Asymmetric Contributions to RNA Binding by the Thr^{45} Residues of the MS2 Coat Protein Dimer*

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A prominent feature of the interaction of MS2 coat protein with RNA is the quasi-symmetric insertion of a bulged adenine (A–10) and a loop adenine (A–4) into conserved pockets on each subunit of the coat protein dimer. Because of its presence in both of these adenine-binding pockets, Thr^{45} is thought to play an important role in interaction with RNA on both subunits of the dimer. To test the significance of Thr^{45}, we introduced all 19 amino acid substitutions. However, we were initially unable to determine the effects of the mutations on RNA binding because every substitution compromised the ability of coat protein to fold correctly. Genetic fusion of coat protein subunits reverted these protein structural defects, allowing us to show that the RNA binding activity of coat protein tolerates substitution of Thr^{45}, but only on one or the other subunit of the dimer. Single-chain heterodimer complementation experiments suggest that the primary site of Thr^{45} interaction with RNA is with A–4 in the translational operator. Either contact of Thr^{45} with A–10 makes little contribution to stability of the RNA-protein complex, or the effects of Thr^{45} substitution are offset by conformational adjustments that introduce new, favorable contacts at nearby sites.

The coat protein of bacteriophage MS2 is a symmetric dimer that serves both as the major structural protein of the virus particle and as a repressor of viral translational activity. Structural analyses of coat protein (1–3) and of its RNA target (4), together with detailed genetic and biochemical dissection of its RNA binding function (5–10), have made this one of the best understood examples of RNA binding by a protein. The structure of the binding site for coat protein is shown in Fig. 1A. In the RNA-protein complex, the bulged A at position –10 and the loop A at –4 project outward from the body of the RNA, with the planes of the two bases aligned roughly parallel to the helix axis. A–4 and A–10 form quasi-symmetric contacts with coat protein, inserting themselves into pockets comprised of Val^{29}, Thr^{45}, Ser^{47}, and Lys^{61} on different subunits of the dimer. Because operator RNA is asymmetric, so too is the RNA-protein complex and we may distinguish the two subunits of the dimer. Here we use the convention of Valegard (11, 12), calling the two subunits A and B. Inspection of the crystal structure of the RNA-protein complex suggests that Thr^{45} makes H-bonds with N6 and N7 of A–4, while on the other subunit Thr^{45} seems to H-bond with N1 and N6 of A–10. However, it is difficult to predict reliably the separate contributions of these individual interactions to binding energy on structural grounds alone.

We previously mapped genetically the MS2 RNA-binding site by isolating a series of repressor-defective mutants in which the ability to bind operator RNA was reduced (7). These mutants were isolated using a two-plasmid genetic system in which coat protein expressed from one plasmid represses translation of a replicase-β-galactosidase fusion expressed from a second plasmid. Repressor-defective mutants make blue colonies on plates containing the chromogenic β-galactosidase substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). To ensure that these mutational defects were confined to the RNA-binding site, each mutant was screened for the ability to assemble into a virus-like particle. This allowed us to discard mutants having gross perturbations of protein structure. In this manner, we identified 10 different amino acids, each residing on the surface of the coat protein β-sheet, whose substitution specifically disrupted RNA binding activity (Fig. 1B). Given the apparent importance of Thr^{45} in RNA binding, however, we were surprised by the absence of substitutions identifying Thr^{45} as a binding site residue. Here we describe the introduction of all 19 amino acid substitutions at position 45. Unexpectedly, every mutation reduced the ability of coat protein to fold correctly, apparently explaining our initial failure to find repressor-defective, assembly-competent Thr^{45} substitutions. At first, this frustrated our efforts to determine the effects of the mutations on RNA binding, but genetic fusion of coat protein subunits reverted these structural defects and allowed investigation of the RNA-binding role of Thr^{45}.

EXPERIMENTAL PROCEDURES

Recombinant DNA—The mutants described in this paper were constructed by site-directed mutagenesis using the method of Kunkel et al. (13) and are simple variants of pCT119 (6, 7). In all cases the complete nucleotide sequences of the mutant genes were determined. The single-chain dimer (i.e. subunit fusion) constructs were produced by methods described earlier (8), and incorporated the various mutations described in the text. Plasmids were introduced into Escherichia coli strain CSH41F (14) for coat protein expression.

Protein Characterization—To determine the relative amounts of coat protein accumulating in soluble form and as insoluble aggregates, cells from overnight 1-ml cultures were pelleted by centrifugation, resuspended in 0.25 ml of 100 mM NaCl, 0.1 mM MgSO4, 0.01 mM EDTA, 10 mM Tris-HCl, pH 7.5, and sonicated three times (on ice) for a total of 30 s. Insoluble material was then pelleted by centrifugation. Pellets were resuspended in 0.25 ml of the above buffer. Five μl of each supernatant or pellet fraction were combined with an equal volume of 2× SDS gel sample buffer, heated to 95 °C for 3 min, and applied to a 17.5% polyacrylamide gel containing SDS (15). The gel was electroblotted to Nytran (Schleicher & Schuell), and coat protein was visualized with anti-MS2 serum and 125I-labeled protein A (16).

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1 The abbreviations used are: X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; WT, wild-type.
The capacities of individual proteins to assemble into virus-like particles were assessed by agarose gel electrophoresis as follows. Cells from 1-ml overnight cultures were pelleted by centrifugation, resuspended in 0.25 ml of 100 mM NaCl, 0.1 mM MgSO4, 0.01 mM EDTA, 50 mM Tris-HCl, pH 7.5, and sonicated. After cellular debris was removed by centrifugation, 10 μl from each lysate were applied to a 1.0% agarose gel in 50 mM potassium phosphate, pH 7.0. Upon completion of electrophoresis, the gel was blotted to a Nytran membrane (Schleicher & Schuell) and coat protein was visualized using anti-MS2 serum and 125I-protein A (NEN Life Science Products).

Measuring Translational Repression—The RNA binding activities of the coat protein variants were assessed by their abilities to repress translation of a replicase-β-galactosidase fusion protein produced from pRZ5 in strain CSH41F2 (see Ref. 6 for details). Cells containing the appropriate plasmids were streaked on LB plates containing X-gal and compared for relative blueness. Assay of β-galactosidase was performed in triplicate by the method described by Miller (14). Activities are reported as percentages of the activity of an unrepressed control.

RNA Binding Assays—Radioactive RNA was produced by the method of Draper et al (17). Protein-excess nitrocellulose filter-binding assays (18) were performed with [32P]RNA at a concentration of 10 pM, which was titrated with coat proteins purified as described by Peabody (6). Binding curves were fitted to a bimolecular equilibrium using the Kaleidagraph program from Abelbeck Software. The concentration of active coat protein in each preparation was determined in a similar filter-binding assay, but one in which 50 nM protein solutions were titrated with excess radioactive RNA.

RESULTS

Effects of Amino Acid Substitutions at Position 45 on the Structure and Translational Repressor Function of Coat Protein—Using site-directed mutagenesis, codons for the 19 amino acid alternatives to threonine were introduced at position 45 in the coat sequence in plasmid pCT119. The effects of amino acid substitutions on RNA binding were monitored by a translational repression assay in which functional coat protein ex-

Fig. 1. A, structure of the MS2 translational operator. Note the adenosines at -4 and -10. B, a schematic illustration of the RNA-binding site of MS2 coat protein as defined by genetic analysis. Shaded circles identify the positions of amino acids whose substitution results in the repressor-defective phenotype. Because the coat protein dimer is symmetric and its RNA ligand is asymmetric, this is actually a composite picture of two overlapping binding sites. C, the heterodimer complementation map of the binding site. Here shaded circles represent a single asymmetric RNA binding site. The basis of heterodimer complementation is explained under “Results” and in Fig. 5.
Thr45 with serine (T45S) resulted in a repressor defect. Significant loss of repressor activity. For example, replacing repressor translation. Even conservative substitutions showed repressor defect. Most mutants showed little or no ability to transcribe (Table II). Every substitution resulted in a translational defect. Its extent of repression is conveniently determined by comparing the blueness of bacterial colonies on solid medium containing the chromogenic substrate X-gal. Table I lists the mutations and shows their effects on translational repression as judged by colony color. The β-galactosidase activities of some mutants were determined using α-nitrophenyl-β-D-galactoside as substrate (Table II). Every substitution resulted in a translational repressor defect. Most mutants showed little or no ability to repress translation. Even conservative substitutions showed significant loss of repressor activity. For example, replacing Thr45 with serine (T45S) resulted in a repressor defect.

In order to conclude with confidence that an amino acid substitution directly disrupts the RNA binding site, it is necessary to exclude the alternative possibility that it grossly perturbs coat protein structure, affecting RNA binding activity only secondarily. This is readily accomplished by measuring the ability of coat protein to assemble into virus-like particles, a property that depends on proper protein folding and is easily monitored in an agarose gel electrophoretic assay (7). Virus-like particles possess a distinctive mobility and may be visualized with anti-MS2 serum and 125I-protein A after blotting to nitrocellulose. Fig. 2A shows that every amino acid substitution at position 45 reduced the yield of virus-like particles. All the mutants were significantly impaired in their abilities to produce capsids; about half were apparently completely defective for capsid synthesis. Since its location in the structure of coat protein indicates no direct role for Thr45 in the assembly of coat dimers into capsids, these results suggest that the mutant proteins fail to fold normally into the dimers that serve both as translational repressors and as the precursors to capsids.

As an additional test of the ability of mutant proteins to acquire native structure, we determined their intracellular fates. It is commonly observed that mutant proteins which fail to fold correctly are either degraded by proteolysis or accumulate as insoluble aggregates in inclusion bodies. Sonicated cell lysates were fractionated by centrifugation into soluble and insoluble fractions, which were then subjected to SDS gel electrophoresis and Western blotting. Coat proteins were visualized using anti-MS2 serum and 125I-protein A. Fig. 2B shows the results. Wild-type coat protein was found mostly in the soluble fraction, and this was also true of T45E, T45Q, T45K, and T45R.

### Table I

Role of Thr45 in MS2 Coat Protein-RNA Interactions

| Mutant  | Activity |
|---------|----------|
| pUCter3 | ++       |
| pCT119  | +        |
| T45P    | +        |
| T45N    | +        |
| T45S    | +        |
| T45A    | +        |
| T45G    | +        |
| T45H    | +        |
| T45L    | +        |
| T45M    | +        |
| T45Q    | +        |
| T45R    | +        |
| T45W    | +        |
| T45E    | +        |

### Table II

β-galactosidase activities produced by selected mutants as single-chain homodimers or as heterodimers with wild-type

| Mutant  | Activity |
|---------|----------|
| pUCter3 | 100      |
| pCT119  | 3        |
| p2CTdl-13 | 1  |
| T45P    | 100      |
| WT-T45P | 100      |
| T45P-WT | 100      |
| 2T45P   | 100      |
| T45N    | 83       |
| WT-T45N | 4        |
| T45N-WT | 38       |
| 2T45N   | 42       |
| WT-T45A | 7        |
| T45A    | 35       |
| T45A-WT | 5        |
| 2T45S   | 12       |
| T45S    | 35       |
| T45S-WT | 1        |
| 2T45S   | 8        |
| WT-T45G | 7        |
| T45G    | 3        |
| T45G-WT | 2        |
| 2T45G   | 16       |
| T45C    | 23       |
| WT-T45C | 3        |
| T45C-WT | 2        |
| 2T45C   | 10       |
| T45V    | 100      |
| T45V-WT | 24       |
| 2T45V   | 100      |
| T45L    | 100      |
| 2T45L   | 100      |

![Fig. 2. A, agarose gel electrophoresis and Western blot of capsids produced by the WT and various T45 mutants (identified using the single-letter amino acid code). B, SDS-polyacrylamide gel electrophoresis and Western blot analysis of the coat proteins produced by WT and various T45 mutants (again denoted by the single-letter amino acid code). Cells were lysed by sonication and separated into soluble and insoluble fractions by centrifugation. In each pair of lanes, the one on the left is the soluble fraction, and the one on the right is the insoluble fraction.](image)
struc
tion of p2CTd1-13, a plasmid in which the coat sequence is duplicated and the two sequences are joined together in a translational fusion (8). Because the N and C termini of different subunits are near each other in space, the single-chain "dimer" folds properly and possesses the RNA binding and capsid assembly functions typical of wild-type coat protein. This fused dimer possesses greatly increased resistance to denaturation by urea and corrects the structural defects imposed by certain peptide insertions (19) and temperature-sensitive mutations.2 Therefore, we wondered whether the defects conferred by the Thr45 substitutions might also be reverted by subunit fusion. We created genetic subunit fusions using a selection of mutants: T45P, T45N, T45V, T45A, T45S, T45L, and T45G. In addition to creating mutant homodimers, which we call 2T45P, 2T45N, etc., many of the mutant sequences were also paired with the wild-type in either the N- or C-terminal half. We call these, for example, WT-T45P when the mutant sequence is in the 3'-half of the fused dimer, and T45P-WT when it is in the 5'-half.

Tests for capsid assembly activity (Fig. 3A) and for production of soluble protein (Fig. 3B) showed that, with one exception, subunit fusion corrected the structural defects of these mutants. This was true whether a mutant was paired with itself in a fused homodimer, or with the wild-type sequence in either half of a fused heterodimer. In their single-chain forms, they were generally produced in normal amounts and in soluble form. They also assemble into virus-like particles, although in a few cases, namely WT-T45V, 2T45V, T45G-WT, and 2T45G, assembly activity was only partially restored (Fig. 3B). Therefore, subunit fusion promotes the formation of native coat protein structure. The one exception was T45P. Although subunit fusion rescued it from proteolysis so that the fused dimer derivatives (WT-T45P, T45P-WT, and 2T45P) were present in normal amounts, they were found almost entirely in the aggregated fraction, and no capsids were detected. The inability of subunit fusion to fully correct the T45P defect is consistent with the difficulty of incorporating prolines into β-sheets.

The translational repressor activities of the proteins were assessed by assays of β-galactosidase activity present in strains containing the appropriate plasmids (Table II). In most cases, subunit fusion apparently restored whatever degree of repressor activity had been lost due to folding defects, allowing us to distinguish the protein-structural and RNA-binding site effects of the amino acid substitutions. Although many mutants were still repressor-defective, others showed complete, or nearly complete, restoration of repressor activity. The WT-T45S and T45S-WT and the WT-T45G and T45G-WT heterodimer fusions repressed translation as well as wild-type, but with the 2T45S and 2T45G homodimers repressor activity was restored only incompletely. Three other mutants we tested (T45A, T45C, and T45N) were nearly as good repressors as wild-type when paired with the wild-type sequence in heterodimers, but, again, each was less active in mutant homodimers.

We also determined the RNA binding activities of some of the fused dimer variants in vitro by measuring their abilities to retain 32P-labeled MS2 operator RNA on nitrocellulose filters. The results shown in Fig. 4 indicate that the WT homodimer and the WT-T45S heterodimer were indistinguishable in their abilities to bind RNA, each giving dissociation constants of 1 nM. The 2T45S homodimer and the WT-T45G heterodimer each bound only a little less well than WT at approximately 2 nM. WT-T45A and 2T45A, the worst binders of the group, gave 3 and 4 nM dissociation constants respectively. We also attempted to determine the binding affinity of 2T45G, but were unable to reconstitute its binding activity after the acid denaturation step used in its purification (6). We assume this to be caused by a failure of this mutant to refold properly. The results of in vivo binding analyses roughly parallel the in vitro translational repression assays, although some of recombinants (e.g., 2T45A) seem to bind RNA better than might be expected from their relatively poor translational repressor activities.

Mapping the Site of Thr45 Interaction to A−4 or A−10—The crystal structure of the coat protein-RNA complex suggests that the two Thr45 residues make contributions to RNA binding by interacting with A−4 and A−10 on different subunits of the dimer. However, the results described above indicate that one of the Thr45 residues is largely dispensable. Which of the two interactions is the important one? Structural analysis of the coat protein-RNA complex seems inconclusive on this point. On subunit A Thr45 is thought to make H-bonds with N6 and N7 of A−4, and on subunit B it is reputed to form similar interactions with N1 and N6 of A−10 (11, 12). However, it is not possible to predict reliably the contributions of these individual interactions to the energy of RNA binding on structural grounds alone. We sought to resolve this question genetically.

In solution coat protein is a symmetric dimer and therefore contains two binding sites for its asymmetric RNA ligand. Since the two sites overlap, only one can be occupied at any instant, accounting for the binding stoichiometry of one RNA per coat protein dimer. A bound RNA molecule makes contacts with both subunits of the dimer, so it is convenient to think of each binding site as being made up of two non-identical half-sites, each representing the contribution of one monomer to a complete binding site. We may label the half-sites of one binding site A and B, and those of the other site A' and B' (Fig. 5A). In the conventional homodimer, any amino acid substitution that inactivates a particular half-site does so on both monomers, thereby inactivating both complete binding sites (Fig. 5B). However, the ability to genetically fuse the two subunits of coat protein makes it possible to create single-chain heterodimers in which the two halves of the molecule may contain different combinations of mutant and wild-type sequences. When a mutation inactivating one half-site, say A, is paired in a heterodimer with a wild-type sequence, one intact, functional
site is restored (Fig. 5C). Likewise, when a mutation inactivating one half-site (for example A) is paired with a mutation inactivating the other half-site (B), one binding site is doubly defective, but one functional site is produced (Fig. 5D). These heterodimers are competent to bind RNA. Of course, the heterodimerization of mutants with different lesions in the same

**Fig. 4.** Nitrocellulose filter-binding assays of the RNA binding activities of wild-type single-chain dimers and the indicated single-chain heterodimers. Dissociation constants are given within each panel.
A–4 contact), and with N55D and T91I from half-site B (where A–10 is bound). These mutants were chosen for the complementation studies because their roles in RNA binding are fairly well defined by both structural and genetic analyses. The ring of TyrA85 stacks on U–5, and its hydroxyl group H-bonds to the phosphate of the same nucleotide. The side chain amide of AsnA87 forms an H-bond to O2 of U–5. AsnA87 interacts with the phosphate of A–7. ThrB39 does not appear to contact RNA, and its substitution in the T91I mutant apparently inactivates the RNA-binding site by steric interference in half-site B. T45A was paired with these mutants to produce the single-chain heterodimers listed in Table III.

Agarose gel electrophoresis showed that each of the heterodimers was competent for capsid assembly, indicating that all are properly folded (results not shown). The RNA-binding activities of the various heterodimers were tested by comparing their abilities to repress β-galactosidase synthesis in bacteria containing the plasmid pTZ5. Table III compares the β-galactosidase activities of bacteria with the indicated heterodimer constructs. T45A is capable of efficient translational repression when paired in heterodimers with N55D or T91I. It complements these mutants as well, or nearly as well, as does a wild-type subunit. But when paired with Y85H or N87S, each of the T45A-containing heterodimers is a poor repressor. This behavior assigns Thr45 to half-site A where it interacts with A–4.

**DISCUSSION**

**Protein Structure Effects of Thr45 Substitutions**—Even before the determination of the structure of the RNA-protein complex, an important role for Thr45 was suggested by its conservation across an evolutionary spectrum of RNA phage coat proteins. An involvement of Thr45 in RNA binding was strongly indicated when the crystal structure of the RNA-protein complex revealed the presence of quasi-symmetric interactions of the bulged adenosine at A–10 and the loop adenosine at A–4 with a site containing Val29, Thr45, Ser47, and Lys63 which is present in both halves of the dimer (11, 12). Therefore, we were surprised by our failure to find substitutions implicating Thr45 as a component of the binding site in our collection of repressor-defective mutants. The results presented here provide an explanation; the structure of coat protein is perturbed by every substitution of residue 45, so that all but a few Thr45 mutants would have been discarded during the screen for capsid assembly. Moreover, those mutants that still produced a significant amount of capsid must have been rare, if not absent, in our mutant library, since only one of them (T45S) could have resulted from a single-nucleotide substitution.

Some other amino acids in the RNA-binding site apparently exhibit a sensitivity to substitution similar to that of Thr45. Recently, we introduced mutations at Val29, Ser47, Thr29, and Lys41, residues also implicated in A–4 and A–10 binding. Ser47 and Thr29 seem to tolerate substitutions, but many different changes at positions 29 and 47 destroyed the ability of the protein to assemble into capsids.

**TABLE III**

| Construct          | Wild-type | pCAT119 | p2CT-113 | T45A-WT | T45A-N55D | T45A-Y85H | T45A-N87S | T45A-T91I |
|--------------------|-----------|---------|----------|---------|-----------|-----------|-----------|-----------|
| **Wild-type**      | 3         | 1       | 30       | 5       | 5         | 18        | 27        | 3         |
| **pCAT119**        | 3         | 1       | 30       | 5       | 5         | 18        | 27        | 3         |
| **p2CT-113**       | 3         | 1       | 30       | 5       | 5         | 18        | 27        | 3         |
| **T45A-WT**        | 3         | 1       | 30       | 5       | 5         | 18        | 27        | 3         |
| **T45A-N55D**      | 3         | 1       | 30       | 5       | 5         | 18        | 27        | 3         |
| **T45A-Y85H**      | 3         | 1       | 30       | 5       | 5         | 18        | 27        | 3         |
| **T45A-N87S**      | 3         | 1       | 30       | 5       | 5         | 18        | 27        | 3         |
| **T45A-T91I**      | 3         | 1       | 30       | 5       | 5         | 18        | 27        | 3         |

| Construct          | Wild-type | pCAT119 | p2CT-113 | T45A-WT | T45A-N55D | T45A-Y85H | T45A-N87S | T45A-T91I |
|--------------------|-----------|---------|----------|---------|-----------|-----------|-----------|-----------|
| **Wild-type**      | 3         | 1       | 30       | 5       | 5         | 18        | 27        | 3         |
| **pCAT119**        | 3         | 1       | 30       | 5       | 5         | 18        | 27        | 3         |
| **p2CT-113**       | 3         | 1       | 30       | 5       | 5         | 18        | 27        | 3         |
| **T45A-WT**        | 3         | 1       | 30       | 5       | 5         | 18        | 27        | 3         |
| **T45A-N55D**      | 3         | 1       | 30       | 5       | 5         | 18        | 27        | 3         |
| **T45A-Y85H**      | 3         | 1       | 30       | 5       | 5         | 18        | 27        | 3         |
| **T45A-N87S**      | 3         | 1       | 30       | 5       | 5         | 18        | 27        | 3         |
| **T45A-T91I**      | 3         | 1       | 30       | 5       | 5         | 18        | 27        | 3         |

**FIG. 5. Illustrations showing the effects on the RNA-binding site of combining mutant and/or wild-type sequences in single-chain coat protein heterodimers.** Asterisks indicate mutational inactivation of a half-site. A, a wild-type homodimer. B, mutant homodimer in which mutation has inactivated half-site A in the picture at left, or half-site B in the picture on the right. C, a wild-type-mutant heterodimer. D, a mutant heterodimer in which the two monomers have mutations in different half-sites.
We find the extreme substitution sensitivity of Thr45 to be surprising, but as our interest is mainly directed toward the RNA-binding site, we have made little effort to characterize the protein structural defects imparted by these mutations. However, several previous studies have systematically examined the effects on protein stability of amino acid changes on the surfaces of β-sheets. They paint a complicated picture. Statistical analysis of amino acid sequences long ago showed that the various amino acids have different tendencies to appear in β-structure (20, 21), and some mutational studies have suggested that the rank order of these statistically defined propensities roughly correlates with the protein stability effects of amino acid substitutions (22–24). Threonine is generally near the top of such rankings (22, 23, 25). Effects of substitutions on stability are observed even when pairs are taken to eliminate interactions of the mutated amino acid with nearby side chains (22, 25), presumably reflecting the intrinsic tendency of an amino acid to appear in a β-strand. However, structural context also plays an important role; mutational analyses demonstrate that the rank order of apparent β-sheet propensities can differ sharply from one protein to another, and even depends on the precise location of an amino acid within the sheet of a single protein (26). The importance of context is also supported by skewing in the frequencies with which certain amino acid pairs are found in neighboring positions on adjacent β-strands (27, 28), and by the experimental determination of stability effects of pairwise amino acid substitutions (24). Inspecting the local environment of Thr45 shows that its non-H-bonded cross-strand neighbor is Glu11, Hutchinson et al. (28), in a statistical survey of favored residues in β-sheets, showed that Thr-Glu pairs are often found in such positions.

Effects on RNA Binding—The crystal structure of the coat protein-RNA complex suggests a key role for Thr45 in both subunits of the coat dimer in stabilizing the RNA-protein complex (11, 12). Specifically it was proposed that Thr45 H-bonds with N6 and N7 of A–4 and Thr445 with N1 and N6 of A–10. However, our finding that several substitutions permit normal RNA binding indicates that Thr45 residues makes little or no contribution to the energy of RNA binding. Since structural studies implicate the Thr45 hydroxyl group in RNA binding, it was no surprise that serine was a reasonable replacement. In fact, the WT-T45S and T45S-WT recombinants, which contain the serine replacement in both halves of the dimer, did not quite attain wild-type activity, suggesting that the methyl group of one of the threonines must play some role in RNA binding. This does not imply necessarily that the methyl group participates in interaction with RNA directly. It may simply engage in interactions that favor the correct positioning of the hydroxyl. The complete, or nearly complete, recovery of translational repressor activity by heterodimer constructs in which one wild-type subunit was paired with T45G, T45N, T45C, or T45A adds weight to the argument that the identity of Thr45 is really crucial to RNA binding on only one of the two subunits. Moreover, the pattern of complementation when the T45A mutant is combined with other RNA-binding site mutations in single-chain heterodimers indicates that it is primarily Thr445’s interaction with A–4 that is sensitive to substitution.

These results have at least three possible explanations. (i) Thr445 interactions with RNA might make only a small contribution to the stability of the RNA-protein complex. Additional support for this idea comes from studies of the effects of nucleotide substitutions of A–10. Wu and Uhlenbeck (29) found that guanine, inosine, and 2-aminopurine were acceptable replacements for the bulged nucleotide. Since these remove the amino group at position C-6 of the base (2-aminopurine) or replace it with a keto group (guanosine or inosine), it seems unlikely that crucial contacts are made at this site. (ii) Some amino acid substitutions might replace lost contacts with new ones. This seems an unlikely alternative. It makes some sense that serine and cysteine should be tolerable replacements of Thr445, but, despite their rather large structural differences, glycine works as well, and alanine and asparagine work nearly as well as serine and cysteine when combined in heterodimers with a wild-type sequence. (iii) Conformational adjustments may allow the formation of new contacts, which compensate for the loss of H-bond interactions that accompany Thr445 substitution. This possibility is suggested by the x-ray structure of the complex of the T45A mutant with RNA, which reveals RNA conformational changes in the vicinity of both A–4 and A–10 (30). Some of these changes, especially those near A–10, seem to offset partially the loss of normal Thr45 contacts by forming additional favorable contacts at other nearby sites. We wonder whether similar, but more extensive, conformational adjustments accompany the substitution of Thr45 with Gly, offsetting the loss of Thr45 side-chain contacts so completely that the WT-T45G heterodimer is as good a repressor as wild-type.

The coat proteins of different RNA phages bind different hairpins (31). However, they do not appear to have acquired the wide range of specificities that characterizes, for example, the ribonucleoprotein class of proteins (32), which also use a β-sheet as the RNA binding site. Our results point out two constraints that must limit the ability of RNA phage coat proteins to evolve new RNA binding specificities. First, the stability of coat protein can be highly sensitive to substitution of RNA-binding site amino acids. This limits its ability to adapt to new ligands. Second, because of the two-fold symmetric nature of the coat protein dimer, any change in one half of the RNA-binding site is accompanied automatically by an identical change in the other half. Therefore, it may be difficult for coat protein to acquire new specificities for intrinsically asymmetric RNA ligands. One wonders what new specificities could be conferred to single-chain coat proteins, where the destabilizing effects of amino acid substitutions are better tolerated, and where the RNA-binding site is free to develop asymmetries.

REFERENCES

1. Valegard, K., Liljas, L., Fridborg, K., and Unge, T. (1990) Nature 345, 36–41
2. Kolmohammadi, R., Valegard, K., Fridborg, K., and Liljas, L. (1993) J. Mol. Biol. 234, 620–629
3. Ni, C.-Z., Syed, R., Kodandapani, R., Wickersham, J., Peabody, D. S., and Ely, K. R. (1995) Structure 3, 255–263
4. Borer, P. N., Lin, Y., Wang, S., Roggenbuck, M. W., Gott, J. M., Uhlenbeck, O. C., and Pellicer, I. (1995) Biochemistry 34, 6488–6503
5. Romanik, P. J., Lowary, P., Wu, H., Stormo, G., and Uhlenbeck, O. C. (1987) Biochemistry 26, 1563–1568
6. Peabody, D. S. (1990) J. Biol. Chem. 265, 5684–5689
7. Peabody, D. S. (1993) EMBO J. 12, 595–600
8. Peabody, D. S., and Lim, F. (1996) Nucleic Acids Res. 24, 2352–2359
9. Lim, F., Spingola, M., and Peabody, D. S. (1994) J. Biol. Chem. 269, 9096–9101
10. Lim, F., Spingola, M., and Peabody, D. S. (1996) J. Biol. Chem. 271, 31839–31845
11. Valegard, K., Murray, J. B., Stockley, P. G., Stonehouse, N., and Liljas, L. (1994) Nature 371, 623–625
12. Valegard, K., Murray, J. B., Stockley, P. G., Liljas, L., Vlcek, V., and Liljas, L. (1994) Nature 371, 623–625
13. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) Methods Enzymol. 154, 367–382
14. Miller, J. H. (1972) Experiments in Molecular Genetics, pp. 352–355, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
15. Laemmli, U. K. (1970) Nature 227, 680–685
16. Burnette, W. N. (1981) Anal. Biochem. 112, 195–203
17. Draper, D. E., White, S. A., and Kean, J. M. (1988) Methods Enzymol. 164, 221–237
18. Carey, J., Cameron, V., de Haseth, P. L., and Uhlenbeck, O. C. (1983) Biochemistry 22, 2601–2610
19. Peabody, D. S. (1997) Arch. Biochem. Biophys. 347, 85–92
20. Chou, P. Y., and Fasman, G. D. (1978) Biochemistry 13, 211–222
21. Chou, P. Y., and Fasman, G. D. (1978) Mol. Biol. 47, 45–448
22. Minor, D. L., and Kim, P. S. (1994) Nature 367, 660–663
23. Kim C. A., and Berg, J. M. (1993) Nature 362, 267–270
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24. Smith, C. K., and Regan, L. (1995) Science 270, 980–982
25. Smith, C. K., Withka, J. M., and Regan, L. (1994) Biochemistry 33, 5510–5517
26. Minor, D. L., and Kim, P. S. (1994) Nature 371, 264–267
27. Lifson, S., and Sander, S. (1980) J. Mol. Biol. 139, 627–639
28. Hutchinson, E. G., Sessions, R. B., Thornton, J. M., and Woolfson, D. N. (1998) Protein Sci. 7, 2287–2300
29. Wu, H.-N., and Uhlenbeck, O. C. (1987) Biochemistry 26, 8221–8227
30. van den Worm, S. H. E., Stonehouse, N. J., Valegard, K., Murray, J. B., Walton, C., Fridborg, K., Stockley, P. G., and Liljas, L. (1998) Nucleic Acids Res. 26, 1345–1351
31. van Duin, J. (1988) in The Bacteriophages (Calendar, R., ed) pp. 117–161, Plenum Press, New York
32. Kenan, D. J., Query, C. C., and Keene, J. D. (1991) Trends Biochem. Sci. 16, 214–220