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FOR THE RECORD

Crystallization of the MS2 translational repressor alone and complexed to bromouridine

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Abstract: The coat protein from the MS2 bacteriophage plays a dual role by encapsidating viral RNA and also by binding RNA as a translational repressor. In order to study the isolated dimer in a conformation not influenced by capsid interactions, a mutant molecule was crystallized that is defective in capsid assembly but is an active repressor. The unassembled dimer crystallized in the space group $P2_12_12$ with $a = 76.2$, $b = 55.7$, and $c = 28.4$ Å. In these crystals, monomers were related by twofold symmetry. When this dimer was co-crystallized with 5-bromouridine, crystals formed in space group $R3$ with $a = b = 155.9$ Å, $c = 29.9$ Å, $\gamma = 120°$; the dimer was the asymmetric unit.

Keywords: crystallization; RNA bacteriophage; RNA hairpin; translational repressor

RNA bacteriophages are spherical viruses that infect *Escherichia coli* and the simple single-stranded genomic RNA serves as mRNA for viral replication. In these viruses, coat protein binds and encapsidates the viral RNA but also acts as a translational repressor by binding to the RNA operator of the replicase gene and blocking synthesis of viral replicase (Romaniuk et al., 1987). The operator is located in an RNA hairpin.

Because of its dual function, the coat protein from the MS2 bacteriophage is a very interesting candidate for the study of protein–RNA and protein–protein interactions. This small protein (M_r = 13,700) is composed of 129 amino acids and self-aggregates to form an icosahedral shell (180 subunits) that binds and encapsidates a single-stranded RNA genome of 3,569 nucleotides. The RNA genome also acts as mRNA and encodes a maturation or A protein, a coat protein protomer, a replicase subunit, and a lysis protein. In addition to forming the shell of the viral particle, coat protein plays a genetic regulatory role. Late during the course of infection, the coat protein binds to the translation initiation region of the replicase cistron and prevents ribosomes from initiating translation there. Interactions of coat protein with the RNA "binding site" for translational repression are well characterized. It has been shown that the active repressor is a dimer and that one RNA operator molecule is bound by a repressor dimer at saturation (Beckett & Uhlenbeck, 1988). Experiments with synthetic RNA fragments revealed the necessity for intact RNA secondary structure (i.e., a 21-nucleotide hairpin) and the requirement of four essential nucleotides, including a bulged base, for binding (Uhlenbeck et al., 1987). This protein–RNA interaction is an important first event in nucleation of virus assembly.

The crystal structure of the intact MS2 virus has been determined (Valegård et al., 1990; Golmohammadi et al., 1993). Because coat protein dimers are the "building blocks" for virus assembly and the active repressor is a dimer, we planned to crystallize the MS2 coat protein as an unassembled dimer. When compared to viral capsid, the structure of this unassembled dimer can be used to address questions of virus assembly. The structure of the isolated dimer can also be compared to the recently determined structure of recombinant MS2 capsids complexed to 19-nucleotide RNA fragments (Valegård et al., 1994). Because binding RNA is a first step in the assembly process, it is important to examine the residues critical for RNA binding in the active repressor, i.e., in a conformation that is not influenced by capsid interactions.

In initial efforts to isolate coat protein dimers, MS2 phage was propagated by a modification of the procedure of Kolakofsky (1971). Cultures of *E. coli* strain K71-18 or K38 were infected

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with phage and incubated in the presence of 2 mM $CaCl_2$ for 3–4 h at 37 °C. Cells were lysed by the addition of lysozyme (30 μM) and deoxyribonuclease (3 nM), and centrifuged at 4,000 rpm. Phage were then precipitated with $(NH_4)_2SO_4$, resuspended in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, and purified by ultracentrifugation through a cesium chloride gradient at 40,000 rpm and 4 °C for 20 h. The isolated phage were further purified using a procedure developed for crystallization of the intact virus (Valegård et al., 1986). Following centrifugation, the phage isolate was dialyzed to remove cesium chloride against 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM $MgSO_4$, 0.1 mM EDTA, and 0.02% sodium azide. The sample was then subjected to molecular sizing chromatography in the same buffer using a 2.5 × 80-cm Sepharose CL-4B column, operated at 4 °C with a flow rate of 20 mL/h. The pooled fractions corresponding to purified phage were precipitated from a solution containing polyethyleneglycol (PEG) 8000 and 0.5 M CaCl. The pellet was recovered by centrifugation at 10,000 rpm at 4 °C, resuspended, and dialyzed at pH 7.0 and 4 °C versus 15 mM sodium citrate–15 mM NaCl. In typical experiments, from 6 L of culture for infection, 100–150 mg purified phage were isolated and stored at 4 °C.

To isolate coat protein, strong acid solutions can be used to disrupt the phage particle (Fraenkel-Conrat, 1957). To ice-cold purified phage stock, nearly equal volumes of cooled glacial acetic acid were added with occasional mixing. After centrifugation, the supernatant was dialyzed against 10 mM acetic acid at 28 °C and finally stored at 19 °C. Typical yields of isolated coat protein were 40–45 mg. All subsequent steps toward crystallization were performed at 19 °C to prevent the formation of higher order aggregates. Gel filtration sizing experiments performed with coat protein solutions in dilute acetic acid indicated that the molecule was present as dimers, even at concentrations (4 mg/mL) used for crystallization.

Crystallization trials were set up with these solutions with care to minimize the storage time between chromatographic elution and crystallization. Small crystals were obtained from these acidic solutions by vapor diffusion at 19 °C with PEG 3350 or PEG 8000 as precipitant. The biggest crystals obtained were only 0.1 mm in the longest dimension. Larger crystals could not be produced, even after numerous attempts to modify the crystallization conditions, probably because the molecular species in mother liquor solutions bathing the crystals shifted to capsids or higher molecular weight complexes with time even in dilute acetic acid. When the protein was co-crystallized with small oligonucleotides, some improvement in crystal morphology was achieved. The best results with these trials were obtained when the protein was crystallized from solutions containing 13% PEG 3350 in the presence of 0.33 mM cytidylyl-adenosine. However, these crystals also did not grow to a large enough size for diffraction experiments.

In order to facilitate crystallization of the MS2 coat protein as dimers rather than capsids, we next tested mutant proteins described previously (Peabody & Ely, 1992) that are defective in viral assembly. These mutants were isolated using a two-plasmid genetic system (Peabody, 1990) that mimics the translational control of replicase synthesis that occurs during MS2 infection. Because the recombinant coat protein assembles into virus-like particles within the bacterium after expression, it is also possible to use this system to test mutants for defects in viral assembly. Mutant proteins that formed dimers but did not assemble into capsids were ideal candidates for crystallization of the isolated dimer. These mutations were originally identified because the failure to assemble causes an elevation in dimer concentration and increased repressor activity. Because these dimers retain the capacity to bind the RNA operator, they are suitable models for the functional dimeric repressor.

Mutant proteins were expressed in *E. coli* strain CSH41F$^-$ under control of the *lac* promoter. Bacterial cells were lysed by sonication (10–20-s bursts) on ice in 50 mM Tris, pH 8.5, 100 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol, and 0.5 mM phenylmethylsulfonylfluoride. DNA was precipitated from the lysate with polyethyleneimine (0.2%) and the pellet was cleared by centrifugation at 11,000 rpm and 4 °C. The soluble fraction was dialyzed against 20 mM Tris-HCl, pH 8.5, at 4 °C and applied to a DEAE-Sepharose Fast-Flow resin (Pharmacia, Inc.) in a column bed appropriate for the volume of the lysate. MS2 mutant proteins, with overall basic charge patterns, washed through the column. Flow-through eluates were dialyzed against 20 mM sodium phosphate, pH 5.8, and applied to a 1.8 × 11-cm column of S-Sepharose Fast-Flow (Pharmacia, Inc.). This ion-exchange chromatography was developed with a linear NaCl gradient from 0 to 0.7 M. The mutant coat protein reported here eluted at 0.2 M NaCl. Pooled fractions were dialyzed against dilute buffers for crystallization trials as described in the following sections. Protein samples were concentrated to approximately 3 mg/mL with Centriprep filtration concentrators (Amicon Corp.).

A mutant protein with a single amino acid interchange at residue 82 (i.e., arginine substituted for tryptophan) crystallized readily. In some cases, crystals were observed at 13 °C during storage after concentration in 5 mM Tris-HCl, pH 7.2, 50 mM NaCl, 7 mM β-mercaptoethanol. Surprisingly, crystals even formed in the dialysis sac during dialysis at 4 °C versus 5 mM Tris-HCl, 50 mM NaCl prior to crystallization. Crystallization conditions were refined and the best crystals were obtained in hanging drops (3 μL) by vapor diffusion at 4 °C from solutions containing 100 mM citrate-phosphate buffer, pH 6.2, and 10% PEG 8000. The use of additives (4% methanol and 10 mM dithiothreitol) was implemented to ensure production of single large crystals. Crystals formed in the orthorhombic space group $P2_12_12$ with $a = 76.2$, $b = 55.7$, and $c = 28.4$ Å, and they diffract to 2.0 Å resolution. Calculations of unit cell volume were consistent with one monomer in the asymmetric unit with V_m of 2.2 Å^3/Da (Matthews, 1968) and solvent content of 44%.

Diffraction data were collected at 4 °C with two San Diego Multiwire Systems multiwire area detectors and a Rigaku RU-200 rotating anode X-ray generator with a graphite monochromator operating at 50 kV and 100 mA. A summary of the data collection statistics is presented in Table 1. Data reduction was done with UCSD area detector data processing programs (Howard et al., 1985). The quality of the data obtained at 4 °C was superior to that collected at room temperature, as expected, because the crystals were grown at 4 °C. These data were used to solve the structure of the unassembled MS2 coat protein dimer (Ni et al., 1995).

When the SU647 mutant protein was used for binding and co-crystallization with mononucleotides, a new crystal form was observed. In order to begin to examine the structural features of the RNA binding site, MS2 SU647 was co-crystallized with bromouridine. In previous studies, this mononucleotide was shown to bind to the closely related R17 repressor (Gott et al.,

Table 1. *Summary of data collection statistics for the SU647 mutant MS2 coat protein*

Minimum resolution (Å)	Average intensity (I)	Average $I/\sigma(I)$	No. of observations	No. of reflections	R_{sym}[a]
3.45	3,348	59.8	9,166	1,635	0.025
2.76	1,207	31.1	6,667	1,648	0.038
2.41	486	12.1	3,359	1,583	0.066
2.19	336	8.0	3,003	1,548	0.093
2.03	245	5.7	1,740	1,033	0.107
Totals	1,210	25.1	23,935	7,447	0.031

[a] $R_{sym} = \sum_h \sum_i |I_{hi} - \bar{I}_h| / \sum \bar{I}_h$.

Table 2. *Summary of data collection statistics for the SU647 mutant MS2 protein complexed to bromouridine*

Minimum resolution (Å)	Average intensity (I)	Average $I/\sigma(I)$	No. of observations	No. of reflections	R_{sym}[a]
4.10	2,919	55.0	7,977	2,062	0.024
3.26	1,334	26.9	4,715	2,060	0.032
2.85	452	12.3	3,608	2,015	0.063
2.59	216	6.3	2,874	1,910	0.113
2.40	140	4.1	2,359	1,739	0.142
Totals	1,056	21.8	21,533	9,786	0.028

[a] $R_{sym} = \sum_h \sum_i |I_{hi} - \bar{I}_h| / \sum \bar{I}_h$.

1991), and the binding of bromouridine inhibited binding of the RNA operator sequence. The mutant protein crystallized in the presence of 20 mM 5-bromouridine at pH 7.2 and 4 °C by vapor diffusion in hanging drops containing 5 mM Tris-HCl, 50 mM NaCl, 4% PEG 8000, 2% glycerol, and 4% methanol. Under the same conditions, the mutant protein alone crystallized with a completely different morphology. In the presence of mononucleotide, crystals formed in the space group $R3$ with $a = b = 155.9$ Å, $c = 29.9$ Å, and $\gamma = 120°$ or, in some cases, in space group $P3_1$ (or $P3_2$). The latter crystals only diffracted to 2.7 Å resolution at the LURE synchrotron source in Orsay, France. Because the $R3$ crystals diffracted to 2.4 Å with a conventional rotating anode X-ray source, they were selected for structure solution.

Diffraction data from the $R3$ crystals were collected to 2.4 Å resolution at 4 °C using the same strategy described above for the protein alone. The data collection statistics are summarized in Table 2. Volume calculations for the $R3$ crystals indicated a solvent content of 52%, $V_m = 2.6$, and a dimer as the asymmetric unit. It is interesting that, in the presence of mononucleotide, the subunits in the dimer are no longer related by an exact crystallographic twofold axis. These results suggest that (1) the brominated-mononucleotide binds to only one monomer, or (2) the protein subunits have conformational differences. It is also possible that both of these events have occurred resulting in loss of crystallographic symmetry. To evaluate the differences, the structure of the dimer complexed to bromouridine is now being determined by molecular replacement methods using the coordinates of the uncomplexed dimer as probe model.

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