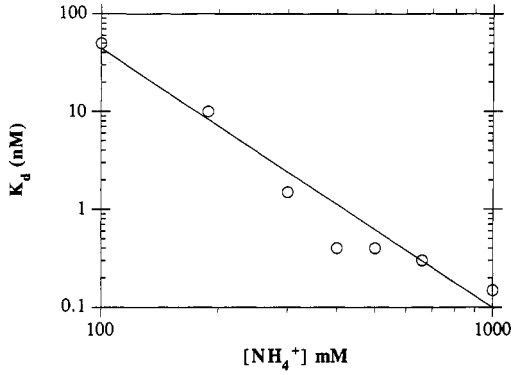

FIGURE 7: Dependence of K_d on ionic strength. Protein excess binding curves were obtained in binding buffer at the concentrations of NH_4-OAc indicated.

a relatively small ΔH (as compared to ΔG) and a positive ΔS. Since the parameters for binding of C5 protein to M1 RNA fit these characteristics, it is tempting to conclude that hydrophobic interactions are the molecular basis of the thermodynamics stability of the complex.

The dissociation constant for the C5 proteins–M1 RNA interaction was essentially independent of pH in the range 7.0–9.5 (data not shown). Below pH 7.0, C5 protein–M1 RNA complexes were poorly resolved in the gel, with significant smearing of the bands, perhaps as a result of being close to the pI of the RNase P holoenzyme (pI = 5.5; Stark, 1977).

DISCUSSION

We have investigated several properties of the interaction between C5 protein and M1 RNA that leads to the formation of the RNase P holoenzyme. The types of experiment and the methods for analysis of data used here have been used previously to study both DNA–protein interactions (e.g., Riggs et al., 1970; Fried & Crothers, 1981; Carey, 1988) and RNA–protein interactions (e.g., Carey & Uhlenbeck, 1983; Ryan & Draper, 1989; Weeks & Crothers, 1991). Many earlier examples have involved regulatory proteins that operate by binding specifically to a relatively small site on a large polynucleotide. In the preceding paper (Talbot & Altman, 1994), we showed that C5 protein requires the presence of a complex three-dimensional structure if it is to bind specifically to M1 RNA to form the RNase P holoenzyme. Thus, the kinetics of formation of the RNase P holoenzyme might be expected to be very different from those that reflect the transient nature of complexes between regulatory proteins and operators.

The rate of dissociation of the C5 protein–M1 RNA complex is relatively low. At 37 °C, in standard binding buffer, the half-life of the complex is approximately 110 min. This value is 2 orders of magnitude higher than the dissociation rates observed for other RNA–protein complexes, such as the HIV-1 Tat-tar (Weeks & Crothers, 1991) or the R17 coat protein–translational operator complex (Carey & Uhlenbeck, 1983). The high on/off ratio of the rates of these latter interactions probably reflects their functions as regulators of translation or transcription *invivo*. A precise determination of the forward rate of complex formation was not possible using the gel retardation assay, but k_{on} could be calculated from the experimentally determined dissociation constant (K_d; 0.4 nM) and the dissociation rate constant (k_{off}; 6×10^{-3} min^{-1}). The value obtained for the association rate constant (k_{on}; 1.5×10^7 M^{-1} min^{-1}) is somewhat lower than that expected (10^8–10^9 M^{-1} min^{-1}; Alberty & Hammes, 1958) for a diffusion-controlled reaction between molecules of the size of C5 protein and M1 RNA. Therefore, it is possible that, rather than a simple bimolecular equilibrium, one or more first-order intermediate steps occur in the association pathway as, for example, in the case of an "induced fit" binding mechanism, wherein a conformational change in M1 RNA would be required before C5 protein could bind specifically. Such a conformational change could be induced by an initial non-specific interaction of basic residues on C5 protein (Figure 8) with the phospho diester backbone of M1 RNA, which then leads to the formation of the specific high-affinity complex. This mechanism of binding is analogous to the "bind and slide" mechanism of binding to DNA by the *lac* repressor (Winter & von Hippel, 1981). In the case of the *lac* repressor, the nonspecific binding is thought to be an important mediator of the subsequent specific binding (Berg et al., 1981).

The values of the thermodynamic parameters that describe the interaction between C5 protein and M1 RNA are summarized in Table 1. The interaction is characterized by a large favorable ΔG and ΔH and a favorable ΔS at 37 °C. A favorable ΔH generally results from the establishment of relatively weak contacts of the van der Waals' and hydrogen-bond types, while ionic bonds and hydrophobic forces contribute favorably to ΔS, via release of bound ions and water molecules (Beaudette & Langerman, 1980). Examination of the ionic strength dependence of the interactions (Figure 7) reveals that the affinity of C5 protein for M1 RNA increases with increasing ionic strength. Taken together with the thermodynamic parameters, these data suggest that hydrophobic and stacking interactions play a more significant role in the formation of the complex than ionic contacts, which would be expected to be disrupted upon an increase in ionic strength. The favorable ΔS could also arise from conformational changes induced in M1 RNA upon binding of C5 if such binding caused the release of ions and water molecules bound to the RNA and/or the protein.

The effect of monovalent ions on the formation of the RNase P holoenzyme is qualitatively quite different from that on the interaction between R17 coat protein and the translational operator (Carey & Uhlenbeck, 1983) or from that on the binding of *lac* repressor to its operator (Riggs et al., 1970). In both these latter cases, the identity of the anion has a large effect on formation of the complex, presumably via the differential interactions of the anions with the protein. By contrast, specific monovalent and divalent cations were required for formation of the RNase P holoenzyme. Cations are known to play an important role in the formation of specific structures by RNAs either by general neutralization of charges,

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M. luteus        M----LPRDR RVRTPAEFRH LGRTG-TRAG RRTVVV---S VATDPDQTRS     42
S. bikiniensis   M----LPTEN RLRRREDFAT AVRRG-RRAG RPLLVVHRLS GATDPH---A     42
E. coli          MVKLAFPREL PLLTPSQFTF VFQQP-QRAG TPQITILGRL NSLGHP----     45
P. mirabilis     MVKLAFPREL RLLTPKHFNF VFQQP-QRAS SPEVTILGRQ NELGHP----     45
Ps. putida       MSQ-DFSREK RLLTPRHFKA VFDSPTGKVP GKNLLILARE NGLDHP----     45
Bu. aphidicola   MLNYFFKKKS KLLKSTNFQY VFSNPCNKNT F-HINILGRS NLLGHP----     45
B. subtilis      MSH--LKKRN RLKKNEDFQK VFKHG-TSVA NRQF-VL--- YTLDQPENDE     43
Consensus        M..-.FPRE. RLLTP..F.. VF..P-.RA. .....IL.R. N.LDHP----     50

M. luteus        TSPSAPRPRA GFVVSKAVGN AVTRNRVKRR LRAVV---AE QMRLPPLRDL     89
S. bikiniensis   PGESAPPTRA GFVVSKAVGG AVVRNQVKRR LRHLV---CD RL--SALPP-     86
E. coli          -RIGLT---- --VAKKNVRR AHERNRIKRL TRESF----- RLRQHELPAM     83
P. mirabilis     -RIGLT---- --IAKKNVKR AHERNRIKRL AREYF----- RLHQHQLPAM     83
Ps. putida       -RLGLV---- --IGKKSVKL AVQRNRLKRL MRDSF----- RLNQQLLAGL     83
Bu. aphidicola   -RLGLS---- --ISRKNIKH AYRRNKIKRL IRETF----- RLLQHRLISM     83
B. subtilis      LRVGLS---- --VSKK-IGN AVMRNRIKRL IRQAFLEEKE RLKEK-----     81
Consensus        -R.GL.---- --V.KK.V.. AV.RNRIKRL .R..F----- RL.Q..L...     100

M. luteus        PVLVQVR-AL PAAAEAD--- -----YALLR RETVGALGKA LKPHLPAASE HA  132
S. bikiniensis   GSLVVVR-AL PGAGDAD--- -----HAQLA.RD----LDAA LQ-RLLGGGT R-  123
E. coli          DFVVVAKKGV ADLDNRALSE ALEKLWRRHC R--------- ----L-ARGS --  119
P. mirabilis     DFVVLVRKGV AELDNHQLTE VLGKLWRRHC R--------- ----L-AQKS --  119
Ps. putida       DIVIVARKGL GEIENPELHQ HFGKLWKRLA RSRPTPAVTA NSAGVDSQDA --  133
Bu. aphidicola   DFVVIAKKNI VYLNNKKIVN ILEYIWSNYQ R--------- ---------- --  114
B. subtilis      DYIIIARKPA SQLTYEETKK SLQHLFRKSS LY-------- ----KKSSSK --  119
Consensus        D.VV.ARK.. ..L.N..... .L..LW.... R--------- ----...... --  150
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FIGURE 8: Alignments of amino acid sequences of homologues of the C5 protein of RNase P from prokaryotic sources. The sequences, deduced from nucleotide sequences are from the following organisms: *Micrococcus luteus* (Fujita et al., 1990); *Streptomyces bikiniensis* (P. D. Morse and F. J. Schmidt, Genebank accession no. M83112, 1992, unpublished work); *Escherichia coli* (Hansen et al., 1985); *Proteus mirabilis* (Skovgaard, 1990); *Pseudomonas putida* (N. Ogasawara, Genebank accession no. X62540, 1991, unpublished work); *Buchnera aphidicola* (Lai & Baumann, 1992); and *Bacillus subtilis* (Ogasawara et al., 1985). The alignments were produced with Geneworks software (Intelligenetics, Inc., Palo Alto, CA). Conserved residues are boxed.

or via interactions with specific binding pockets formed within the tertiary structure of the RNA. The identity of the anion had only a slight effect on K_d (up to 3-fold variation; Table 2), whereas the various monovalent cations tested had significant effects on K_d (14-fold variation; Table 2). The identity of the divalent cation in the binding buffer had an even greater effect on the K_d (50-fold variation; Table 2). Our data suggest that, in the absence of these divalent metal ions, or in the presence of metal ions that are incapable of filling specific pockets, M1 RNA cannot adopt the specific three-dimensional structure required for recognition by the C5 protein.

The RNA subunit of eubacterial RNase P has a strict requirement for Mg^{2+} ions for maximal catalytic activity (Guerrier-Takada et al., 1983; Gardiner et al., 1985). It has been reported that Ca^{2+} or Mn^{2+} ions can substitute for Mg^{2+} ions in the specific cleavage of precursor substrates by M1 RNA (Kazakov & Altman, 1991). However, Co^{2+}, Ni^{2+}, Cu^{2+}, and Cd^{2+} ions are all effective as inhibitors of the M1 RNA catalyzed cleavage reaction (Kazakov & Altman, 1991), in general agreement with the binding data presented here. It is possible to define three distinct roles for divalent cations in the formation of the RNase P holoenzyme and the cleavage of substrates: (i) the cations are involved in folding of the RNA subunit into a tertiary structure that can be recognized by the protein subunit; (ii) they are involved in binding of the substrate (Perreault & Altman, 1992); and (iii) they play a direct role in catalysis (Guerrier-Takada et al., 1983; Kazakov & Altman, 1991).

The amino acid sequence alignments of homologues of RNase P protein subunits from several prokaryotic sources is shown in Figure 8. The primary sequences do not exhibit any of the RNA-binding motifs found in other RNA-binding proteins [e.g., an RNA-recognition motif (RRM), Mattaj, 1989; an arginine-rich motif, Lazinski et al., 1989]. It is demonstrated previously that the protein moieties of the RNase P holoenzymes from *E. coli* and *Bacillus subtilis* can be mixed with the heterologous RNAs to form functional hybrid holoenzymes (Guerrier-Takada et al., 1983) and that each protein has a measurable affinity for the RNA from the other organism (Talbot & Altman, 1994). Therefore, although the primary sequences of these two proteins exhibit only 25% homology, their higher order structure and mode of recognition of the catalytic RNA must be quite similar. Analysis of the alignment of sequences for the seven known proteins from prokaryotic homologues of RNase P reveals only 7% sequence identity. However a core of highly conserved basic and hydrophobic residues, as well as a number of conserved and semiconserved aromatic residues, can be identified from the alignments. The presence of conserved aromatic and hydrophobic residues would appear to support the hypothesis that hydrophobic interactions play a critical role in formation of the RNase P holoenzyme. Indeed, preliminary evidence from site-directed mutagenesis of the C5 protein (V. Gopalan and S. Altman, in preparation) suggests that a Trp → Ala^{109} or a Phe → Ala^{18} mutation inhibits formation of an active RNase P holoenzyme. A working model describing the interaction of C5 protein with M1 RNA could involve initial binding of the two subunits via ionic interactions of basic amino acid side chains with the phospho diester backbone of M1 RNA, followed by formation of specific hydrophobic contacts between the aromatic/hydrophobic residues and the bases. This latter type of interaction would confer the high specificity observed for binding of C5 protein, and any disruption of the tertiary structure of M1 RNA would significantly effect recognition (Talbot & Altman, 1994). The interaction of U1 snRNP A

with U1 RNA has been shown to involve a cluster of aromatic residues that form part of the RNP1 and RNP2 domains of the so-called RNA-recognition motif (RRM), which is conserved between the U1 A and U2 B″ proteins (Nagai et al., 1990; Jessen et al., 1991). In addition to these aromatic residues, which are thought to stabilize the complex by van der Waals interactions, there are also a number of basic amino acids which are essential for the interaction (Jessen et al., 1991). Although C5 protein does not have any sequence homology with the RRM identified in these snRNPs, the mechanism of binding of M1 RNA to C5 protein would appear to be somewhat similar.

Extensive mutational analysis of C5 protein is currently being undertaken in an attempt to determine the role that aromatic and hydrophobic amino acid residues play in the formation of the RNase holoenzyme. This detail of the interaction between C5 protein and M1 RNA serve to underline the unique nature of RNase P for the study of both RNA–protein complexes and catalytic RNA.

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