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An RNA mutation that increases the affinity of an RNA–protein interaction

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ABSTRACT

The introduction of a cytidine in place of one of the two single stranded uridines in the R17 replicase translational operator results in a much tighter binding to R17 coat protein. The complex containing the variant RNA is stable to gel electrophoresis and has a binding constant about 50 times greater than the one with wild type RNA. The nearly thirty percent increase in the free energy of binding for the variant RNA is primarily due to a more favorable enthalpy of interaction. A possible explanation for this surprising result is that the U to C change leads to a greater extent of formation of a transient covalent complex between the protein and the RNA.

INTRODUCTION

The interaction of R17 coat protein with a synthetic RNA fragment containing the translational operator of the R17 replicase gene has been extensively studied as an example of a sequence specific RNA-protein interaction (1). Among the large number of variant RNA fragments tested for coat protein binding (2), one was found that appeared to bind the protein about five-fold tighter than the wild type sequence. This variant has a cytidine substituted for one of the two single stranded uridines in the wild type sequence. Changing the adjacent single stranded uridine to a cytidine had no effect on binding to the protein. A possible explanation for why the C containing fragment binds the protein more tightly comes from the observation that high concentrations of a variety of 5 halogenated nucleotides inactivate coat protein for RNA binding (3). The inactivation requires incubation and is reversed by dithiothreitol. Similar nucleotide inactivation has been observed for thymidylate synthetase (4) and isoleucine tRNA synthetase (5) and was interpreted to be the consequence of the formation of a

transient covalent intermediate between a cysteine on the protein and the C-6 of a pyrimidine. In the case of the tRNA synthetase, tritium exchange experiments suggested that the uridine at position 8 in $tRNA^{Ile}$ is the site of the adduct. If a similar transient covalent intermediate occurs between R17 coat protein and a U in its binding site, changing that U to a C could alter the interaction in such a way that the affinity of the RNA to the protein is increased. Additional support for this view comes from the observation that high concentrations of CMP also inactivate coat protein for RNA binding while similar concentrations of UMP have little effect (3).

These considerations prompted a more careful examination of the interaction of the C containing fragment with R17 coat protein. The kinetics and thermodynamics of the binding reaction were studied in greater detail to determine how the U to C change altered the interaction. Several experiments were designed to test for the presence of a transient covalent intermediate of the type proposed.

MATERIALS AND METHODS

The enzymatic synthesis of the 21 nucleotide fragment of R17 RNA corresponding to the translational operator of the R17 replicase gene has been described previously (6). The fragment forms a hairpin loop with seven base pairs as shown in Figure 1. This molecule will be termed U-loop. The synthesis of a variant containing a C in one of the single stranded positions (position -5) was carried out using the pentamer CAC_3 for the preparation of the 3' half molecule. This molecule will be termed C-loop. C-loop and U-loop were synthesized with an internal ^{32}P label in the phosphate 5' to the C or U by including [γ-^{32}P] ATP in the polynucleotide kinase reaction prior to the final ligation.

Coat protein binding experiments were carried out in a buffer of 0.1 M Tris-HCl pH 8.5, 80 mM KCl, 10 mM magnesium acetate, 80 μg/ml bovine serum albumin unless otherwise indicated. The methods for determining the equilibrium constant and rate constants for the protein-RNA interaction using the nitrocellulose filter binding assay are described in detail in Carey et al. (7) and Carey and Uhlenbeck (8). When β-mercaptoethanol

or dithiothreitol was present in the buffer and large volumes were filtered, the ability of nitrocellulose filters to retain the complex was substantially reduced. Thus, k_{off} could not be reliably determined by the dilution method in these buffers and the method using competition by non radioactive loop had to be used.

Gel electrophoresis of protein-RNA complexes were carried out on a 10 percent polyacrylamide slab gel (2 x 135 x 100 mm) containing 50 mM Tris-acetate pH 7.6 and 1 mM magnesium acetate (9). Electrophoresis was at 150 volts for 2 h at 4°C.

RESULTS AND DISCUSSION

A Tight Binding Variant

The filter retention assay was used to measure the binding of a low concentration of U and C-loops to varying concentrations of R17 coat protein in the standard manner (8). As seen in Figure 1, C-loop clearly binds coat protein better than U-loop as previously reported (2). However estimating the exact difference in K_a is complicated by the fact that while the data for U-loop closely fits a theoretical first order binding curve, the C-loop data does not. When compared with a theoretical curve chosen somewhat arbitrarily to have $K_a = 5 \times 10^9\ M^{-1}$, it is clear that the C loop data produces a sharper binding curve.

The poor fit of the C loop data to the theoretical binding curve prompted a study of the kinetics of the C-loop coat protein interaction. The initial rate of complex formation between C-loop and coat protein was determined by mixing 10 pM RNA and several different coat protein concentrations (0.08-1.0 nM) and filtering aliquots at short intervals. If it is assumed that the filtration effectively stops the association process and little dissociation occurs at early times, these data can be used to calculate an approximate bimolecular association rate constant (8). The value of k_{on} for C-loop is $0.6 \times 10^8\ M^{-1}\ min^{-1}$ which is slightly less than the value of $1 \times 10^8\ M^{-1}\ min^{-1}$ for U-loop. It is unlikely that the difference is very significant since the higher protein concentrations required to form a complex with U-loop results in a very rapid association rate that is difficult to determine accurately. The similar association rate for the two

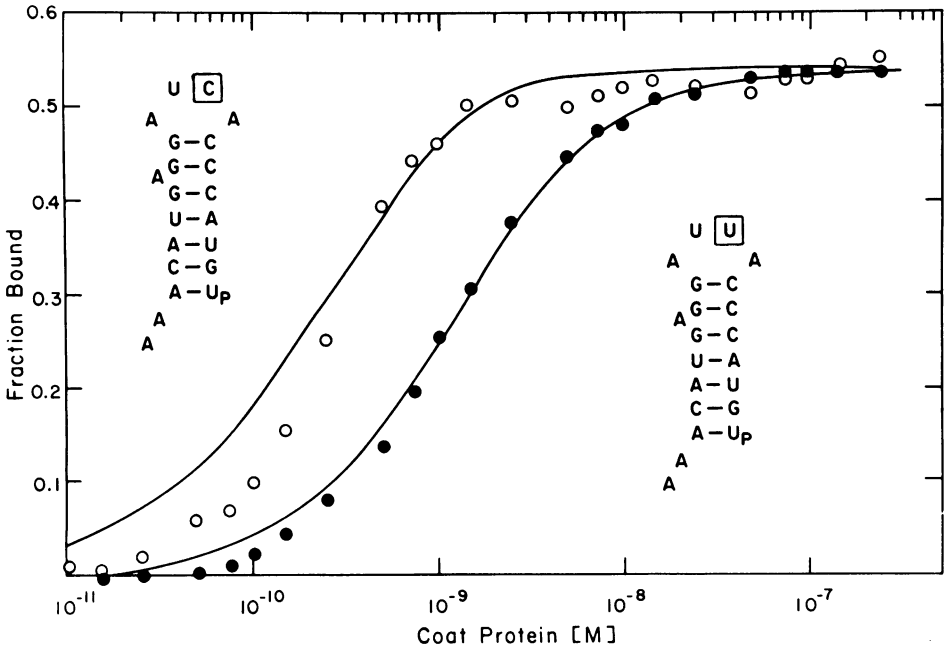

Fig. 1. Coat protein excess binding curves for C-loop (left structure, open circles) and U-loop (right structure, closed circles) at 0°C. Samples were incubated 2 h prior to filtration. Solid lines are theoretical pseudo first order binding curves calculated for K_a = 5 x 10^9 M^{-1} and 8 x 10^8 M^{-1}.

loops suggests that the difference in K_a must lie in the dissociation rate.

The dissociation rate of the complex between the C-loop and coat protein was determined by two different methods. In the first, a complex between ^{32}P-labeled C-loop and an excess of coat protein was formed and dissociation was initiated by diluting the reaction. In the second, a stoichiometric complex between ^{32}P-labeled C-loop and coat protein was formed and dissociation of the labeled loop was measured by adding an excess of nonradioactive C-loop. In both cases, samples were withdrawn and filtered at intervals until a new equilibrium was reached. As shown in Figure 2, both methods gave very similar results. The C-loop complex dissociates very slowly with a half life of 408 min. The half life of the U-loop complex was redetermined using

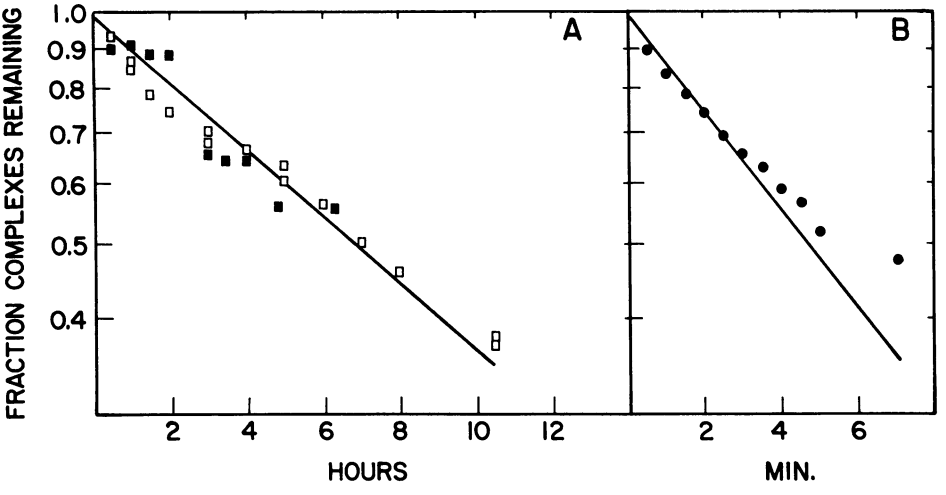

Fig. 2. A. Dissociation kinetics of C-loop coat protein complexes. Open squares: Complex between 10 pM C-loop and 0.8 nM coat protein was diluted 25-fold to start dissociation. Solid squares: Complex between 1 nM ^{32}P labeled C-loop and 1 nM coat protein was mixed with 4 nM nonradioactive C-loop at zero times. The line corresponds to k_{off} = 0.0017 min^{-1}.
B. Dissociation Kinetics of U-loop coat protein complex. 10 pM U-loop and 8 nM coat protein was diluted 25-fold to start dissociation. The line corresponds to k_{off} = 0.15 min^{-1} although a second, slower component may be present.

the dilution method and determined to be 4.8 min., or about 90 times faster than C-loop.

In Table 1, the K_a values deduced from the kinetic data are compared to the K_a values from Fig. 1. In the case of U-loop, the K_as determined by the two different methods are in excellent agreement. For C-loop the K_a determined from the ratio of the rate constants is 3.5 x 10^{10} or about 50-fold tighter than U loop. An equilibrium binding curve with this K_a would result in an even poorer fit of the data in Fig. 1. We believe that the anomolously low K_a values observed for the protein excess equilibrium method is a consequence of using very dilute protein concentrations. This is either a consequence of protein adhering to the reaction tube or of the protein dimer dissociating into monomers at low concentrations (10).

Experiments measuring the binding of C-loop to coat protein

TABLE 1
Comparison of kinetic and equilibrium binding data

	U-loop	C-loop
k_{on}	1×10^8 M^{-1} min^{-1}	0.6×10^8 M^{-1} min^{-1}
k_{off}	0.15 min^{-1}	0.0017 min^{-1}
k_{on}/k_{off}	6.7×10^8 M^{-1}	3.5×10^{10} M^{-1}
K_a	8×10^8 M^{-1}	—

at different temperatures are summarized in Figure 3. In most cases the equilibrium data give an unnaturally steep binding curve as described above. If the midpoint of these curves is considered to be the K_a, the van't Hoff plot is curved. In contrast, when K_a is determined by a ratio of forward and reverse rate constants, a linear van't Hoff plot is obtained with ΔH = -22 kcal/mole.

The single nucleotide change has a remarkably large effect on the thermodynamic properties of the protein-RNA interaction. Values for ΔH, ΔG and ΔS at 24°C for the two loops are compared in Table 2. The nearly thirty percent increase in the free energy of binding of C-loop over U-loop is primarily a result of a more favorable enthalpy of the C-loop interaction. If the different contacts between the protein and RNA are assumed to act independently, it is possible to estimate the contribution of U_{-5} or C_{-5} to the total free energy of binding. This is done by also assuming that when an A is present at position -5, no favorable or unfavorable contributions to the -5 position is made. Since the ΔG of the A_{-5} variant is -8.5 kcal/mole (2), the U_{-5} contributes -1.3 kcal/mole while the C_{-5} contributes -4.1 kcal/mole. Thus as much as a third of the total free energy of binding derives from the C_{-5} contact.

The interaction of C-loop with coat protein was also examined in a variety of solvent conditions. The slope of the log K_a *versus* log ionic strength for C-loop is very similar to U-loop. When the data is analyzed using the ion displacement model (11), about 5 nucleotide phosphates are involved in ion pairs with the coat protein for both loops. The pH dependence of K_a for C-loop also closely resembles U-loop (8), with a broad optimum centering about pH 8.5. Thus, it is unlikely that C-loop

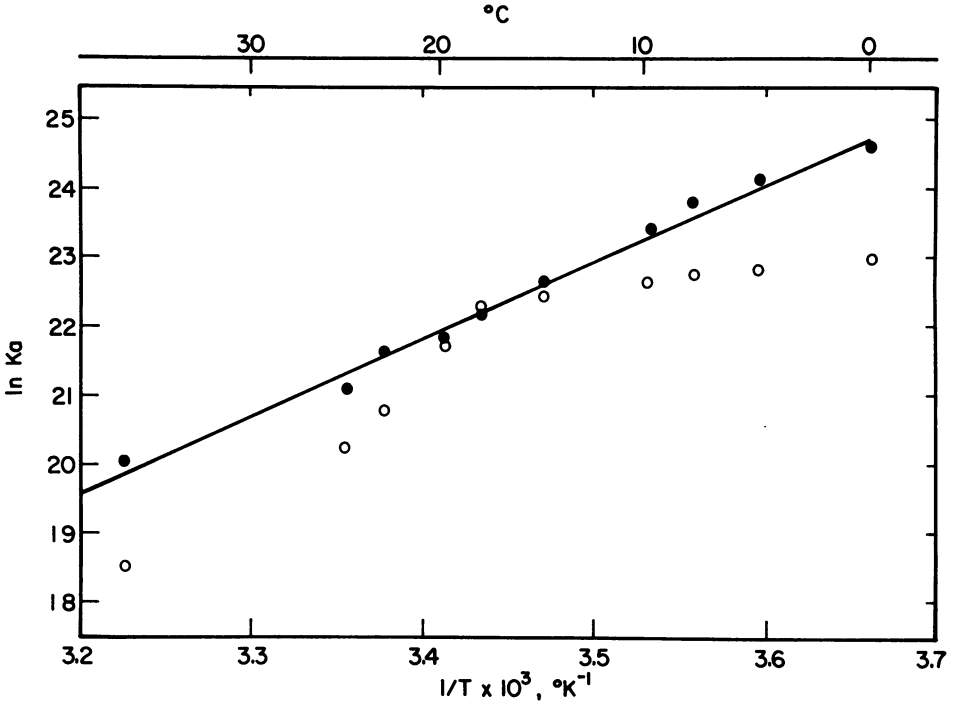

Fig. 3. Temperature dependence of K_a of the C-loop coat protein complex. K_a at each temperature was determined by either a protein excess binding curve (open circles) or by the ratio of the forward and reverse rate constants (closed circles). The line corresponds to a ΔH = -22 kcal/mol.

interacts with coat protein in a substantially different way. The much tighter binding is more likely to be the result of a local change at the site of the substitution.

Gel Retardation Assay

The RNA-protein complex can be detected by polyacrylamide

TABLE 2
Thermodynamic properties of Coat Protein binding to both loops

	C loop	U loop*
ΔH	-22 k/cal/mol	-19 kcal/mol
ΔG (24°C)	-12.6 kcal/mol	- 9.8 kcal/mol
ΔS	-31.5 cal/mol deg	-30 cal/mol deg

*Data taken from reference 8.

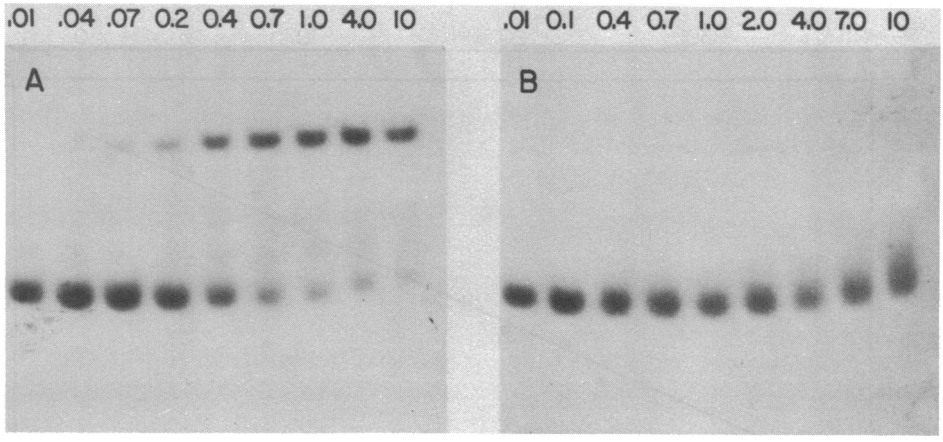

Fig. 4. Analysis of complexes between coat protein and C-loop (panel A) or U-loop (panel B) by polyacrylamide gel electrophoresis. 10 pM ^{32}P labeled loop was incubated for 3 h at 0°C with coat protein at concentrations (in nM) indicated at the top of each lane. The faster moving band on the autoradiogram corresponds to free loop and the slower one to complex.

gel electrophoresis. Reactions containing 10 pM ^{32}P labeled C-loop or U-loop and varying concentrations of coat protein were applied to the gel after incubation. The autoradiogram of the gel with C-loop shows a slower moving band which appears at protein concentrations similar to those where a complex was detected in the filter binding experiment (Figure 4A). Complexes formed between 49 nM coat protein and C-loop concentrations varying from 10 to 100 nM also migrate at the same position in the gel (data not shown). Thus a unique complex between C-loop and coat protein forms at all protein to RNA ratios tested. If sodium dodecyl sulfate is added to the reaction either before or after incubation, no complex is observed on the gel, suggesting that no permanent covalent bond occurs between the protein and the RNA.

It is interesting to note that at high protein concentrations nearly all the C-loop migrated as a complex on the gel despite the fact that only about half of the radioactive C-loop was retained on the millipore filter (Figure 1). This supports the contention (7) that the millipore filter retains only a

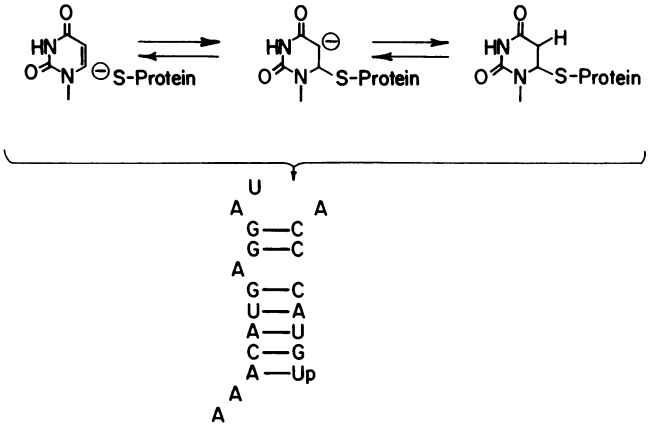

Fig. 5. Proposed mechanism of transient covalent intermediate between coat protein and U-loop.

fraction of the complex and the assumption of a filter retention efficiency is justified.

In contrast to C-loop, the complex between U-loop and coat protein cannot be observed on the gel at any protein concentration (Figure 4B). Presumably the less stable U-loop complex dissociates during the course of the electrophoresis while the C-loop complex remains stable. Indeed, at the highest protein concentration some broadening of the U-loop band can be observed suggesting gradual dissociation of the complex during the separation.

A Possible Transient Covalent Complex

The formation of a transient covalent complex between the protein and the pyrimidine ring at position -5 offers a possible explanation for the tight binding of C loop. Based on nucleotide inactivation data (3), it has been hypothesized that a cysteine anion on the coat protein attacks the 6 carbon of the pyrimidine ring to give a delocalized anion intermediate (Figure 5). Protonation of the anion could follow to give a dihydrouridine adduct. The first step of this mechanism has been established for thymidylate synthetase (4) and the second step is believed to be responsible for the catalysis of ^{3}H from H_2O into position U_8 in tRNA by tRNA synthetase (5). The extent to which the equilibrium is shifted towards the covalent complex would determine the

contribution of the contact to the total K_a of the RNA. A greater proportion of covalent complex occurring with C-loop than U-loop is consistent with the fact that cytidine is more susceptible than uridine to nucleophilic attack.

In order to provide evidence for the protonation step, the dissociation rates of complexes formed in different percentages of D_2O were determined. If a substantial proportion of protonated nucleotide forms, one would expect that the complex formed in D_2O would be more stable since the increased strength of the C-D bond over the C-H bond would make deprotonation more difficult. It was found that the k_{off} for both C-loop and U-loop is not effected when the complex was formed in zero, 50 or 90 percent D_2O. The absence of an isotope effect indicates that either protonation does not occur or the second equilibrium in Figure 5 does not contribute substantially to K_a.

It has been suggested that the inactivation of R17 coat protein by high concentrations of CMP may be related to the tight binding of C-loop (3). While the mechanism of CMP inactivation is unclear, the inactivated protein can be reactivated by dithiothreitol or β-mercaptoethanol. We therefore investigated the equilibrium binding of C-loop coat protein and the dissociation kinetics of the complex in differing concentrations of these two sulfhydryl reagents. As had previously been found with U-loop (8) these compounds had no effect on K_a or k_{off} at any concentration tested. Thus the tight binding of C-loop differs from CMP inactivation of coat protein in at least one significant fashion.

In conclusion, our inital report of an RNA mutation which increases the affinity of an RNA-protein interaction has been substantiated by further experiments. This is the first example reported of a tight binding mutant in an RNA-protein interaction although such a mutant has been described for a DNA-protein interaction (12-13). The K_a for C loop to R17 coat protein can be extraordinarily high. At low ionic strengths and temperatures, K_a values greater than 10^{11} M^{-1} have been estimated from values of the $t_{\frac{1}{2}}$ of dissociation of greater than 24 hrs. These values are comparable to antigen-antibody interactions and much higher than previously determined simple RNA-protein complexes. The origin of this tight binding remains unexplained. Although

the formation of a transient covalent complex of the type shown in Figure 5 remains an attractive possibility, we have not succeeded in obtaining additional support for it. It will be interesting to see whether U to C substitutions in the tRNA-synthetase interaction will also increase the stability of the complex.

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