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Localization of Coliphage MS2 A-Protein

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The purification of coliphage MS2 dinitrophenol (DNP) conjugates provided a system for localization of the single molecule of A-protein in the capsid of the MS2 phage particle. Three A-protein preparations isolated from unconjugated MS2, overconjugated DNP-MS2, and purified 78*S* DNP-MS2 were tested for the presence of covalently bound DNP. The binding characteristics to Dowex 1-X8 and rabbit anti-DNP bovine serum albumin (DNP-BSA) immunoglobulin G of the 78*S* DNP-MS2 and overconjugated DNP-MS2 A-protein preparations indicate that the A-protein is located on the surface of the phage particle where it can be covalently conjugated with hapten. Extensive enzymatic iodination of the A-protein of intact unconjugated MS2 substantiates this conclusion.

The A-protein, or maturation protein of the icosahedral RNA bacteriophages, has been shown to play an important role during infection and is a necessary constituent in the assembly process of the virus (21). However, the exact function of the A-protein is not known, nor has its location in the physical arrangement of the intact phage been determined. The "core" model predicts that the A-protein is located on the interior of the phage particle and functions during folding of the RNA molecule (9). The "tail" model predicts that the A-protein is located on the surface of the phage particle where it facilitates adsorption to the bacterial pilus and the injection of viral RNA (2, 28). O'Callaghan has postulated its location on the vertex of the icosahedron (17). These models have been difficult to test because of the nature of the A-protein, namely its presence in very small amounts, one copy per phage particle (26), extreme insolubility requiring the presence of denaturing solvents, and its stickiness to glass and dialysis membranes making handling procedures difficult (28).

The preparation and purification of coliphage MS2 conjugated with dinitrophenol (DNP) has provided a new and relatively simple system to determine the location of the A-protein on the MS2 particle (4). Using CsCl density centrifugation, intact MS2 particles conjugated with DNP (DNP-MS2) can be obtained. These conjugates have the same relative infectivity, sedimentation rate, density, and physical stability as the unconjugated phage particle (4). In this report, we describe the isolation of the A-protein from these DNP-MS2-conjugated particles and show that it has covalently attached DNP, lending support to the model which places the A-protein on the outer surface of the phage particle (2, 17, 28). This location is confirmed by extensive lactoperoxidase iodination of the A-protein of intact unconjugated MS2 phage particles.

MATERIALS AND METHODS

Preparation of phage. MS2 phage was prepared as described previously (4). MS2 phage labeled with [^{3}H]histidine was prepared according to the method of Steitz (26). *Escherichia coli* AB261 was grown in 100 ml of an amino acids-salts medium lacking histidine to 2×10^8 bacteria/ml, phage at a multiplicity of 20, and 1 μCi of [^{3}H]histidine per ml was added. The culture was then incubated for 4 h, and the resulting lysate was purified as described previously (4). [^{14}C]amino acid-labeled MS2 phage was prepared according to the method of Vinuela (29). *E. coli* AB261 was grown in 100 ml of MPTA media at 37 C to 2×10^8 bacteria/ml, phage at a multiplicity of infection of 20, and 25 μCi each of ^{14}C-labeled valine, isoleucine, leucine, and alanine was added per ml. After 4 h of incubation, the resulting lysate was purified as described previously (4).

Preparation of conjugated phage. DNP-MS2 was prepared as previously described (4). The extent of conjugation of DNP-MS2 banded on CsCl gradients and unbanded phage was determined by adsorbance at 358 nm (7) after exhaustive dialysis against buffer B (0.1 M NaCl, 0.01 M Tris, pH 7.6, at 4 C).

^{125}I-labeled MS2. Pure intact MS2 was iodinated by using lactoperoxidase (purity $A_{412}/A_{280} > 0.875$; Affinity Systems, Inc.). To 0.1 ml of MS2 (500 μg) in buffer A (0.1 M NaCl, 0.01 M EDTA, and 0.05 M Tris, pH 7.6) were added 5 μg of lactoperoxidase, 2 nmol of NaI, and 100 μCi of carrier-free Na^{125}I (Amersham Searle). Hydrogen peroxide (10 μmol) was added to initiate the reaction, and an additional 10 μmol was added at 5 min. The reaction was continued for

another 5 min at room temperature. Self-iodination of lactoperoxidase was done exactly as described above with the elimination of MS2 phage from the reaction mixture. Unreacted iodine and lactoperoxidase were removed by three exchanges of buffer A in an Amicon Minicon S-125 prerinsed with bovine serum albumin (BSA) (1 mg/ml) and dialysis overnight at 4 C against buffer A.

Polyacrylamide gel electrophoresis of phage proteins. Phage proteins were prepared as described by Steitz (26). One volume of phage in 0.1 M Tris-0.1 M NaCl (pH 7.6) was added to 3 volumes of 8.0 M guanidine-hydrochloride, and 0.1 M Tris-hydrochloride (pH 8.5) containing 1% mercaptoethanol. The mixture was incubated for 2 h at 45 C and then dialyzed into one change of Tris-glycine buffer and three changes of Tris-glycine buffer containing 1% mercaptoethanol and 0.1% sodium dodecyl sulfate (SDS) at room temperature. A precipitate formed in the dialysis tubing which could be solubilized by adding 1 μg of boiled RNAse per ml and incubating at 37 C for 1 h. The dissociated phage proteins were subjected to electrophoresis on 15% acrylamide gels in Tris-glycine buffer (pH 9.5) containing 0.1% SDS and 1% mercaptoethanol (5). Ten micrograms of sample were loaded per gel, and electrophoresis was carried out for 70 min at 3 mA/gel. The gels were frozen on dry ice and sliced into 1-mm slices with a Mickle gel slicer. ^{14}C and ^{3}H gels were digested overnight at 70 C in 3% H_2O_2, placed in toluene containing 10% TLA (Beckman) and 10% BBS-3 (Beckman) and counted in a Beckman LS-355 liquid scintillation counter to 2% error. ^{125}I radioactive gel slices were placed in glass tubes and counted for 1 min with background subtracted in a Nuclear Chicago auto-gamma counter. Non-radioactive gels were also frozen on dry ice and sliced into 1-mm slices. The A-protein fractions were eluted from the slices with three changes of Tris-glycine buffer containing 0.1% SDS at room temperature for 24 h.

Dowex binding assay. [^{3}H]histidine-labeled phage proteins were dissociated in the presence of guanidine as described. The protein was concentrated and exchanged three times in an Amicon Minicon A-25 with Tris-glycine containing 1% mercaptoethanol and 0.1% SDS (pH 8.5). Approximately 500 counts/min of each protein preparation as well as [^{3}H]DNP-lysine (3.22 Ci/mmol; New England Nuclear) were added separately to an excess of Dowex 1-X8 anion exchange resin (BioRad) equilibrated in the same buffer, incubated 1 h at 37 C with mixing, and centrifuged 500 $\times$ *g* at room temperature to sediment the Dowex. Then 0.1-ml samples of the supernatant were placed in toluene containing 10% TLA and 10% BBS-3, and counted in a Beckman LS-355 liquid scintillation counter using automatic quench calibration to 1% error.

Immunoassay. [^{3}H]histidine-labeled phage proteins were prepared as described for the Dowex binding assay, and all other reagents were diluted in Tris-glycine buffer. The various labeled antigen preparations (0.01 ml) containing approximately 500 counts/min and 0.01 ml of rabbit immunoglobulin G (IgG) anti-DNP-BSA (2.85 mg/ml) were mixed in glass tubes and incubated overnight at room temperature. Then, 0.01 ml of normal rabbit serum (diluted 1:10) was added, and the rabbit IgG was optimally precipitated with 0.01 ml of goat anti-rabbit IgG (Microbiological Associates, Inc.). After 24 h of incubation at room temperature, the mixture was vortexed, the precipitate was collected on membrane filters (0.45 μm pore size; HAWP, Millipore Corp.), and washed three times with Tris-glycine buffer. The dried precipitate was hydrolyzed with 0.25 ml of 0.2 N NaOH, neutralized with 3 drops of 5% acetic acid, and counted in toluene containing 10% TLA and 10% BBS-3, as previously described, using automatic quench calibration. Negative controls consisting of normal rabbit IgG (2.9 mg/ml) in place of specific antibody, and whole normal rabbit serum in place of precipitating antibody, were treated in a similar manner. [^{3}H]DNP-lysine was used as a positive antigen control. The binding efficiency of this SDS mercaptoethanol system was tested by comparing the binding of [^{3}H]DNP-lysine in the presence and absence of 0.1% SDS and 1% mercaptoethanol. The results reported for [^{3}H]DNP-lysine represent an 80% reduction in the overall efficiency of the system due to the presence of SDS and a reducing agent.

RESULTS

In our attempts to obtain a maximally conjugated DNP-MS2 phage particle, we found a limit to the number of DNP groups which could be covalently attached to an intact infectious phage particle (Curtiss et al. manuscript in preparation). As previously shown, conjugation of MS2 with DNP results in the generation of altered phage particles (4). Characterization of the conjugation reaction mixture in the model E analytical ultracentrifuge revealed a minimum of two peaks: one 78*S* peak identical to that of the unconjugated phage, and a slower more heterogeneous peak of 61*S*. Purified, unconjugated MS2 capsids sediment at 43*S* (22). The noninfectious, slower-sedimenting particles could be separated from the infectious MS2 by isopycnic CsCl density centrifugation (4). The intact 78*S* DNP-MS2 phage particles of density 1.46 g/ml contained 150 DNP groups per phage particle (0.8 DNP groups per coat protein monomer [15]) and were of the same percent infectivity as MS2. A number of simple tests performed on the unaltered 78*S* DNP-MS2 conjugate (DNP_{150}-MS2) to determine its stability relative to the unconjugated phage showed that it and MS2 were equally resistant to 60 C (5 min in phosphate-buffered saline) (pH 10.0), increased salt, RNase, 5% mercaptoethanol, and light, and equally sensitive to pH 3.0, water (37 C, 24 h), and 5.0 M urea (4). No differences have ever been detected between 78*S* DNP_{150}-MS2 and 78*S* MS2 with the exception of the absorbance at 358 nm (maximum adsorp-

tion of DNP-lysine attached to proteins [3]) and the immunologically detectable DNP (DNP_{150}-MS2 can be neutralized with anti-DNP-BSA IgG and MS2 cannot (Curtiss et al. manuscript in preparation). The altered particles with density of 1.44 g/ml or less ($s_{20,w}$ = 61) are noninfectious (4) and contain 80% less RNA and greater than two DNP groups per coat protein monomer (Curtiss et al. manuscript in preparation).

The lysine residues of the MS2 coat protein in monomer are distributed like those of other globular proteins on the surface, as well as in crevices and the interior of the molecule (6). Modification (conjugation) of the accessible lysine residues on the surface of the monomer is likely to cause only local conformational changes in the monomer. These local changes would result from changes in the bulk sizes and the states of ionization of the exposed side chains. In contrast, conjugation of the less accessible lysine residues which conceivably occurred during extreme conditions of DNP conjugation would be expected to cause profound overall conformational changes. These overall changes in the conformational integrity of the monomer would result from the increased bulk size of the internal side chains where proper folding of the polypeptide chain is prevented (13). Because the conformational features of the bacteriophage subunits determine the integrity of fit and maintain the particle as a thermodynamically stable, biologically active virus, it was not surprising to observe a maximum in the extent to which MS2 could be conjugated with DNP. An intact infectious DNP_{150}-MS2 phage particle can be prepared, whereas an intact particle containing greater than 150 DNP groups cannot. We believe this limit is directly related to the modification of only accessible residues of the 78S conjugated particle, whereas modification of the less accessible residues involved in subunit interaction or located in crevices and the interior of the particle were responsible for the generation of the biologically inactive 61S particle. Dinitro benzene sulfonic acid (DNBS), because of its small molecular weight, is capable of penetrating the phage particle and labeling all phage proteins whether located on the surface or in the interior of the particle. However, penetration of the phage by DNBS results in the generation of altered phage particles, and hence its use as a molecular probe to study viral structure is feasible if a means of separating infectious from noninfectious phage is available, as described previously (4).

The A-protein from 78S MS-2, 78S DNP_{150}-MS2, and the altered 61S DNP-MS2 particles was isolated. It was thought that the A-protein from the altered DNP particle should contain covalently attached DNP, whereas unconjugated MS2 A-protein would not. The presence or absence of DNP on the A-protein isolated from the intact 78S DNP-MS2 should indicate whether or not the A-protein is exposed on the surface of the MS2 particle.

Dissociation of phage particles with guanidine proved the most feasible method for A-protein isolation. The gel patterns of [^{14}C]amino acid and [^{3}H]histidine-labeled MS2 are shown in Fig. 1. Less than 1% of the total ^{14}C radioactiviy in the gel appears in fractions 30 to 40, whereas 70% of the [^{3}H]histidine radioactivity was found in these same fractions. Thus, the A-protein, which contains histidine and has an estimated molecular weight of 37,000 ± 10% (26) or 41,500 ± 3% (9), can be readily separated from the coat protein monomers which do not contain histidine (15) by electrophoresis on 15% acrylamide gels in the presence of SDS and mercaptoethanol. The presence of a reducing agent during electrophoresis is essential. The nature of the double peak from MS2 labeled with [^{3}H]histidine is unknown; however, it appears repeatedly in these labeled gels.

It was thought that the presence of DNP on the A-protein of MS2 would alter its combining

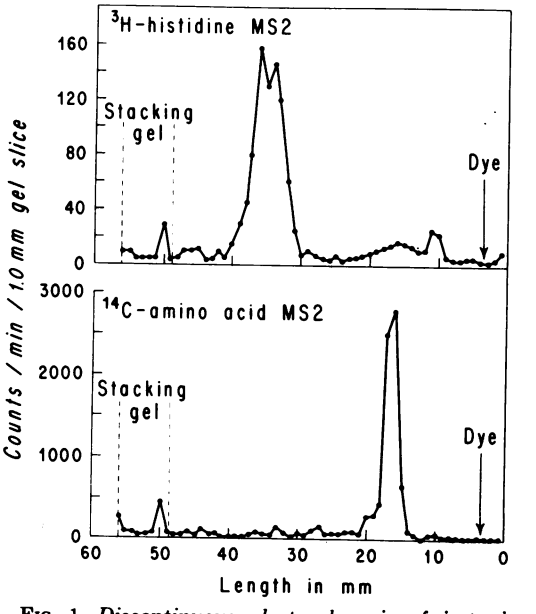

FIG. 1. *Discontinuous electrophoresis of isotopically labeled MS2-proteins on 15% acrylamide gels containing 0.1% SDS and 1% mercaptoethanol. The position of the running dye was noted and measured before H_2O_2 digestion.*

properties. Various binding assays were performed in an attempt to distinguish between preparation I ([^{3}H]histidine MS2) and preparation II (overconjugated [^{3}H]histidine DNP-MS2) and, ultimately, which of these preparation III ([^{3}H]histidine 78S DNP-MS2) more closely resembled. Isotopically labeled preparations II and III were prepared from two separate purified lysates of preparation I. Table 1 summarizes the results of labeled A-protein binding to Dowex. All of the A-protein preparations were bound quite effectively to Dowex; however, repeated experiments showed that preparation III had a significantly greater affinity than preparation I for the anion exchange resin. A statistical test of equality performed on this Dowex binding data, percent binding of preparation II equaling percent binding of preparation I ($P < 0.01$), and percent binding of preparation III equaling preparation I ($P < 0.01$), substantiates these differences.

The immunoassay proved to be more conclusive (Table 2). Although the overall efficiency of the system was greatly reduced, a sixfold increase in percent binding of preparation II relative to preparation I could be detected in this system. Preparation III displayed a 5.5-fold increase in percent binding over that of preparation I. In all experiments performed to distinguish the binding capacities of the various A-protein preparations, preparation III always resembeled preparation II and was significantly different from preparation I, indicating that the 78S DNP-MS2 A protein did contain covalently attached DNP.

To confirm the work done with the DNP conjugates of MS2, a separate method was sought of potentially labeling the A-protein of MS2 if indeed it were located on the surface rather than in the interior of the phage particle, in close association with RNA. Lactoperoxidase (molecular weight ~70,000) (23) has been shown to catalyze the iodination of only the surface proteins of red blood cells (19), influenza virus (25), and Sindbis virus (24). As a preliminary test, we used the classical method of dissociating MS2 using acetic acid. Osborn (18) showed that the treatment of intact phage with cold 66% acetic acid results in the coprecipitation of A-protein and RNA, but not the coat protein. One volume of intact [^{125}I]MS2 (2.45×10 counts/min) was added to 2 volumes of acetic acid and incubated 15 min at 4 C, and the precipitate was pelleted at $12,000 \times g$ for 30 min, washed, and counted. Sixteen percent (3.94×10^4 counts/min) of the total radioactivity was recovered in the A-protein RNA precipitate. This A-protein RNA radioactivity was subsequently shown to be RNase resistant and cold trichloroacetic acid precipitable (not shown). When the iodinated MS2 was disrupted and analyzed by electrophoresis on 15% polyacrylamide gels in the presence of 0.1% SDS and 1% mercaptoethanol, only two major peaks were seen in the separating gel (Fig. 2): the small-molecular-weight coat-protein monomers representing 51% of the total label, and the higher-molecular-weight A-protein. The peak seen in the stacking gel is thought to consist of aggregated coat protein. Surprisingly, the A-protein represented not something less than 2% of the total label as would be suspected, but 13%. It is thought that this difference must be related to the accessibility to enzymatic iodination of the 13 tyrosine residues on the A-protein (26) relative to the three tyrosine residues of the coat protein monomer (15). This data does correlate well with the immunoassay for DNP. In all cases, the 78S DNP-MS2 (preparation III) gave values approaching those of preparation II, indicating that the extent of DNP conjugation

TABLE 1. *Dowex binding of A-Proteins*[a]

Prepn	Mean ($n = 4$)	Standard deviation	99% Confidence interval
I ([^{3}H]A-protein)	70.15	0.7964	±2.324
II (DNP-[^{3}H]A-protein)	86.91	0.7106	±2.075
III (?[^{3}H]A-protein)	79.37	0.5050	±1.475
[^{3}H]DNP-lysine	95.78	0.8865	±2.589

[a] Dowex binding is expressed as mean percent of input bound of four trials done in duplicate. All preparations contain 2.8×10^6 counts per min per mg of MS2. The 99% confidence interval was calculated by using the *t*-distribution with 3 degrees of freedom.

TABLE 2. *Radioimmunoassay for DNP*[a]

Prepn	Mean ($n = 5$)	Standard deviation of the mean
I ([^{3}H]A-protein)	5.18[b] 5.63[c]	0.3182
II (DNP-[^{3}H]A-protein)	36.82[b] 31.50[c]	3.7614
III (?[^{3}H]A-protein)	29.80[b] 29.55[c]	0.1769
[^{3}H]DNP-lysine	43.00	

[a] The radioimmunoassay is expressed as mean percent of input specifically precipitated in five samples. All samples were assayed in the presence of 0.1% SDS and 1% mercaptoethanol as described.

[b] Specific activity, 2.8×10^6 counts per min per mg of MS2.

[c] Specific activity, 1.9×10^6 counts per min per mg of MS2.

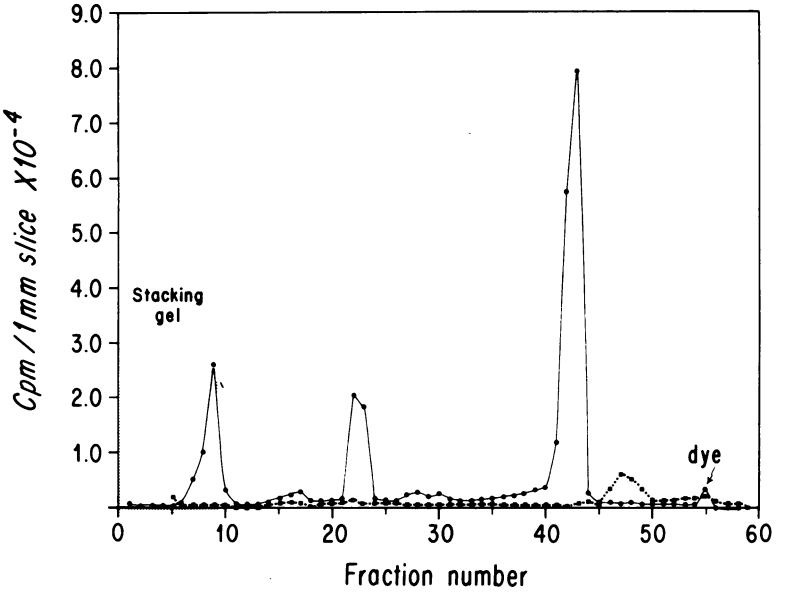

FIG. 2. *Discontinuous electrophoresis of lactoperoxidase ^{125}I-labeled MS2. [^{125}I]MS2 (——) was dissociated in guanidine and run on 5.5-cm 15% polyacrylamide gels containing 0.1% SDS in the presence of 1% mercaptoethanol. -----, Simultaneous electrophoresis of lactoperoxidase self-iodinated and purified under the same conditions.*

in these two preparations was of the same magnitude. Thus, it appears that a larger proportion of the tyrosine and lysine residues of the A-protein are accessible and exposed to the environment relative to those same residues of the coat protein monomer.

DISCUSSION

Purification of coliphage MS2 hapten conjugates has provided a system for determining that the A-protein of MS2 is located on the surface of the particle. Three A-protein preparations obtained from unconjugated MS2 (I), overconjugated DNP-MS2 (II), and purified 78*S* DNP-MS2 (III) have been used to show this location. The ability of Dowex and anti-DNP-BSA IgG to bind with greater affinity to A-protein with DNP attached than to A-protein containing no DNP even in the presence of SDS has provided a simple system to test for the presence of DNP on these purified 78*S* DNP-MS2 A-protein preparations. Thus, the A-protein appears to be exposed on the surface of the icosahedral particle to the extent that DNP can be covalently attached to the ϵ-amino group of some of the 16 lysine residues (26) in the A-protein, and an intact infectious particle recovered by rebanding in CsCl. Enzymatic iodination of the intact MS2 phage particle results in extensive A-protein labeling and confirms the work with the DNP-MS2 hapten conjugates.

This finding has new implications for various aspects of the study of these simple RNA bacteriophages including the self-assembly process, phage adsorption, and phage neutralization by specific antibody. Kaerner (11) has provided evidence that the A-protein is needed early during assembly for proper packaging and the production of infective particles. Early assembly complexes have been reported between the RNA molecule and the A-protein as well as coat protein (20). It is known that the A-protein functions in protecting the intact phage particle from nuclease digestion (8) and may in fact be attached to the 5'-terminus of the RNA, which extrudes from phage particles lacking the A-protein, and is injected first during infection of the host bacterium (16). All of these facts provide for the hypothesis of an assembly pathway that involves early interaction of the A-protein and the 5'-terminus of the RNA molecule with subsequent or concomitant folding of the RNA and the addition of coat protein. The particle would then be formed not around the A-protein but conceivably as postulated by O'Callaghan (17) in such a spatial arrangement that might involve using it as one of the twelve major vertices of the icosahedral capsid.

Phage particles which lack A-protein are unable to adsorb to the F pili of male cells (27).

Experiments discussed by Valentine (28) initially suggested that although A-protein was initially bound to the pilus, it did not enter the bacterium and was ultimately found free in the supernatant unattached to phage protein. Krahn et al. (14) subsequently showed that the A-protein was cleaved upon interaction with the pilus and the two smaller components transferred into the cell along with the phage RNA. The position of the A-protein on the surface of the particle therefore supports the notion that the A-protein is required for adsorption and that its presence facilitates a specific point of attachment and the correct orientation for release and transfer of viral RNA into the infected cell.

Neutralization of icosahedral bacteriophages by specific antibody has been shown to follow a first-order reaction in antibody excess (1), and this has been interpreted to imply a "single hit" model of neutralization. Hornick and Karush (10, 12), using hapten conjugated bacteriophage ϕX174 and antihapten antibody, concluded that there is one critical site per particle for neutralization. This has been further confirmed by Witte et al. (30), who presented direct evidence for a single critical site for neutralization of haptenated bacteriophage f2 by antihapten antibody. The presence of a single A-protein on the surface of these icosahedral viruses implies a region of asymmetry and in turn could be "the critical site" for viral neutralization. It is interesting to speculate on the ability of antibody generated against the purified A-protein to neutralize an intact phage particle.

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