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Bacteriophage T4 regA protein binds RNA as a monomer, overcoming dimer interactions

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ABSTRACT

The stoichiometry of the complex formed between the T4 translational repressor protein regA and the 16 nt gene 44 recognition element (gene 44RE) RNA has been determined. Under quantitative binding conditions, the association of wild-type regA protein with gene 44RE RNA exhibits saturation at a 1:1 ratio of protein to RNA. It is known that regA protein exists as a dimer in protein crystals. Thus, the stoichiometry may be indicative of a regA dimer bound to two RNAs or a regA monomer bound to one RNA. Gel filtration through Sephadex G-75 revealed that wild-type and R91L regA proteins (14.6 kDa) elute at a mass of 29 kDa, consistent with the mass of a dimer. However, wild-type regA preincubated with gene 44RE (1:1) resulted in a complex that eluted at ~20 kDa, consistent with a regA monomer–RNA complex. Covalent crosslinking of surface lysines with glutaraldehyde confirmed that wild-type and R91L proteins exist as dimers and higher oligomers in solution. However, the addition of RNA to wild-type regA protein prior to crosslinking inhibited the formation of crosslinked dimers. Thus, the regA protein–protein interactions observed in solution are disrupted or blocked in the presence of gene 44RE RNA. Together, these studies demonstrate that regA protein binds RNA as a monomer, although free protein exists predominantly as a dimer.

INTRODUCTION

The bacteriophage T4 regA protein is an unusual translational repressor that regulates the expression of 15–30 early T4 genes, including the regA gene. It does so by binding to the translation initiation region (TIR) of target mRNAs and inhibiting formation of ribosome–mRNA initiation complexes (1,2). Although the regA recognition element has been studied in detail for gene 44 (3) and the rIIB gene (1,4) and has been mapped by RNase protection in three other mRNAs (3), it is still not clear what the common features for regA protein repression are. Nor is it known what domains or residues in regA protein function in the recognition and discrimination of target mRNAs. Thus, the structural basis for multiple regA protein–RNA interactions is largely unknown.

The recent solution of the crystal structure of regA protein (5) revealed that it contains an α-helical core and two regions of antiparallel β-sheets. Interestingly, in the crystal structure, regA protein exists as a dimer. The observation by Y. Kyogoku (cited in 1) that in relatively dilute solution (30 μM) regA protein exists as a dimer, suggested to Kang and co-workers that regA may bind RNA as a dimer (5). The relatively large and variable regA protein binding site sizes observed on some T4 early mRNAs (2) also suggests that multiple regA proteins may bind some mRNAs. Miller *et al.* (6) estimated regA protein concentration in the early stages of T4 infection to be 0.02–0.1% of total protein, corresponding to ~1.5–7.5 μM. Spicer and co-workers (7,8) were able to demonstrate functional activity of purified regA protein *in vitro* in translational repression assays at concentrations ranging from 0.5 to 20 μM. However, it is unclear if regA protein exists as a dimer or monomer at these lower physiological concentrations. To address this question, we have used saturation binding analysis to measure the stoichiometry of protein–RNA complex formation (at 0.2–5 μM) and have employed gel filtration (at 1–12 μM) and glutaraldehyde crosslinking (at 10–12 μM) to assess the quaternary structure of regA protein.

The determination of whether regA binds RNA as a dimer or monomer is crucial to ongoing efforts to localize the RNA binding site on regA protein. Likewise, correct interpretation of the mapping of protein binding sites on mRNAs depends on knowing whether regA protein binds as a monomer or a dimer.

MATERIALS AND METHODS

Reagents and strains

Oligo ribo- and deoxyribonucleotides were synthesized on an Expedite (Model 8909) Nucleic Acid Synthesis System by the MUSC Oligosynthesis Facility. Oligoribonucleotides were deprotected as previously described (9) and then purified by perfusion chromatography using Poros™ HQ and R1 columns essentially as described (9). Poly(U) and poly(U)–agarose were purchased from Pharmacia LKB Biotech. *Escherichia coli* AR120 (λcI^+, N^+) was obtained from A. Shatzman (Smith, Klein and French). Construction of pAS_1regA was as previously described (9).

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RegA mutagenesis of the dimerization domain

Site-directed mutagenesis was carried out by annealing mutagenic oligonucleotides (Table 1) to a double-stranded plasmid carrying the wild-type *regA* gene (pAS_1regA) (8). Primer annealing, chain extension and transfection of *E.coli* AR120 was carried out via the Chameleon protocol (Stratagene). Mutations were confirmed by DNA sequence analysis, using [^{35}S]dATP and a Sequenase (v.2) kit (US Biochemicals).

Table 1.

Mutation	Size	Oligonucleotide (5′→3′)
E68A	33	ATG ACA GAA GAA CAT **GC**A GTT CGT CGT GAT TCG
E68Q	33	ATG ACA GAA GAA CAT **C**AA GTT CGT CGT GAT TCG
R91L	33	ATC GTT CCT GCT CAA CTA A**CT** TTT ATG AAA GAT

regA protein purification

Purification of wild-type regA protein has been described previously (7). RegA mutant R91L protein was purified from AR120/pAS_1regA-R91L cells. After induction of regA transcription from λ P_L with nalidixic acid, cells were pelleted and lysed by sonication (VirSonic 475 Ultrasonic Cell Disrupter; VirTis) at a setting of 4–6 with six 1 min bursts. The lysate was centrifuged at 118 727 *g* (Beckman 45Ti rotor) at 4°C for 1 h. The supernatant was dialyzed into buffer A (40 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol and 0.1 mM DTT) overnight. The dialysate was applied to a Poros™ HQ column (4.6 × 100 mm, 1.66 ml) equilibrated with buffer A, using a BioCAD/SPRINT Perfusion Chromatography System (PerSeptives Biosystems). Proteins were eluted with a gradient of 0.1–2 M NaCl over 15 column volumes in buffer A. Fractions were collected, concentrated and analyzed for regA protein content by SDS–PAGE. Fractions containing regA protein were pooled and dialyzed against buffer B (20 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM DTT, 10% glycerol and 20 mM NaCl) for 20 h. The dialysate was applied to a poly(U)–agarose (Pharmacia) column and a gradient of 0–1 M NaCl in buffer B (>10 column volumes) was used to elute R91L protein. Fractions were assayed for absorbance at 280 nm and analyzed for regA protein content by SDS–PAGE. Appropriate fractions were pooled, dialyzed into buffer A and chromatographed through a Poros™ HQ column as described above, to remove contaminating poly(U). R91L was then concentrated under nitrogen (30–50 p.s.i.) through an Amicon concentrator with stirring (Amicon Diaflo Ultrafiltration membrane YM3, 43 mm). The sample was dialyzed overnight against 20 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 20 mM NaCl, 0.1 mM DTT and 50% glycerol and stored at –80°C.

Protein concentrations were determined by duplicate amino acid analyses, performed by the W. M. Keck Biotechnology Foundation (Yale University). Protein concentrations are cited as concentration of monomer regA protein. The purity of regA protein, as assessed by SDS–PAGE, was >95%.

Titration binding analyses

Increasing concentrations (0.2–5 μM) of purified wild-type and R91L regA proteins were incubated with [^{32}P]gene 44RE RNA (5′-GAA UGA GGA AAU UAU G-3′) at 1 μM in 10 mM Tris–HCl, pH 7.5, 50 mM NaCl and 1 mM EDTA. Samples were incubated for 15 min at 25°C and then for 15 min on ice. Tracking dyes (0.025% bromophenol blue and 0.025% xylene cyanole FF) were added, the reaction products were applied to a 6% polyacrylamide gel in 0.5× TBE and electrophoresed at 100 V for 4 h at 8°C. Products were visualized by autoradiography and quantitated by storage phosphor imaging (Molecular Dynamics Phosphor Imager™ SI). The data obtained were in the linear range of signal detection.

Gel filtration analysis of regA protein

RegA protein was chromatographed on a Sephadex G-75 (Pharmacia Biotech) column (60 × 0.75 cm) equilibrated with 10 mM Tris–HCl, pH 7.5, 150 mM NaCl and 1 mM β-mercaptoethanol. Three reference proteins (Sigma Chemical Co.) were used to generate a standard curve: ovalbumin (44 kDa), carbonic anhydrase (29 kDa) and lysozyme (14.4 kDa) (see Fig. 3, inset). A regression analysis of the elution volumes of the standards was found to be linear ($y = -1.9698x + 75.096$, where y = molecular weight and x = fraction number) with $r^2 = 0.996$. Based on this equation, a monomer (14.6 kDa) should elute at fractions 30–31, a dimer (29.2 kDa) at fraction 23 and a trimer (43.8 kDa) at fraction 16. Similarly, a regA monomer bound to one RNA (19.9 kDa) should elute at fraction 28 and a dimer bound to one RNA should elute at fractions 20–21.

Wild-type regA and R91L proteins were chromatographed at 24, 12, 6, 2 and 1 μM. Collected fractions were analyzed for absorbance at 280 nm (Shimadzu UV160 Spectrophotometer). However, when protein concentrations were < 12 μM, detection by spectrophotometry was not accurate. A Coomassie Plus™ Protein Assay (Pierce Chemical Company), based on the traditional Lowry and Bradford Assays (10,11), was employed to detect the lower concentration of protein in the column fractions. A standard curve over the range 0–0.21 μM was generated using purified regA protein. The plot of protein concentration versus $A_{595\ nm}$ was linear, with the equation $y = 1.3841x - 0.0121$, $r^2 = 0.988$, where y = concentration of protein (μM) and $x = A_{595\ nm}$.

Glutaraldehyde crosslinking of regA protein

Wild-type regA (11.4 μM) and R91L (12 μM) were incubated with 0.1% glutaraldehyde (12) in 10 mM sodium phosphate, pH 7.5, at 25°C for 30 min. Samples were then diluted into Laemmli sample buffer (13), boiled and analyzed by SDS–15% PAGE. Crosslinking was also carried out in the presence of gene 44RE RNA (16mer) and oligo$(U)_{24}$. Unlabeled RNA was incubated with wild-type and R91L proteins at an RNA:protein (monomer) ratio of 2:1 to ensure saturation of binding sites on the protein. Wild-type and R91L regA proteins (final concentrations 11.4 and 12 μM respectively) were preincubated with gene 44RE or oligo$(U)_{24}$ RNA (final concentrations 22.8 and 24 μM respectively) in 10 mM sodium phosphate, pH 7.5, on ice for 15 min (total reaction volume 13 ml). A one third volume of 0.3% glutaraldehyde was added to the reaction mixture (final concentration of glutaraldehyde 0.1%) and samples were incubated at 25°C for 30 min. The samples were treated as described above and analyzed by SDS–PAGE.

RESULTS

In the crystal structure of regA protein, two polypeptides are seen associated as a dimer (5). The crystal dimer is stabilized by symmetrical hydrogen bonds between the side chains of Arg91 on

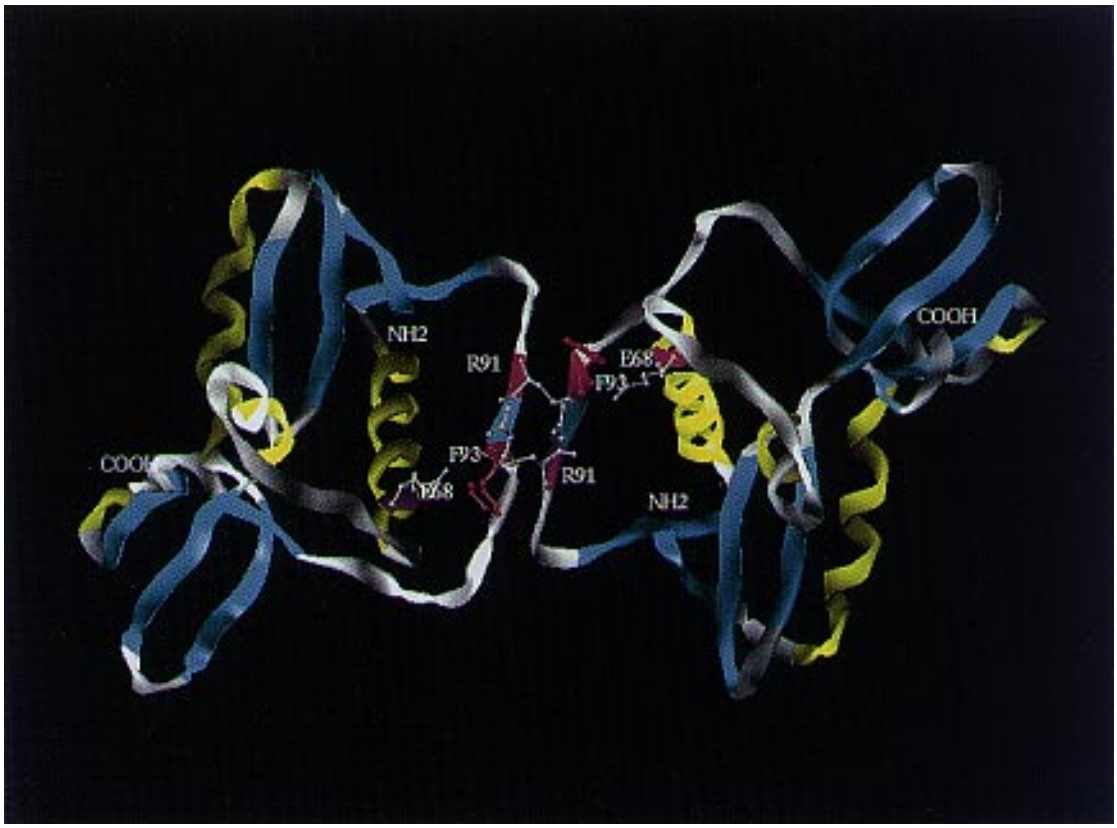

Figure 1. Ribbon diagram of regA protein dimer as observed in the crystal structure. β-Sheets are shown in light blue and α-helices are shown in yellow. Residues Glu68 and Arg91, which form hydrogen bonds that stabilize dimer formation, are displayed. Partial chymotryptic proteolysis of regA protein results in cleavage at Phe93, also shown (22).

one subunit and Glu68 on the other subunit (5), as illustrated in Figure 1. Additional stabilization comes from symmetrical intersubunit hydrogen bonds between the carbonyl group of Thr92 on one subunit and the amino group of Thr92 on the other subunit. The observation that regA protein exists as a dimer in 30 μM solutions (Y. Kyogoku; see 1) suggests that the dimer form may be the biologically active form of regA protein. To test this hypothesis, substitutions were introduced into regA protein at residues Arg91 and Glu68 to eliminate hydrogen bonding between the residues, with the expectation that a reduction in the stability of the dimer would reduce regA protein's RNA binding affinity if regA protein binds RNA as a dimer.

Mutations Arg91→Leu (R91L), Glu68→Ala (E68A) and Glu68→Gln (E68Q) were introduced into the inducible regA expression vector pAS_1regA (8) (see Materials and Methods). Following induction of mutant regA expression in *E.coli* AR120/pAS_1regA at 37°C, cells were harvested, sonicated, centrifuged and examined for soluble regA protein (see Materials and Methods). RegA protein R91L exhibited solubility equal to that of wild-type regA protein (~60–80% solubility) as determined by SDS–PAGE and was subsequently purified by perfusion chromatography and poly(U)–agarose chromatography (see Materials and Methods). In contrast, both E68A and E68Q proteins remained in the cell pellet following lysis and centrifugation. Some mutant forms of regA have been found to be insoluble (presumably misfolded) when protein expression is carried out at 37°C, but are soluble when expression is carried out at 25°C. To test this possibility, E68Q and E68A were expressed at 25°C and re-examined for solubility. Although both mutant regA proteins were highly expressed at the lower temperature, they remained insoluble.

Binding stoichiometry of regA protein

To evaluate the stoichiometry of regA protein–RNA complexes, RNA gel mobility shift assays (14) were performed with a synthetic 16mer RNA corresponding to the gene 44RE. 5′-^{32}P-End-labeled gene 44RE RNA (1 μM) was incubated with increasing concentrations of wild-type regA protein and the reaction products were separated by polyacrylamide gel electrophoresis, as shown in Figure 2A. In earlier studies (3), the affinity of regA protein for a synthetic RNA corresponding to the T4 gene 44 recognition element (gene 44RE) was found to be 10^7 M^{-1} (in 150 mM NaCl). Thus, a 1 μM concentration of RNA is 10-fold above the stoichiometric point for regA protein binding. The amount of protein–RNA complex and free RNA was determined by storage phosphor imaging (15). A plot of the formation of complex as a function of protein concentration is shown in Figure 2B. Analysis of the stoichiometric point by linear regression indicated saturation at 1.19 μM protein. The affinity of wild-type regA (in 50 mM NaCl) for gene 44RE RNA calculated from double reciprocal analysis of the binding data is $K_{app} = 3.5 \times 10^6$ M^{-1}. [Note that this value represents a minimal estimate of the affinity

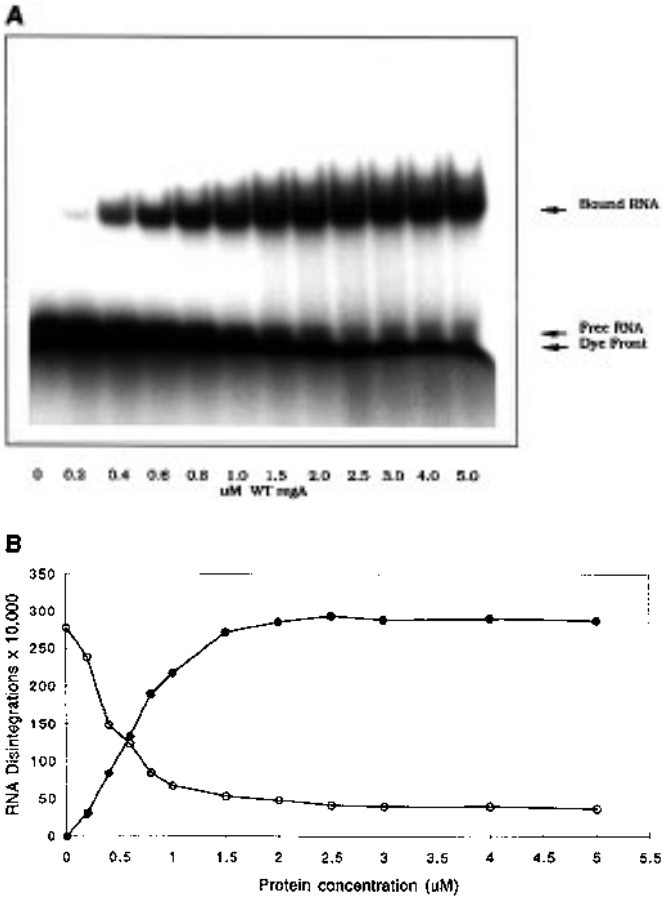

Figure 2. Gel mobility shift assays of regA protein binding to gene 44RE RNA. (**A**) Titration of 1 µM ^{32}P-labeled gene 44RE RNA with increasing concentrations of wild-type regA protein. Increasing protein concentrations of regA (0–5 µM, lanes 1–12) were incubated with RNA in 10 mM Tris–HCl, pH 7.5, 50 mM NaCl and 1 mM EDTA at 25°C for 15 min and then at 0°C for 15 min. Reactions were analyzed by electrophoresis on a native 6% polyacrylamide–0.5× TBE gel and visualized by autoradiography. The radioactivity of the ion front is presumed to contain unincorporated [^{32}P]ATP and short oligoribonucleotides. (**B**) Plot of bound (●) and free (○) RNA as a function of regA protein concentrations, as determined by phosphor image analysis of the polyacrylamide gel (RNA disintegrations are arbitrary units). A maximum of 88% of the RNA disintegrations were bound by regA protein.

and is lower than the affinity measured by fluorescence quenching assays (4)].

A similar titration binding analysis was performed with regA R91L. Initial titration analysis revealed less efficient complex formation, suggesting that R91L has a weaker affinity for gene 44RE RNA than wild-type regA. To determine the binding stoichiometry and relative binding affinity of R91L, gel shift binding assays were performed in lower salt concentrations (10 mM NaCl). Under these conditions, R91L–gene 44RE complex formation was more efficient. Estimation of the affinity constant from the half saturation point on the titration curve revealed that R91L binds gene 44RE RNA with an affinity of ~2×10^6 M^{-1}. Taking into account the differences in NaCl concentration between the gel shift assays (10 versus 50 mM NaCl for wild-type) and the measured effect of ionic strength on regA protein's RNA affinity (3), the affinity of R91L regA protein for gene 44RE is estimated to be 10-fold lower than wild-type regA protein affinity.

For both wild-type and R91L, the saturation of RNA binding occurs at a 1:1 ratio of protein to RNA. This stoichiometry can arise from the binding of a monomer to gene 44RE RNA or from a dimer of regA protein binding two gene 44RE RNAs. To distinguish between these possibilities, the molecular masses of regA protein and regA protein–RNA complexes were examined.

Gel filtration of regA protein

Since the Stokes radius of regA protein (35 × 35 × 35 Å; C.-H. Kang, personal communication) determined from the crystal structure indicates that regA protein is globular (i.e. not elongated), gel filtration chromatography is a valid method to evaluate molecular mass. To assess the native molecular size of regA protein in solution, chromatography of regA protein through Sephadex G-75 was carried out at concentrations of 24, 12, 2 and 1 µM. At each of these concentrations, wild-type regA protein (14.6 kDa) eluted at the same volume as carbonic anhydrase (29 kDa), indicating that it was present predominantly as a dimer (Fig. 3A). A small shoulder was present in the regA elution profile (Fig. 3A) that co-migrated with lysozyme (14.4 kDa), suggesting the presence of some monomer at low regA concentrations. The absence of peak trailing into the lower mass region of the chromatogram suggests that regA protein does not interact with the column matrix. In addition, a small peak preceding the major (dimer) peak was observed, which appears to be regA protein trimers. To evaluate the effect of ionic strength on dimer formation, gel filtration chromatography of 12 µM regA protein was performed at low (10 mM Tris, 20 mM NaCl) and moderate (10 mM Tris, 150 mM NaCl) salt concentrations (data not shown) and the dimer was the predominant form under both conditions.

Chromatography of regA R91L on Sephadex G-75 was also performed at concentrations of 1–2 µM. Surprisingly, the majority of mutant R91L regA protein also eluted as a dimer (Fig. 3B), even though the side chain interactions between Arg91 and Glu68 have been eliminated by amino acid replacement.

To assess the molecular size of regA protein–RNA complexes and further evaluate the binding stoichiometry, wild-type regA complexed to [^{32}P]gene 44RE (1:1 ratio) was chromatographed on Sephadex G-75. Protein elution was monitored by Coomassie Plus™ assays (measured as $A_{595\ nm}$) and RNA was detected by Cerenkov counts. As can be seen in Figure 4, regA protein and gene 44RE RNA co-eluted at a position corresponding to a molecular mass of ~20 kDa. A regA monomer bound to gene 44RE RNA has a molecular mass of ~20 kDa and a dimer bound to gene 44RE RNA has a mass of ~34 kDa. Thus, the regA–gene 44RE complex eluted at a position consistent with a complex stoichiometry of one regA protein (monomer) bound to one 16mer RNA.

Crosslinking of regA protein

To further evaluate the oligomerization of wild-type and R91L regA proteins, the ability of glutaraldehyde to induce covalent crosslinks between subunits was assessed. Glutaraldehyde was chosen as a crosslinking reagent because it efficiently forms covalent bonds between lysine residues on the surface of proteins (11) and regA protein has a number of surface lysines (5). Crosslinkage allows for the stabilization of oligomeric forms of protein and subsequent visualization by SDS–PAGE (12). As shown in Figure 5A, exposure of wild-type regA protein to 0.1% glutaraldehyde resulted in formation of SDS-resistant dimers, trimers and higher oligomers (lane 3). RegA protein dimers have

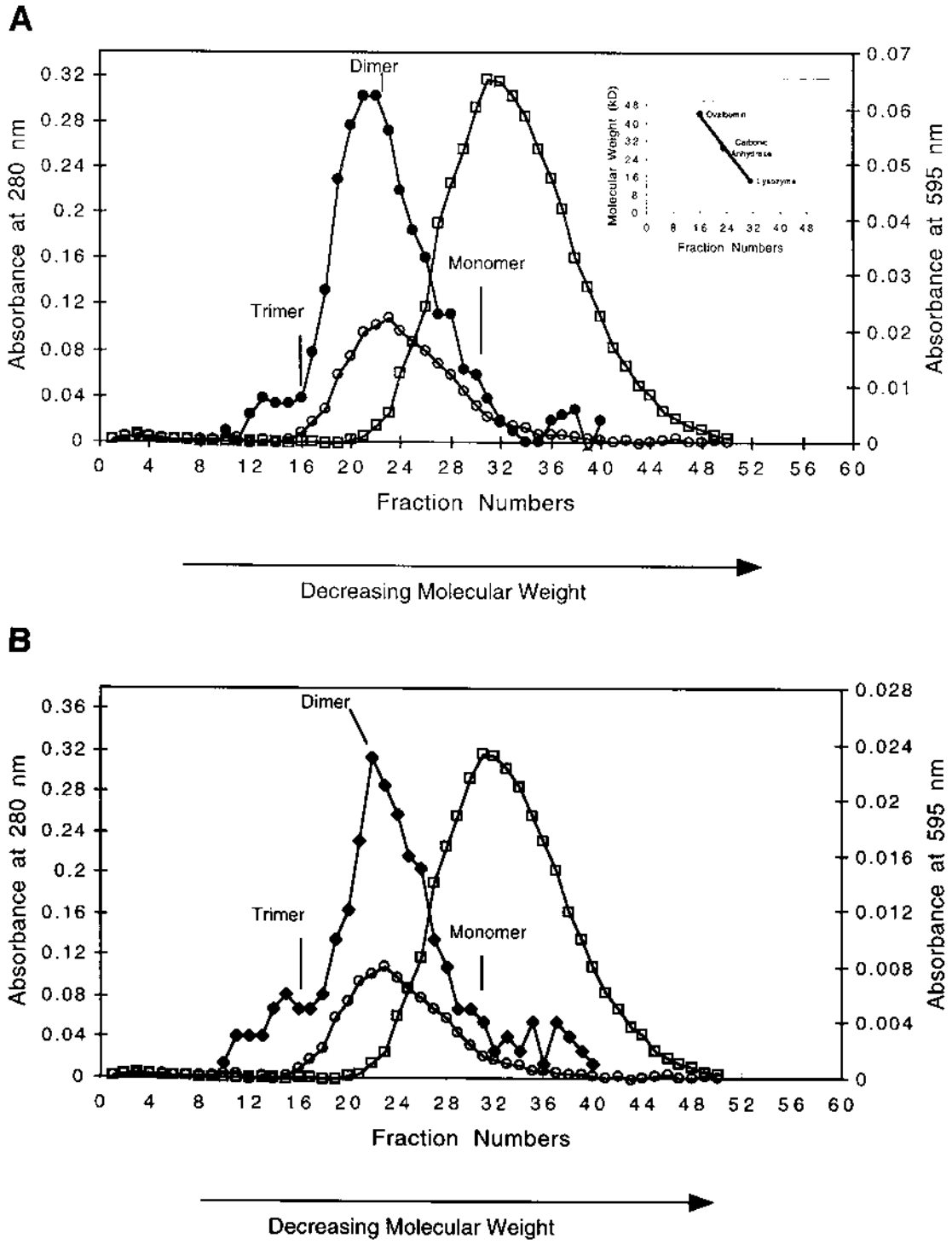

Figure 3. Gel filtration of regA protein through Sephadex G-75. Chromatography was performed in 10 mM Tris–150 mM NaCl at 8°C. (Inset) Chromatography of reference proteins ovalbumin, carbonic anhydrase and lysozyme, exhibiting a linear relationship between molecular weight and elution volume. (**A**) Chromatography of 1 µM regA protein (●). RegA protein concentrations were monitored by Coomassie Plus™ assays, plotted as $A_{595\ nm}$ on the right *y*-axis. (**B**) Gel filtration of 1 µM R91L regA (♦). Elution of reference proteins carbonic anhydrase (○) and lysozyme (□) was detected by absorbance at 280 nm, plotted on the left *y*-axis. Arrows indicate the expected elution of regA oligomers, based on the standard curve.

a molecular weight of 29 kDa, however, the first crosslinked band migrates in SDS–polyacrylamide gels at a mass of ~35 kDa. The reason for this anomalous migration is unknown, but may be due to crosslink-induced formation of a more rigid conformation of the dimer that retards its mobility. Based on the conclusion from gel filtration analysis that regA protein exists predominantly as a dimer in 10 µM solution, we estimate the efficiency of regA crosslinking by glutaraldehyde to be ≥60% in these assays.

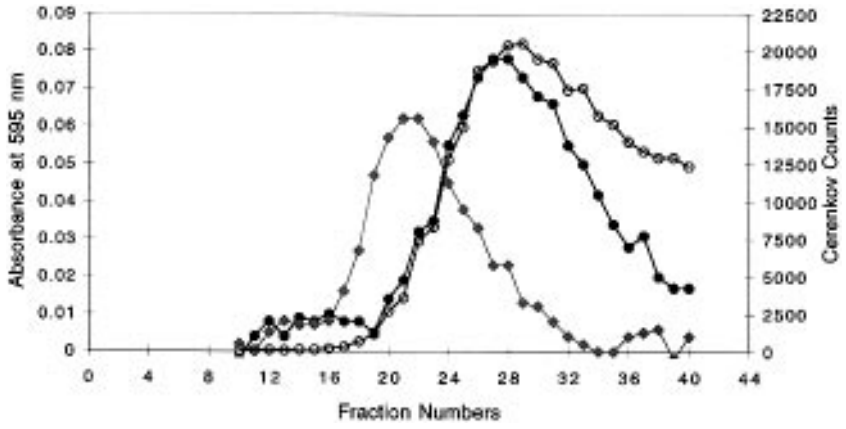

Figure 4. Gel filtration of regA protein–gene 44RE RNA complexes through Sephadex G-75. Chromatography was performed in 10 mM Tris, 150 mM NaCl at 4°C. Wild-type regA protein (1 µM) was preincubated with ^{32}P-labeled gene 44RE RNA (1 µM). RegA protein fractions (●) were monitored by Coomassie Plus™ assay and are plotted as absorbance at 595 nm on the left *y*-axis. RNA elution (○) was monitored through detection of Cerenkov counts, plotted on the right *y*-axis. Elution of regA protein in the absence of RNA (♦, gray lines) is superimposed as a reference. A protein monomer complexed to one RNA (19.9 kDa) was expected to elute at fraction 28, whereas a dimer complexed to one RNA (34.5 kDa) was expected to elute at fractions 20–21.

Interestingly, when gene 44RE RNA was preincubated with wild-type regA protein prior to crosslinking with glutaraldehyde, ≥90% of regA protein was observed as a monomer by SDS–PAGE (Fig. 5A, lane 4). Although not readily seen in Figure 5A, a faint band at a molecular weight of ~20 kDa was present in lane 4. The observation that glutaraldehyde can crosslink nucleic acids to proteins (with low efficiency) (12) suggests that this band may be a crosslinked complex of a regA monomer with gene 44RE. In fact, when wild-type regA protein was preincubated with [^{32}P]gene 44RE RNA, gel autoradiography revealed radioactivity associated with the 20 kDa band (Fig. 5A and B, lane 7), but not with higher molecular weight bands, consistent with the association of RNA only with monomer regA protein.

As shown in Figure 5B, crosslinkage of R91L regA protein with 0.1% glutaraldehyde also resulted in the formation of dimers, trimers and higher oligomers (lane 3). As was the case with wild-type regA, preincubation of R91L protein with gene 44RE RNA significantly reduced protein–protein crosslinking, such that ≥90% of regA protein migrated as a monomer (lane 4). Thus, although both wild-type and R91L regA exist as dimers and higher oligomers in solution, binding to RNA significantly inhibits the regA protein–protein interactions that enable glutaraldehyde crosslinking.

To test the possibility that regA protein may require longer RNAs to bind as a dimer, wild-type and R91L regA proteins were preincubated with oligo(U)$_{24}$, which contains two potential binding sites [based on the poly(U) binding site size of 9 ± 1 nt] (4) and then reacted with glutaraldehyde (Fig. 5A and B, lane 5). Again, formation of crosslinked dimers as well as other oligomers was significantly reduced by addition of RNA.

DISCUSSION

The mechanism regA protein uses to distinguish between target mRNAs and other T4 mRNAs present in the phage-infected cell is largely unknown. One interesting feature of this regulatory system is that T4 mRNAs vary in their sensitivity to regA repression *in vitro* (1,17) and *in vivo* (16). In addition, RNase protection assays (2) indicate a range of binding site sizes (from 16 to 48 nt) in different mRNAs, suggesting variation in the number of regA proteins bound to different mRNAs.

The observation that regA protein exists as a dimer in 30 µM solutions and is present as a dimer in protein crystals suggests that regA may bind RNA as a dimer. If so, regA protein dimers could have two RNA binding sites, which could contribute to the large binding sites observed in some mRNAs. Alternatively, RNA may bind to a single site formed across the dimer interface, as is the case with the MS2 coat protein (17). A third possibility is that regA protein binds RNA as a monomer and that some T4 mRNAs contain multiple monomer binding sites. Distinguishing between these possibilities is critical for interpretation of RNA binding site studies and to understanding how regA protein distinguishes between mRNAs.

As a first step towards determining whether regA protein binds RNA as a monomer or dimer, the stoichiometry of regA protein–RNA complex formation was examined by gel mobility shift assays. These assays demonstrated the saturation of RNA binding at a 1:1 ratio of protein to 16mer RNA, consistent with the binding of a monomer to RNA or the binding of two RNAs per protein dimer (i.e. ruling out one RNA per dimer).

Gel filtration analysis of wild-type regA protein revealed that even in dilute solutions (1 µM) regA protein exists as a dimer, with a mass of ~29 kDa. However, addition of gene 44RE RNA to regA protein prior to chromatography shifted the elution of regA protein to a position corresponding to a mass of 20 kDa, consistent with the formation of a monomer–RNA complex. Thus, regA protein binds RNA as monomer, although in the absence of RNA, protein–protein interactions lead to dimer formation. Interestingly, the R91L regA protein also exists in dilute solutions as a dimer. This observation suggests that the protein–protein interactions formed in solution may be different from the intersubunit bonds observed in regA protein crystals or that the symmetrical bonds formed between Thr92 (observed in the crystal dimer) contribute significant strength to maintain dimer formation in solution.

The conclusion that regA protein binds RNA as a monomer is supported by the glutaraldehyde crosslinking studies reported here. In the absence of RNA, glutaraldehyde treatment of regA

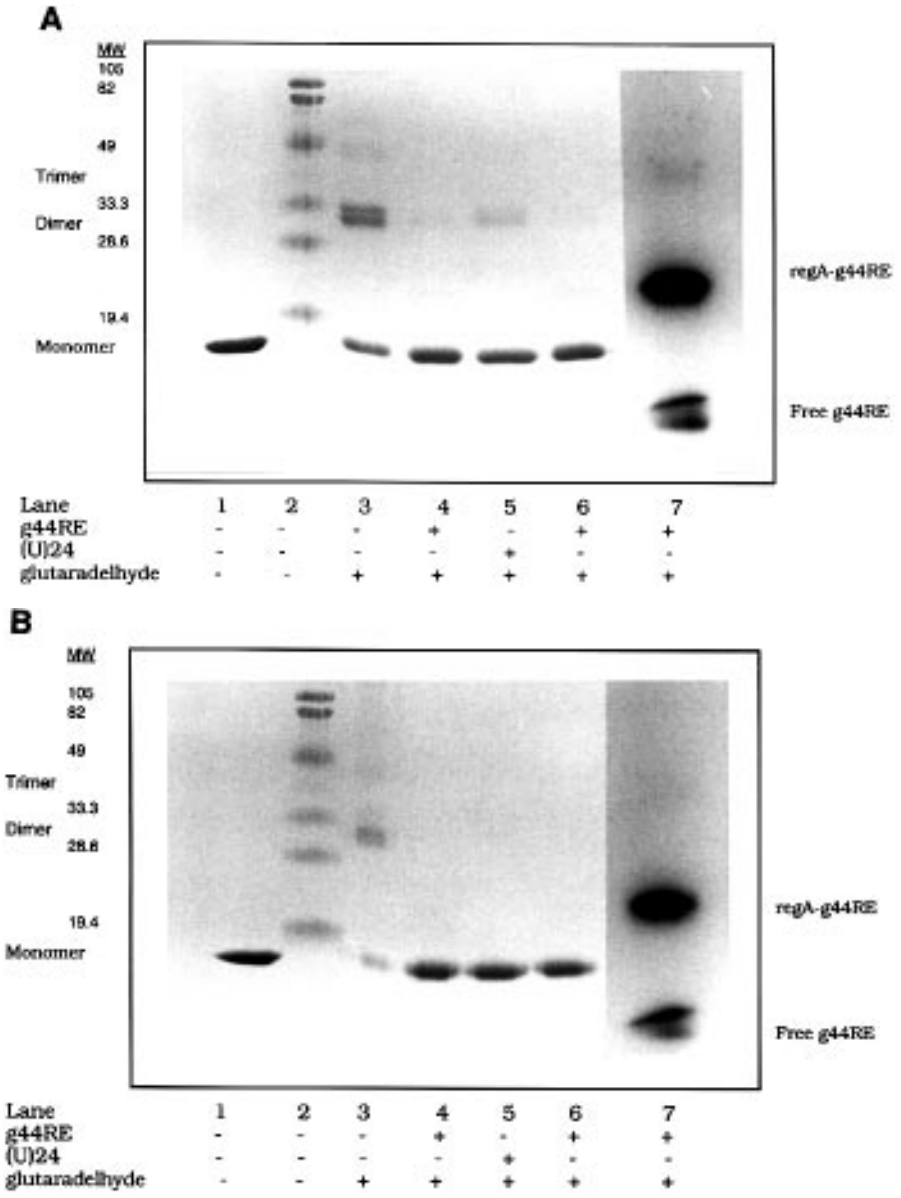

Figure 5. Glutaraldehyde crosslinking of regA protein. (**A**) Crosslinking of wild-type regA protein (11.4 µM). Lane 1, wild-type regA, untreated; lane 2, molecular weight standards; lanes 3–7, samples treated with 0.1% glutaraldehyde; lane 3, wild-type regA protein; lane 4, wild-type regA + gene 44RE RNA (22.8µM); lane 5, wild-type regA + oligo(U)$_{24}$ RNA (22.8 µM); lane 6, wild-type regA + [^{32}P]gene 44RE RNA (22.8 µM); lane 7, autoradiograph of lane 6 (note that the majority of unbound RNA ran off the gel). (**B**) Crosslinking of R91L regA protein (12 µM). Lane 1, R91L regA, untreated; lane 2, molecular weight standards; lanes 3–7, samples treated with 0.1% glutaraldehyde; lane 3, R91L regA protein; lane 4, R91L regA + gene 44RE RNA (24µM); lane 5, R91L regA + oligo(U)$_{24}$ RNA (24 µM); lane 6, R91L regA + [^{32}P]gene 44RE RNA (24 µM); lane 7, autoradiograph of lane 6 (note that the majority of unbound RNA ran off the gel). Wild-type and R91L proteins were preincubated in the presence or absence of RNA in 10 mM sodium phosphate, pH 7.5, on ice for 15 min. Samples were then reacted with 0.1% glutaraldehyde for 30 min at 25°C. Samples were diluted with Laemmli buffer, boiled and run on an SDS–15% polyacrylamide gel.

protein efficiently produces SDS-resistant dimer, trimer and higher oligomers. However, in the presence of RNA, protein–protein crosslinking is essentially eliminated. Thus, protein–protein interactions are inhibited by RNA binding. This inhibition could arise from a conformational change in regA protein that buries residues that form intersubunit bonds or from a direct shielding by RNA of residues that otherwise form protein–protein interactions. The finding that R91L regA protein has a reduced affinity for RNA suggests that the site of RNA binding may overlap with residues that form the protein–protein interactions. This hypothesis is consistent with the observation that RNA binding protects the C-terminal region of regA protein, including the peptide bond between Phe93 and Met94, from proteolytic cleavage (18). Current efforts to obtain the structure of regA protein–RNA co-crystals (C.-H. Kang, personal communication) should help to localize the site of RNA binding and the relationship between the sites of protein–protein and protein–RNA interactions.

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REFERENCES

1 Winter,R.B., Morrissey,L., Gauss,P., Gold,L., Hsu,T. and Karam,J. (1987) *Proc. Natl. Acad. Sci. USA*, **84**,7822–7826.
2 Unnithan,S., Green,L., Morrissey,L., Binkley,J., Singer,B., Karam,J. and Gold,L. (1990) *Nucleic Acids Res*., **18**, 7083–7092.
3 Webster,K.R. and Spicer,E.K. (1990) *J. Biol. Chem*., **265**, 19007–19014.
4 Karam,J., Gold,L., Singer,B.S. and Dawson,M. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 4669–4673.
5 Kang,C.-H., Chan,R., Berger,I., Lockshin,C., Green,L., Gold,L. and Rich,A. (1995) *Science*, **268**, 1170–1173.
6 Miller,E.S., Winter,R.B., Campbell,K.M., Power,S.D. and Gold,L. (1985) *J. Biol. Chem*., **260**, 13053–13059.
7 Adari,H.Y. and Spicer,E.K. (1986) *Proteins: Structure, Function, Genet.*, **1**, 116–124.
8 Webster,K.R., Adari,H.Y. and Spicer,E.K. (1989) *Nucleic Acids Res*., **23**, 10047–10068.
9 Webster,K.W., Shamoo,Y., Konigsberg,W.H. and Spicer,E.K. (1991) *Biotechniques*, **11**, 658–661.
10 Sedmak,J.J. and Grossberg,S.E. (1977) *Anal. Biochem*., **79**, 544–552.
11 Bradford,M. (1976) *Anal. Biochem*., **72**, 248.
12 Kim,D., Lee,Y.J. and Corry,P.M. (1992) *J. Cell Physiol*., **153**, 353–361.
13 Laemmli,U.K. (1970) *Nature*, **277**, 680–685.
14 Fried,M. and Crothers,D.M. (1981) *Nucleic Acids Res*., **9**, 6505–6525.
15 Johnston,R.F., Pickett,S.C. and Barker,D.L. (1990) *Electrophoresis*, **11**, 355–360.
16 Miller,E.S., Karam,J., Dawson,M., Trojanowska,M., Gauss,P. and Gold,L. (1987) *J. Mol. Biol*., **194**, 397–410.
17 Peabody,D.S. (1993) *EMBO J*., **12**, 595–600.
18 O'Malley,S.M., Sattar,A.K.M., Williams,K.R. and Spicer,E.K. (1995) *J. Biol. Chem*., **270**, 5107–5114.