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Cooperative Binding of R17 Coat Protein to RNA†

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ABSTRACT: The binding of the R17 coat protein to synthetic RNAs containing one or two coat protein binding sites was characterized by using nitrocellulose filter and gel-retention assays. RNAs with two available sites bound coat protein in a cooperative manner, resulting in a higher affinity and reduced sensitivity to pH, ionic strength, and temperature when compared with RNAs containing only a single site. The cooperativity can contribute up to −5 kcal/mol to the overall binding affinity with the greatest cooperativity found at low pH, high ionic strength, and high temperatures. Similar solution properties for the encapsidation of the related fr and f2 phage suggest that the cooperativity is due to favorable interactions between the two coat proteins bound to the RNA. This system therefore resembles an intermediate state of phage assembly. No cooperative binding was observed for RNAs containing a single site and a 5′ or 3′ extension of nonspecific sequence, indicating that R17 coat protein has a very low nonspecific binding affinity. Unexpectedly weak binding was observed for several RNAs due to the presence of alternative conformational states of the RNA.

R17 coat protein binds tightly to a single hairpin in the 3600-nt R17 genomic RNA, resulting in repression of phage replicase synthesis. This system has been extensively studied as an example of a specific RNA–protein interaction (Romaniuk et al., 1987). The RNA–protein complex has also been shown to act as a precursor for assembly of the phage capsid, with the translational operator acting as a specific nucleation site (Hung et al., 1969; Ling et al., 1969). Qualitative studies have shown that capsid formation is very cooperative with no observed intermediates, presumably due to extensive protein–

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protein contacts in the shell (Hohn, 1969). Here we develop an artificial system to measure the amount of cooperative binding of R17 coat protein using a RNA containing two adjacent binding sites. The solution properties of the cooperativity are quantitated and discussed in relation to capsid assembly.

Materials and Methods

Materials. Oligodeoxynucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer. [γ-^{32}P]ATP (5000 Ci/mmol) and [α-^{32}P]CTP were purchased from Amersham. Calf intestine alkaline phosphatase was purchased from Boehringer Mannheim and T7 DNA polymerase (Sequenase) from United States Biochemical Corp. DNA restriction enzymes, mung bean nuclease, T4 DNA ligase, and T4 polynucleotide kinase were purchased from New England Biolabs. Manufacturer's protocols were followed for all reactions.

RNAs. Plasmids were constructed by cloning synthetic DNA fragments into plasmid vector pUC13 (Messing, 1983) to create transcription templates for RNA fragments **4**, **5**, **7**, **8**, and **9**, into pT71 (United States Biochemical Corp.) to create fragment **2** template, or into pMT7-2 (Lowary et al., 1985) to create fragment **14** template. *Escherichia coli* JM109 was used as a host in each case (Yanisch-Perron et al., 1985). Restriction sites were removed by cleavage, mung bean nuclease treatment, and religation with T4 DNA ligase. Transformants harboring plasmid DNA were screened by ampicillin resistance and restriction analysis of minilysate plasmid DNA (Maniatis et al., 1982). Plasmids were sequenced in the region of interest with T7 DNA polymerase using a 5′-^{32}P-labeled oligomer primer.

Oligoribonucleotides were prepared by in vitro transcription from synthetic DNA (**1**, **3**, **6**, **10**, **11**, **12**, and **13**) or from restricted CsCl-purified (Maniatis et al., 1982) plasmid DNA (**2**, **4**, **5**, **7**, **8**, **9**, and **14**) by procedures previously described (Milligan et al., 1987; Sampson & Uhlenbeck, 1988). Transcription reactions were terminated by adding EDTA to 50 mM, phenol extracted, $CHCl_3$/isoamyl alcohol (24:1) extracted, and ethanol precipitated. Transcripts were purified by electrophoresis on denaturing 10% polyacrylamide gels and eluted from the gel in 0.1 M Tris, pH 8.0, and 1 mM EDTA overnight at 4 °C, followed by ethanol precipitation.

RNAs were labeled either by the addition of [α-^{32}P]CTP (5 μCi) into the transcription reaction or by removing the 5′-triphosphate with calf intestine phosphatase, gel purification of the RNA, and labeling the T4 polynucleotide kinase and [γ-^{32}P]ATP. Labeled RNA was gel-purified and stored in 10 mM Tris/0.1 mM EDTA, pH 8.0 at −20 °C.

Coat Protein. Bacteriophage R17 was propagated on *E. coli* S26 and purified according to Carey et al. (1983). Purified phage was stored at 43 mg/mL in 150 mM NaCl/15 mM sodium citrate, pH 6.7, at 4 °C. R17 coat protein was purified according to Witherell and Uhlenbeck (1989), except that no dithiothreitol was added to the dialysis buffer. The coat protein was stored at a dimer concentration of 50 μM in 1 mM acetic acid, pH 3.2, at 4 °C. Stored protein maintained its activity for greater than 8 months. Protein concentrations were determined by using a molar extinction coefficient of 15 400 at 280 nm (Weber & Konigsburg, 1975).

Filter Binding Assays. Protein excess filter binding assays were performed according to Witherell and Uhlenbeck (1989) under various buffer and temperature conditions described in the text. Typical reactions contained either MMK buffer [0.1 M MOPS, pH 7.5, 0.1 M KCl, 10 mM $MgCl_2$, and 80 μg/mL BSA] or TMK buffer [0.1 M Tris, pH 8.5, 0.08 M KCl, 10 mM $MgCl_2$, and 80 μg/mL BSA] and were incubated at 25 °C for 10–30 min before filtration.

RNA excess assays were performed as described in Witherell and Uhlenbeck (1989) except that reactions were incubated in 100 μL of TMK buffer at 4 °C, 90 μL was filtered, and the filters were washed with 500 μL of TMK buffer. Assays contained 50 nM coat protein dimer and 10–70 nM ^{32}P-labeled RNA. The amount of RNA bound was corrected for the retention efficiency, which varied from 40% to 80%, and the activity of the coat protein, which varied from 50% to 80%.

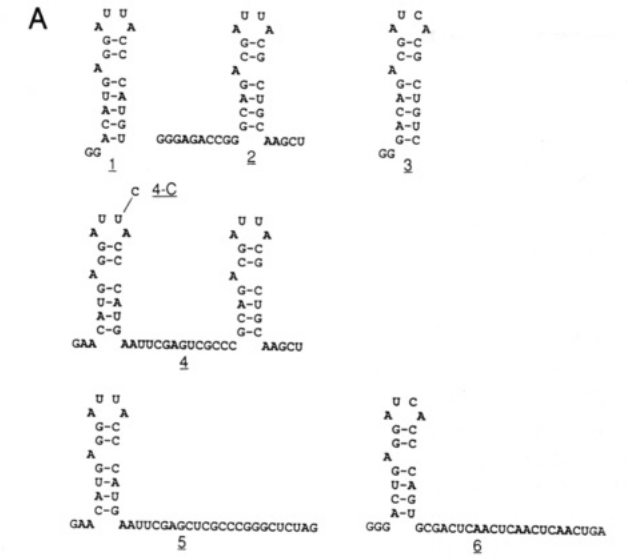
Gel-Retention Assays. ^{32}P-Labeled RNA (~10 pM) was incubated with coat protein dimer concentrations from 15 to 150 nM in 10 μL of MMK buffer or TMK buffer at 4 °C. All RNAs were heated to 90 °C and quick-cooled on ice before use. After 10 min, 2 μL of loading buffer [30% glycerol, 0.25% Bromophenol Blue, and 0.25% Xylene Cyanol] was added, and the reactions were loaded on a 10% native polyacrylamide gel (24 × 15 cm) containing 50 mM MOPS, pH 7.5, and 1 mM $MgCl_2$. Gels were run overnight at 4 V/cm, 4 °C, and the bands were visualized by autoradiography.

Results

Cooperative Binding to RNA. Seven RNA fragments were prepared to investigate whether R17 coat protein could bind cooperatively to RNA (Figure 1A). All seven RNAs contain the necessary elements of sequence and secondary structure defined by the consensus R17 coat protein binding site (Romaniuk et al., 1987). **1**, **2**, and **3** are hairpins which differ in base-paired residues and 3′- and 5′-terminal sequences which do not affect binding. **4** contains two hairpins having the core sequences of **1** and **2** that are connected by a 15-nucleotide spacer. **5** and **6** approximate the length of **4** but only contain a single coat protein binding site near the 5′ terminus. The interaction of four of these RNAs with R17 coat protein was examined with a nitrocellulose filter binding assay. As shown in Figure 1B, **1**, **2**, and **5** bind coat protein in a manner consistent with a simple bimolecular equilibrium and a $K_a = 80$ μM^{-1}. In contrast, **4** binds at much lower coat protein concentrations and shows a steeper binding curve, suggesting cooperative binding. It is clear that the second coat protein binding site, rather than the extra nucleotides, is responsible for the tighter binding of **4**, since **5** binds with the same affinity as **1** and **2**, despite the presence of the extra 3′-nucleotides.

The stoichiometry of protein binding to **4** was determined by titrating a solution of 100 nM protein monomer with increasing RNA concentrations and determining the amount of complex bound to filters. Saturation occurs at 20 nM **4**, corresponding to five coat protein monomers per RNA molecule, which is reasonably consistent with the expected value of four, or one coat protein dimer per hairpin. A control experiment using fragment **1** gave the expected stoichiometry of two coat protein monomers bound per RNA.

A polyacrylamide gel-retention assay was also used as an alternative method for establishing the stoichiometry of the complex between coat protein and **4**. Since it has previously been shown that coat protein complexed to the wild-type binding site does not form a stable complex on polyacrylamide gels, it was necessary to use tight binding variants of the RNA that contained a cytidine in the loop (**3**, **4-C**, and **6**) (Lowary & Uhlenbeck, 1987). As shown in Figure 2, when **3** and **6** are mixed with saturating coat protein, a band corresponding to the RNA–protein complex is observed. In contrast, **4-C** migrates at a similar position to **3** and **6** only at low coat protein concentrations, migrating more slowly at higher concentrations. Presumably at the lower coat protein concen-



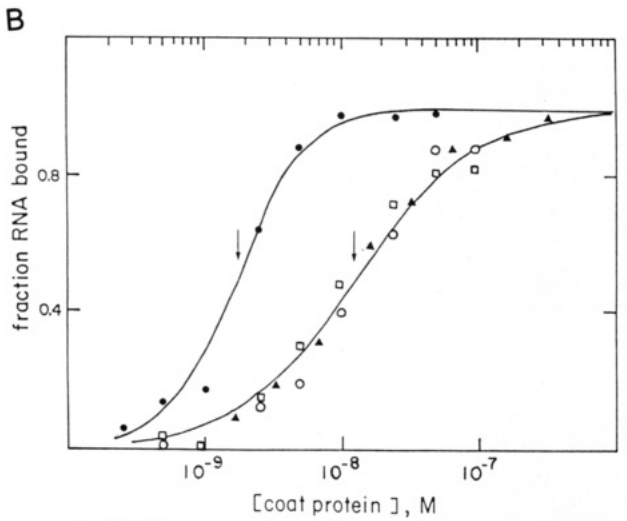


FIGURE 1: (A) Sequence and possible secondary structures of RNA fragments **1**, **2**, **3**, **4**, **4-C**, **5**, and **6**. (B) Coat protein excess binding curves for ^{32}P-labeled fragments **1** (○), **2** (▲), **4** (●), and **5** (□), assayed in TMK buffer at 25 °C. Binding curves for fragments **1**, **2**, and **5** fit a theoretical bimolecular binding curve calculated by using K_a = 80 μM^{-1}. The binding curve for **4** fits a theoretical cooperative binding curve using K_a = 80 μM^{-1} and K_c = 59. Arrows indicate the half-saturation point ($[P]_{0.5}$) for RNA binding.

trations one coat protein dimer binds to **4-C** and as the concentration is increased, the second site is filled. Interestingly, **4** also forms the slower migrating complex at high coat protein concentrations, indicating that both sites are filled (Figure 2). The increased affinity of **4** presumably results in a complex stable enough to survive the gel electrophoresis.

Since the two binding sites on **4** have the same intrinsic affinity to coat protein, the binding data in Figure 1B can be fit to a model involving cooperative binding to two identical sites:

$$Y = (K_a[P] + K_a^2[P]^2K_c)/(1 + 2K_a[P] + K_a^2[P]^2K_c)$$

where Y is the fraction of RNA bound, K_a is the equilibrium association constant for binding to either single hairpin, [P] is the concentration of the coat protein, and K_c is a cooperativity factor (Ackers et al., 1983). As shown in Figure 1B, this equation accurately fits the data with K_c = 59, corresponding to a cooperative free energy of −2.3 kcal/mol. The cooperative binding energy therefore makes a substantial contribution to the overall binding affinity of **4** to coat protein since under these conditions the free energy for coat protein binding to a single hairpin is −10.8 kcal/mol.

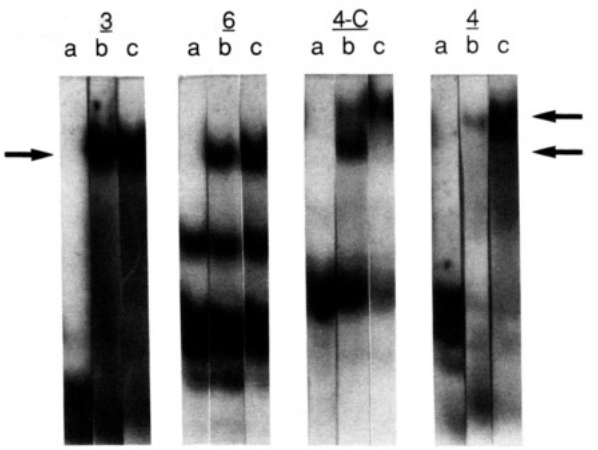


FIGURE 2: Gel retention assay of fragments **3**, **6**, **4-C**, and **4**. Binding assays were performed in MMK buffer at 4 °C and contained ^{32}P-labeled RNA (~10 pM) and no protein (lane a), 15 nM coat protein (lane b), or 150 nM coat protein (lane c). The migration positions of complexes containing a single protein dimer (lower arrow) or two dimers (upper arrow) are indicated. Free RNA migrates more rapidly at a position that depends on size and conformation. Two conformational isomers of **6** are seen.

Solution Properties of the Cooperative Interaction. The effects of ionic strength, temperature, and pH on the interaction between the R17 coat protein and an isolated binding site are well established (Carey & Uhlenbeck, 1983). Since cooperative binding presumably involves protein–protein interactions, the effect of these extrinsic variables on cooperativity is expected to be different. The affinity of coat protein binding to the double hairpin **4** was compared to the single hairpins **1** and **2** under a variety of conditions. The data for each variable are presented in Figure 3 as both the coat protein concentration required for half-saturation, $[P]_{0.5}$ (lower panels), and the cooperative free energy, in K_c (upper panels). The former allows a general comparison of the affinities of the double site with the single sites while the latter provides a direct measure of the contribution of the cooperativity to the interaction.

As has been previously reported, R17 coat protein binding to a single site such as **1** or **2** showed greatly reduced binding at high ionic strengths. In contrast, the molecule with two sites (**4**) showed relatively little salt dependence on binding, indicating much greater cooperativity at higher ionic strengths and suggesting that ionic interactions actually destabilize the cooperative binding interactions. The affinity of the coat protein for single hairpins was drastically reduced at high magnesium concentrations while the double hairpin affinity was much less sensitive (data not shown). Identical results were seen with calcium, suggesting that divalent cations, even at low concentrations, increase the cooperative binding of the RNA containing a double hairpin.

In contrast to single hairpins which bind protein less well as the temperature is increased, the affinity of coat protein binding to the double hairpin showed very little temperature dependence. The double hairpin therefore bound with the highest cooperativity at the highest temperature, opposite to the temperature dependence of protein–RNA binding. From the slope of a van't Hoff plot of the data (Figure 3), an unfavorable cooperative ΔH = +23.0 kcal/mol was obtained. The cooperative ΔS was calculated to be +85.7 cal mol^{-1} K^{-1}. Cooperative binding is therefore driven by a favorable entropy change.

R17 coat protein binding to a single site showed a broad pH dependence of K_a with an optimum around pH 8. The double hairpin showed relatively little pH dependence on

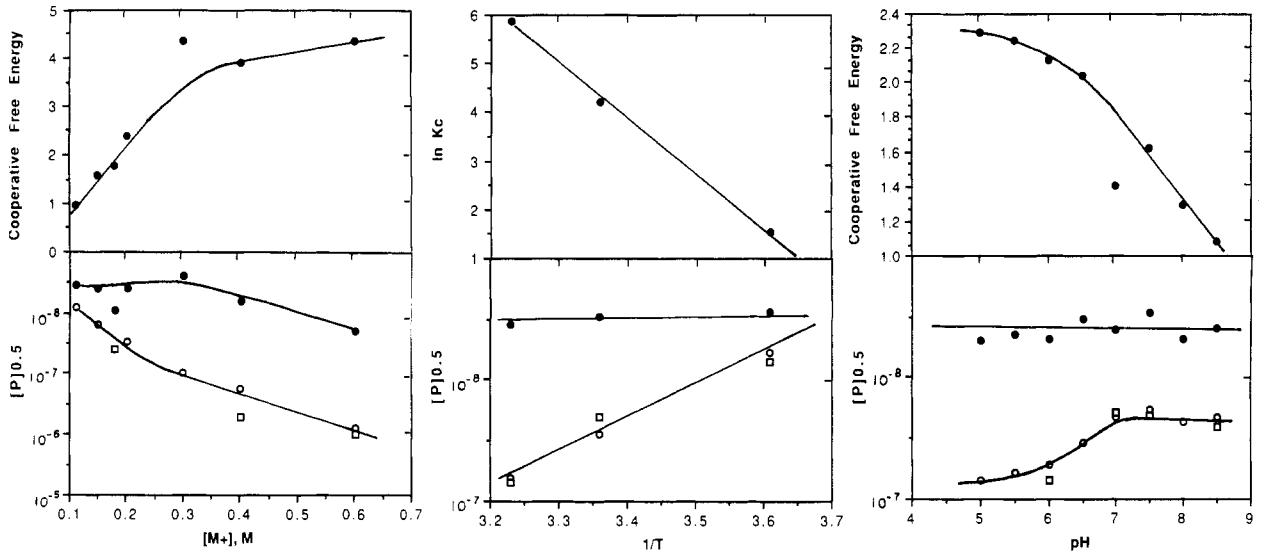


FIGURE 3: Ionic strength dependence, temperature dependence, and pH dependence of $[P]_{0.5}$ for **4** (●), **1** (○), or **2** (□) (lower panels) and cooperative free energy in kilocalories per mole or ln K_c (upper panels). Standard filtration assays were performed in MMK buffer: ionic strength dependence determined by adjusting the KCl concentration to the total cation concentration $[M^+]$ indicated, temperature dependence determined by adjusting the temperature as indicated, and pH dependence determined by using either NaOAc (pH 5.0), MES (pH 5.5–6.5), MOPS (pH 7.0–7.5), or Tris (pH 8.0–8.5) adjusted to the indicated pH.

FIGURE 4: Sequence and possible secondary structures of RNA fragments **7R**, **7B**, **7X**, and **7XΔ**. Δ indicates the four nucleotides removed from **7X** to create **7XΔ**. K_a's for coat protein binding in TMK at 4 °C are indicated below each fragment.

binding, indicating much greater cooperativity at lower pH. Presumably one or more groups on the protein must be protonated for efficient cooperative binding.

Alternative Conformations. A variety of RNAs containing one translational operator and either 5′ or 3′ extensions were synthesized as controls for the cooperative assay. A survey of the coat protein binding properties of these RNAs revealed that the nonspecific sequences either had no effect or actually reduced the affinity of binding to the RNA. In one experiment, summarized in Figure 4, a series of RNAs were prepared containing a 5′-terminal binding site and different lengths of 3′ extensions. These molecules were all prepared by run-off transciption of the same plasmid DNA template, cleaved with different restriction enzymes. Whereas the 24-nt **7R** and the 42-nt **7B** RNAs showed tight binding to coat protein, the 48-nt **7X** RNA bound very poorly. **7X** RNA could not be activated for protein binding by heating to 95 °C and either quick-cooling on ice or slow-cooling to 25 °C in the presence or absence of magnesium. Incubation of **7X** with protein for up to 50 min also did not restore binding. An alternative secondary structure for the **7X** RNA (Figure 3) provides a reasonable explanation of how the six extra 3′-nucleotides could abolish binding. In order to test this hypothesis, four nucleotides, believed to be involved in stabilizing the alternative structure, were deleted. The resulting RNA (**7XΔ**) bound coat protein normally. Thus, the presence of a "poison" sequence elsewhere in the RNA molecule was shown to disrupt the secondary structure of the coat protein binding site.

A second example where alternative RNA structures affect coat protein binding was observed in fragments **8-3**, **8-12**, and **8-16** where two identical coat protein binding sites are separated by varying numbers of nucleotides (Figure 5A). In this case, the shorter and longer RNAs (**8-3** and **8-16**) show tight, cooperative binding identical with **4**, while the intermediate **8-12** binds in a manner consistent with coat protein binding to a single site (Figure 5B). Only one coat protein dimer was found to bind per molecule of **8-12** in RNA excess assays, consistent with this interpretation (data not shown). **8-12** did not bind cooperatively when denatured under a variety of conditions or after prolonged incubation with coat protein. Presumably **8-12** forms an alternate conformation that blocks one of the two binding sites. The four additional nucleotides in **8-16** reverse this effect. While potential alternate secondary structures may be drawn to explain these data, they have not been confirmed.

In the two examples discussed thus far, the alternative conformation was sufficiently stable to prevent any coat protein binding. However, if the inactive conformation can equilibrate with the active one during the time scale of the binding experiment, the resulting binding curve will be similar to fully active RNA but have a lower apparent K_a. This situation was observed for several RNAs containing a single binding site and either 5′ or 3′ extensions (Figure 6). Only one of six molecules tested (**10**) showed a K_a value similar to a control RNA (**9R**) consisting of an isolated binding site. The remaining five molecules (**9B**, **11**, **12**, **13**, and **14**) showed normal binding curves with K_a values which were from 2-fold to 34-fold less than **9R**. The most likely explanation for these differences in K_a is that each one of these molecules can form an inactive conformation in rapid exchange with the active one. However, the possibility that the single-stranded 3′ or 5′ tail can interfere with protein binding more directly cannot be ruled out.

Discussion

In the process of investigating the cooperative binding of R17 coat protein to RNA, a subtantial number of RNAs containing one or two coat protein binding sites were prepared.

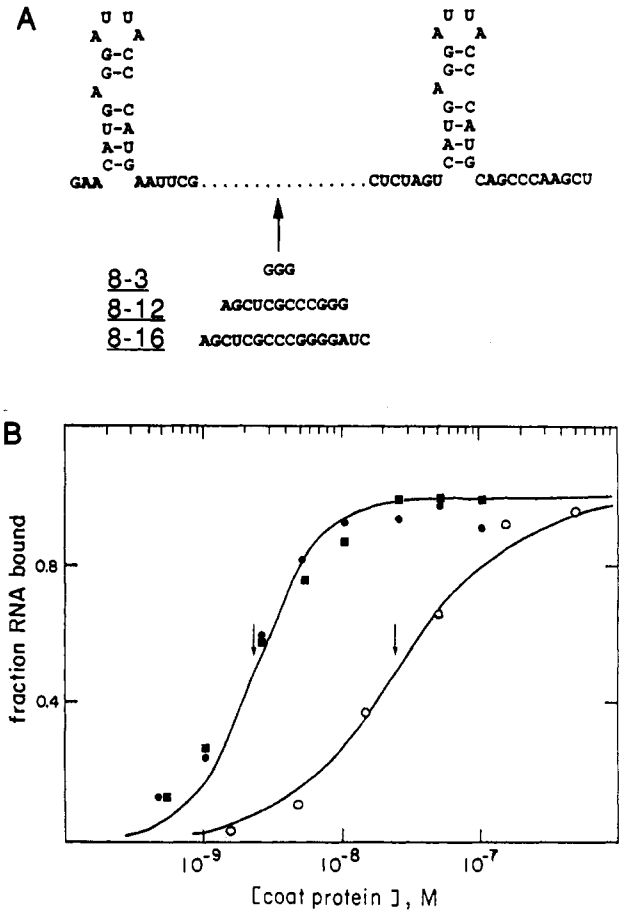


FIGURE 5: (A) Sequence and possible secondary structures of RNA fragments **8-3**, **8-12**, and **8-16** showing differences in sequence and length of linker RNA between binding sites. (B) Coat protein excess binding curves for ^{32}P-labeled fragments **8-3** (●), **8-12** (○), and **8-16** (■), assayed at 25 °C in 0.1 M MES, pH 5.5, 0.1 M KCl, and 10 mM $MgCl_2$. Binding curves for fragment **8-12** fits a theoretical bimolecular binding curve calculated by using K_a = 40 μM^{-1}. The binding curves for **8-3** and **8-16** fit a theoretical cooperative binding curve using K_a = 28 μM^{-1} and K_c = 242.

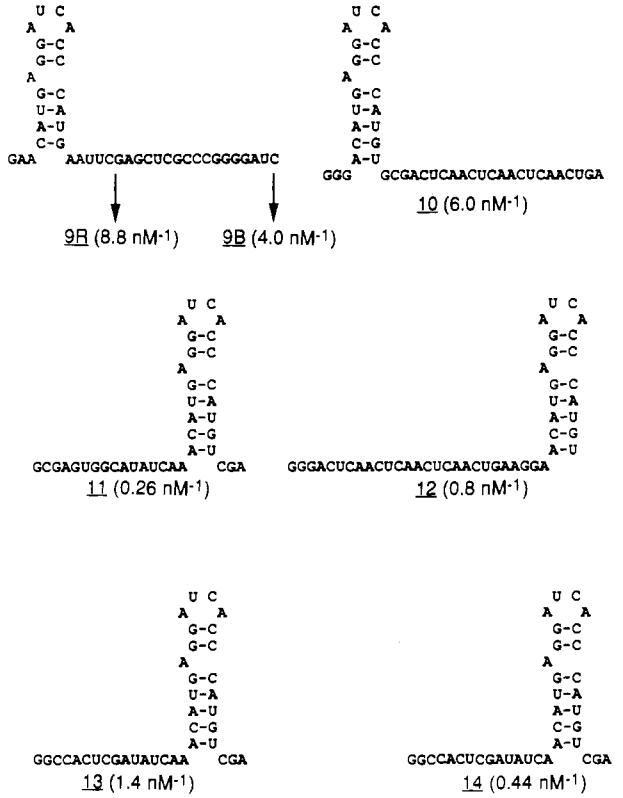


FIGURE 6: Sequence and possible secondary structures of RNA fragments **9R**, **9B**, **10**, **11**, **12**, **13**, and **14**. K_a's for coat protein binding in TMK at 4 °C are indicated below each fragment.

Somewhat surprisingly, many of these RNAs did not show the expected protein binding properties. Sites either were unavailable for coat protein binding or showed unexpectedly low K_a values. It appears that the explanation for these observations is that the RNAs fold into structures which differ from those designed. In the two cases where coat protein did not bind at all to a site, it is clear that an alternative structure was thermodynamically preferred over the designed structure. A variety of different renaturation procedures did not result in activation of the coat protein binding potential. The kinetic barrier between the inactive structure(s) and the active one must also be substantial since coat protein was not able to alter the equilibrium after prolonged incubation. In the cases where the coat protein binding affinity was reduced, the exchange between active and inactive conformations must be more rapid since most of the RNA was active in protein binding.

The propensity for RNA to adopt alternative conformations is well documented (Hawkins et al., 1977; Kao & Crothers, 1980; Altman & Guerrier-Takada, 1986; Fedor & Uhlenbeck, 1990). Nevertheless, it is striking that several relatively short RNA molecules capable of forming one or two well-defined stable hairpins did not fold as anticipated. Analysis of the sequences did not always permit identification of convincingly more stable alternative secondary structures, emphasizing our limited capacity to predict and design RNA structures. Finally, it is worth noting that an alternate RNA structure is believed to affect the accessibility of R17 coat protein binding in vivo as well. A sequence element in the coat protein gene pairs with the replicase initiation region (Min Jou et al., 1972; Berkhout & Van Duin, 1985) and is believed to be responsible for the timed expression of R17 replicase (Engelhardt et al., 1967).

For RNA molecules with two available sites, clear evidence for cooperative binding of two coat protein dimers to the RNA was obtained. Although data could be fit by using a single cooperative binding energy, the number may reflect an average of values for coat protein dimers interacting along different interfaces. In the viral capsid, three distinctly different protein–protein contacts are formed (Casper & Klug, 1962). The amount of cooperative binding free energy is substantial. Values as high as −5 kcal/mol have been observed, although under physiological conditions −4 kcal/mol is a more likely value. This compares with the cooperative binding free energies of −3.6 kcal/mol for λ repressor (Senear et al., 1986), −3.6 kcal/mol for *E. coli* single-stranded binding protein (Bujalowski & Lohman, 1987), and −4.7 kcal/mol for T4 gene 32 protein (von Hippel et al., 1982).

The presence of a second coat protein binding site substantially alters the observed properties of the RNA–protein interaction. While R17 coat protein binds single hairpins tightly under a limited set of conditions, it binds double hairpins tightly under nearly any condition tested. This is due to the fact that the properties of the cooperative protein–protein interaction are very different from the RNA–protein interaction and happen to compensate for the relatively large effects of temperature and ionic strength on the RNA–protein interaction. This situation is also likely to occur in more complex systems where several different proteins interact with an RNA molecule to form a ribonucleoprotein particle. An individual protein may interact with the RNA very differently in the presence of the other proteins than it does when it binds by itself.

In an attempt to estimate the binding affinity of R17 coat protein to nonspecific RNA, several RNA molecules were prepared containing a single binding site with a 5′ or 3′ extension long enough to bind a second coat protein dimer. If we assume that the same amount of cooperativity is obtained between the specific and nonspecific sites:

$$Y_1 = (K_1[P] + K_1K_2K_c[P]^2)/[1 + (K_1 + K_2)[P] + K_1K_2K_c[P]^2]$$

where K_1 and K_2 are the equilibrium association constants for specific and nonspecific binding sites, respectively (Ackers et al., 1983). The protein excess filter assay allows measurement of binding to the high-affinity site (Y_1). However, if binding to the nonspecific site is strong enough, it will enhance binding to the specific site, allowing K_2 to be determined. No such enhancement was observed among the molecules tested. RNA excess filter binding experiments and gel-retention experiments did not detect coat protein binding to the nonspecific site. Since we could detect a 2-fold increase in affinity by the protein excess assay, an upper limit of $K_2 = 60\ \mu M^{-1}$ can be calculated under optimal R17 binding conditions using the above equation. Other estimates of K_2 suggest a much greater value. Attempts to measure the binding of the Qβ operator (Witherell & Uhlenbeck, 1989) to R17 coat protein indicate $K_2 < 0.2\ \mu M^{-1}$ (data not shown), and competition of the R17 operator with various nonspecific RNAs indicate K_2 to be less than $0.6\ mM^{-1}$ (Carey et al., 1983). A more accurate determination of K_2 may come from experiments that detect occupancy at the second site directly.

When long RNAs are incubated with high concentrations of coat protein under a variety of conditions, virallike capsids containing RNA and 90 coat protein dimers are formed (Matthews & Cole, 1972). As would be expected, this encapsidation reaction is highly cooperative, occurring within a very narrow range of coat protein concentrations (Beckett et al., 1988). Encapsidation is clearly dominated by cooperative interactions since in long RNAs virtually no preference for the presence of a tight binding site is observed and the high-affinity site only improves encapsidation slightly in shorter RNAs (Beckett et al., 1988). Like the cooperative interactions studied in this work, capsid formation in the related f2 (Matthews & Cole, 1972) and fr (Hohn, 1969) phage occurs most efficiently at high ionic strength, high temperatures, and low pH, suggesting that the cooperative interactions and encapsidation reactions are closely related.

It is reasonable to consider the encapsidation reaction as an extension of the 2-site cooperative model by assuming that capsid formation involves a single high-affinity site with affinity K_1, 89 equivalent nonspecific sites with affinity K_2, and 90 equivalent cooperative interactions, each contributing K_c. We can derive an equation for the encapsidation reaction such that

$$\nu = (K_1K_2^{89}K_c^{90}[P]^{90}[R])/([P] + K_1K_2^{89}K_c^{90}[P]^{90}[R])$$

where ν is the percent of protein in capsid, [P] is the free coat protein concentration, and [R] is the free genomic RNA concentration. Beckett et al. (1988) have determined the fraction of capsid formed as a function of coat protein concentration under buffer conditions where $K_1 = 600\ \mu M^{-1}$. The best fit of their data is when $K_2K_c = 4\ \mu M^{-1}$. While it is quite possible that the affinity to nonspecific sites may be different on capsid formation than when measured with free protein, it is likely that K_2 will not be tighter than $0.2\ \mu M^{-1}$. If this is the case, K_c must be considerably greater than the value of 10 obtained with RNAs binding two dimers. The enhanced amount of cooperativity in capsid assembly is expected, since more protein–protein contacts occur in the capsid than in the complex with two dimers.

Registry No. **1**, 129811-36-5; **2**, 129811-37-6; **3**, 129811-38-7; **4**, 129811-39-8; **4-C**, 129811-41-2; **6**, 129811-40-1; **7B**, 129811-43-4; **7R**, 129811-42-3; **7X**, 129811-44-5; **7XΔ**, 129811-45-6; **8-3**, 129811-46-7; **8-12**, 129811-47-8; **8-16**, 129811-48-9; **9B**, 129811-50-3; **9R**, 129811-49-0; **10**, 129811-51-4; **11**, 129811-52-5; **12**, 129811-53-6; **13**, 129811-54-7; **14**, 129811-55-8; Mg, 7439-95-4; Ca, 7440-70-2.

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Structural Features of the Protoporphyrin–Apomyoglobin Complex: A Proton NMR Spectroscopy Study†

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ABSTRACT: The structural properties of the complex formed by apomyoglobin and protoporphyrin IX (des-iron myoglobin) were studied to probe the influence of iron-to-histidine coordination on the native myoglobin fold and the heme binding site geometry. Standard two-dimensional proton nuclear magnetic resonance spectroscopy methods were applied to identify porphyrin and protein signals. A pronounced spectral resemblance between carbonmonoxymyoglobin and des-iron myoglobin was noticed that could be exploited to assign a number of resonances by nuclear Overhauser spectroscopy. Protoporphyrin IX was determined to bind in the same orientation as the heme. Most residues in contact with the prosthetic group were found in the holomyoglobin conformation. Several tertiary structure features were also characterized near the protein termini. It was concluded that the protoporphyrin–apomyoglobin interactions are capable of organizing the binding site and the unfolded region of the apoprotein into the native holoprotein structure.

Myoglobin (Mb)[1] is a *b* heme protein naturally used for oxygen storage. Its single prosthetic group resides in a tight crevice lined with hydrophobic residues (Takano, 1977a,b). The interactions that fasten the heme to the protein matrix fall in three classes. There are van der Waals contacts between the hydrophobic side chains and the heme π system, coordination of the central iron atom to the proximal histidine, and electrostatic interactions between the heme propionate side chains and residues bordering the cavity. Sperm whale apomyoglobin studies have demonstrated that the heme group not only plays a functional role but also allows Mb to adopt and maintain the correct native fold: disruption of the heme–protein interactions results in a structure that is ca. 25% less helical (Harrison & Blout, 1965) and ca. 23 kJ less stable (Griko et al., 1988a,b) than the holoprotein.

The relative importance of the various heme–protein interactions in forcing the unwound elements into the native holoMb conformation has been a matter of interest for many years. In 1967, Breslow and co-workers investigated the structural role of the Fe–His bond by using an artificial Mb containing protoporphyrin IX (PrIX) as prosthetic group (Breslow et al., 1967). This protein differs in composition from native Mb by the absence of the iron and is therefore referred to as des-Fe Mb. It was then shown that coordination of the proximal His stabilizes the holoprotein but does not affect the extent of secondary structure measured by CD spectroscopy. Most tertiary interactions also appear to be formed in des-Fe Mb, since it behaves as the holoprotein with respect to pH titration and bromoacetate modification of histidine side chains (Breslow & Koehler, 1965). However, fluorescence depolarization (Albani & Alpert, 1986) and immunoassay experiments (Atassi, 1967) reflect subtle dynamic and structural differences between the two complexes.

The novel ability to engineer mutations in myoglobin and the interest in the structure–function relationship manifested by these altered proteins (Braunstein et al., 1988; Springer et al., 1989; Varadarajan et al., 1989a,b) prompted us to examine the interactions holding PrIX in the heme cavity and determine the position of binding-site residues in the absence of coordination to a central ion. We chose ^{1}H NMR spectroscopy as the most promising method for analyzing the structural details of this modified protein. For a complete description of the structural role of the prosthetic group, complementary information must be gathered on the apoprotein. A parallel study of apomyoglobin was undertaken, whose results are presented in the companion paper (Cocco & Lecomte, 1990).

Materials and Methods

Apomyoglobin. Apomyoglobin was prepared from horse skeletal myoglobin (Sigma Type I) or sperm whale myoglobin (Sigma) by using the methyl ethyl ketone method introduced by Teale (1955). Residual heme content was lower than 1%

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[1] Abbreviations: apoMb, apomyoglobin; CD, circular dichroism; des-Fe Mb, des-iron myoglobin; COSY, correlated spectroscopy; 2D, two-dimensional; DQF-COSY, double-quantum-filtered COSY; Eq, equine; holoMb, holomyoglobin; Mb, myoglobin; MbCN, metcyanomyoglobin; MbCO, carbonmonoxymyoglobin; met, ferric form of myoglobin; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, two-dimensional proton nuclear Overhauser spectroscopy; PrIX, protoporphyrin IX; SW, sperm whale; TOCSY, total correlation spectroscopy; 2Q, two-quantum.

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