JOURNAL OF VIROLOGY, Feb. 1977, p. 445-450
Vol. 21, No. 2

Printed in U.S.A.

Effect of Spermidine on the RNA-A Protein Complex Isolated from the RNA Bacteriophage MS2

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Received for publication 31 August 1976

The polyamine spermidine has recently been reported to be a substantial component of the RNA phage particle. Its effect on the isolated RNA-A protein complex of the phage MS2 is investigated here. This complex infects intact *Escherichia coli* cells via F-pili, as does the whole phage. It is shown that the infectivity of the complex on intact *E. coli* cells was enhanced by incubation with spermidine. Optimal stimulation (20-fold) of the complex infectivity was achieved by incubation with 3×10^{-4} M spermidine for 20 to 30 min at 37°C. This gave a more compact structure to the complex, as could be seen by its faster sedimentation in sucrose gradients. Although spermidine and Mg^{2+} are known to partially replace one another in several systems, no enhancement of the infectivity of the complex, but only its considerably faster sedimentation in sucrose gradients, occurred after incubation with 3×10^{-4} M Mg^{2+}. Only if the Mg^{2+} concentration was raised by more than one order of magnitude could increased infectivity of the complex be observed. At concentrations of spermidine and Mg^{2+} that maximally stimulated the infectivity of the complex on intact *E. coli* cells, no increase in infectivity of phenol-extracted RNA to *E. coli* spheroplasts was detected. From these in vitro results, the role of the polyamine spermidine in the RNA phage particle for the infecting RNA-A protein complex molecules in phage infection is discussed.

Viruses containing DNA and RNA have been reported to have large amounts of the polyamine spermidine (1-3, 7, 15). This polyamine has been shown recently also to be present in RNA phage R17 and in its isolated RNA (6). From these reports, it might be deduced that spermidine is also associated with the RNA of the RNA-A protein complex that is present in viable phage (10) and is injected into the host cell during RNA-virus infection (8, 9).

As we have demonstrated recently, the RNA-A protein complex can be isolated from purified RNA phage by treatment with 66% acetic acid (10). When this procedure was done, the phage was dissociated, displacing the coat protein into the supernatant and leading to a precipitate of RNA and A protein (13). After this acetic acid precipitate was dissolved in an aqueous buffer, infectivity toward whole bacterial cells was observed. This infectivity has been shown to be due to an RNA-A protein complex with a molecular ratio of 1:1 (10, 11).

In this paper, I studied whether the spermidine molecules in the virus may fulfill a biological function during infection of host cells with the RNA-A protein complex. These studies were carried out with the isolated RNA-A protein complex from the phage MS2, which is able to infect intact *Escherichia coli* cells via F-pili as does the whole original phage (10, 14).

MATERIALS AND METHODS

E. coli K-12 AB301 (Hfr λ^+ *met*$^-$ RNase I$^-$) was obtained from R. Gesteland. Phage MS2 was a gift from W. Fiers. Unlabeled and ^{32}P- and ^{35}S-labeled phage were grown as previously described (10). Buffer A contained 0.01 M Tris, 0.001 M EDTA, and 1% mercaptoethanol, pH 7.4.

Preparation of acetic acid pellets. Acetic acid pellets were prepared essentially as described previously (10, 11), except that the phage concentration was lowered to 0.4 mg/ml for acetic acid precipitation. The small amounts of coat protein detected previously in the redissolved aceti c acid pellet when higher phage concentrations were used were not observed at this lower phage concentration. This can be shown by polyacrylamide gel electrophoresis of ^{35}S-labeled proteins from the acetic acid pellet (unpublished data), suggesting that all the coat protein is displaced into the supernatant if the phage concentration is sufficiently low during acetic acid treatment.

Isolation of RNA. Isolation of RNA with phenol from the virus or the acetic acid pellet was done as described (10).

Test for infectivity of the complex on intact *E.*

coli AB301 cells. The tests were done as described previously except that the cells were washed two times with antibiotic medium 3 (Difco) (11).

Test for infectivity of RNA on *E. coli* AB301 spheroplasts. The tests were done essentially as described by Delius and Hofschneider (5). The competence of the spheroplasts was about 10^{-6}.

RESULTS

Effect of spermidine on the infectivity of the RNA-A protein complex. To study the effect of spermidine on the infectivity of the RNA-A protein complex, I used a solution of an unlabeled acetic acid pellet in buffer A, which was supplemented with appropriate concentrations of spermidine as described in the legend to Fig. 1. In the first experiment, the infectivity in the solution was measured after incubation of six aliquots for 10 min in 0, 10^{-4}, and 5×10^{-4} M spermidine at 0 and 37°C as described in Materials and Methods. In each of the three aliquots, incubated at 0°C, the same number of plaques was counted, suggesting that the infectivity of the complex is not affected by spermidine under these conditions. However, in the aliquots that were incubated at 37°C, considerably increased infectivity in the spermidine-containing samples compared with the control in buffer A without spermidine occurred. Thus, after 10 min at 37°C, the infectivity of the complex was enhanced 4- and 10-fold over that of the control solution by 10^{-4} and 5×10^{-4} M spermidine, respectively.

The kinetics of infectivity of the complex were then measured as a function of molarity of spermidine in the buffer at 37°C. The infectivity was increased continuously from 10^{-4} to 3×10^{-4} M spermidine, leading maximally to a 20-fold enhancement at the latter concentration after 20 to 30 min of incubation (Fig. 1). At higher spermidine concentrations the infectivity of the complex was stimulated to a lesser extent and was finally reduced below the infectivity of the control without spermidine, the infectivity of which was not significantly changed during the incubation time.

Since it is well known that in some functions an apparent interchangeability of spermidine and Mg^{2+} ions exists (4), I now asked whether the stimulatory effect of spermidine on the infectivity of the RNA-A protein complex could be replaced by Mg^{2+}. For this purpose, I studied the kinetics of infectivity of the complex as a function of different concentrations of Mg^{2+} ions after incubation at 37°C. At 10^{-4} and 3×10^{-4} M Mg^{2+}, the infectivity of the complex was similar to that of the control solution (Fig. 2). At 10^{-3} M Mg^{2+}, the infectivity of the complex was raised only slightly (twofold) above that of the control, but increased continuously as the Mg^{2+} concentration was increased from 2×10^{-3} to 5×10^{-3} M; it reached the maximum of 20-fold within 20 min of incubation at 37°C at the latter concentration. At higher concentrations, enhancement of the complex infectivity was reduced.

Taken together, the results show that the infectivity of the complex can be increased 20-fold both by spermidine and by Mg^{2+}. However, this enhancement of infectivity by Mg^{2+} can only be achieved by a concentration that is higher by more than one order of magnitude

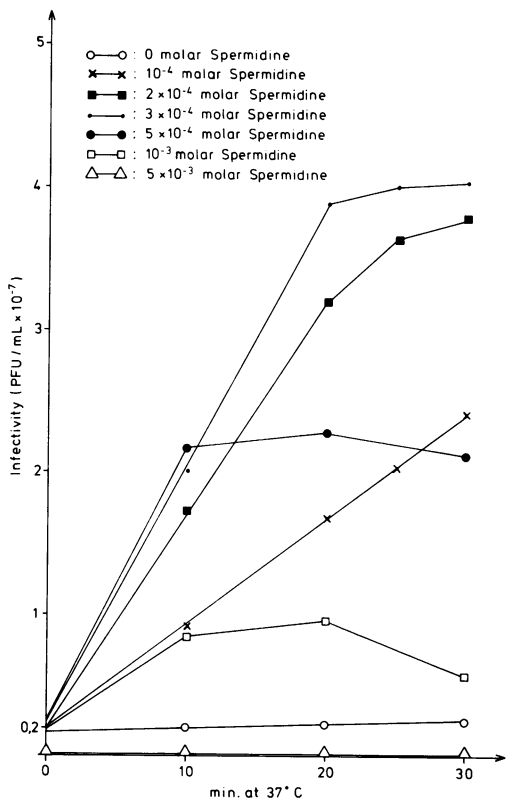

FIG. 1. *Kinetics of infectivity of the RNA-A protein complex during incubation with spermidine at 37°C. An acetic acid pellet, containing the RNA-A protein complex, was prepared from 2×10^{12} infections phage MS2 (6×10^{13} physical particles) as described in Materials and Methods. The pellet was dissolved in 1 ml of buffer A for 5 min at 37°C. Aliquots of 45 μl were taken and made to 1, 2, 3, 5, 10, and 50 $\times 10^{-4}$ M spermidine by addition of 5 μl of buffer A containing different appropriate concentrations of spermidine. To the control solution, 5 μl of buffer A was added. Incubation was performed at 37°C in polypropylene tubes. At the times indicated, 5-μl aliquots were taken, diluted in the same ice-cold buffer in which incubation was done, and tested for infectivity on intact E. coli cells as described in Materials and Methods.*

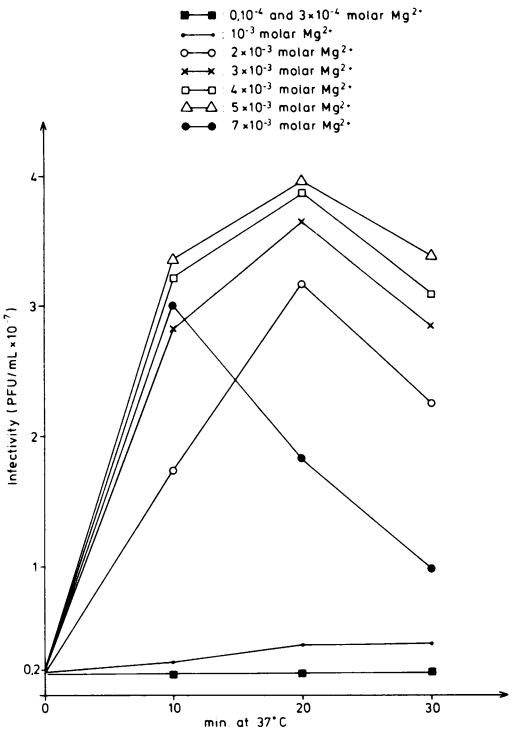

FIG. 2. *Kinetics of infectivity of the RNA-A protein complex during incubation with Mg^{2+} at 37°C. The sample preparation and the tests were as described in Fig. 1. Aliquots of 45 μl were made to 1, 3, 10, 20, 30, 40, 50, and 70 × 10^{-4} M magnesium chloride in excess of the EDTA concentration by addition of 5 μl of buffer A with different appropriate concentrations of magnesium chloride and incubated at 37°C. At the times indicated, aliquots were tested for infectivity on intact E. coli cells.*

than that of spermidine. These findings demonstrate the much greater capacity of spermidine compared with Mg^{2+} to increase the infectivity of the complex.

Effect of spermidine and Mg^{2+} on the infectivity of RNA on *E. coli* spheroplasts. The concentration of spermidine and Mg^{2+} for optimal enhancement of the infectivity of the complex for intact *E. coli* cells was now used to investigate their effect on the infectivity of RNA on *E. coli* spheroplasts. For this purpose, equivalent amounts of RNA were extracted with phenol from the acetic acid pellet and from the virus, as described in Materials and Methods. The two RNA samples were dissolved in buffer A (0.1 mg/ml). Aliquots were made to 3 × 10^{-4} M spermidine and to 5 × 10^{-3} M Mg^{2+}, as described in the legends to Fig. 1 and 2. RNA control aliquots were in buffer A. The infectivity of the RNA samples on *E. coli* spheroplasts was measured before and after incubation for 30 min at 37°C, as described in Materials and Methods. The result obtained revealed that the infectivity of both the phage RNA and the acetic acid pellet RNA remained unchanged after incubation with spermidine or Mg^{2+} and was in both cases like that of the control. This means that under conditions where the infectivity of the RNA-A protein complex on intact *E. coli* cells is stimulated mostly by spermidine or Mg^{2+}, no effect on the infectivity of the RNA alone on spheroplasts is measurable.

Effect of spermidine on the sedimentation of the RNA-A protein complex in sucrose gradients. Various RNAs, such as tRNA and phage RNA, have been reported to be converted to a more stable and compact structure by polyamines (4). Therefore, it might be expected that the RNA-A protein complex is also converted into a more compact structure by the spermidine incubation used here, where maximal enhancement of its infectivity occurred.

To test this, a ^{35}S-, ^{32}P-labeled RNA-A protein complex was analyzed by sucrose gradient centrifugation before incubation with no spermidine and after incubation with no and 3 × 10^{-4} M spermidine, respectively (Fig. 3). The complex in the control solution without spermidine sedimented in fraction no. 15 both before and after incubation at 37°C for 30 min (Fig. 3a, b). Also the whole, unchanged infectivity banded in this position. However, after incubation for 30 min at 37°C in 3 × 10^{-4} M spermidine, a considerably increased sedimentation rate of the complex was observed (Fig. 3c), indicating a more compact structure of the complex. Now the complex peak banded in fraction no. 10, where the 20-fold increased infectivity compared with the control solution was also detectable.

When the complex was analyzed by sucrose gradient centrifugation after incubation with 3 × 10^{-4} and 5 × 10^{-3} M Mg^{2+}, respectively, the sedimentation rate of the complex increased considerably, as indicated in Fig. 3d and e by shift of the position of the complex peak to fraction no. 11 and 9, respectively. However, only in the latter case did a 20-fold increase in infectivity occur, as had already been observed for the unlabeled complex.

Since equivalent amounts of complex molecules were layered on the sucrose gradients in Fig. 3 a–e, it can be seen directly from the reduced ^{35}S radioactivity, present in the A protein of the complex peak, that some of the complex molecules were dissociated upon incubation with 3 × 10^{-4} M spermidine or with 5 × 10^{-3} M Mg^{2+}. The dissociated A protein molecules, which were insoluble in the gradient buffer, appeared to sediment to the bottom of

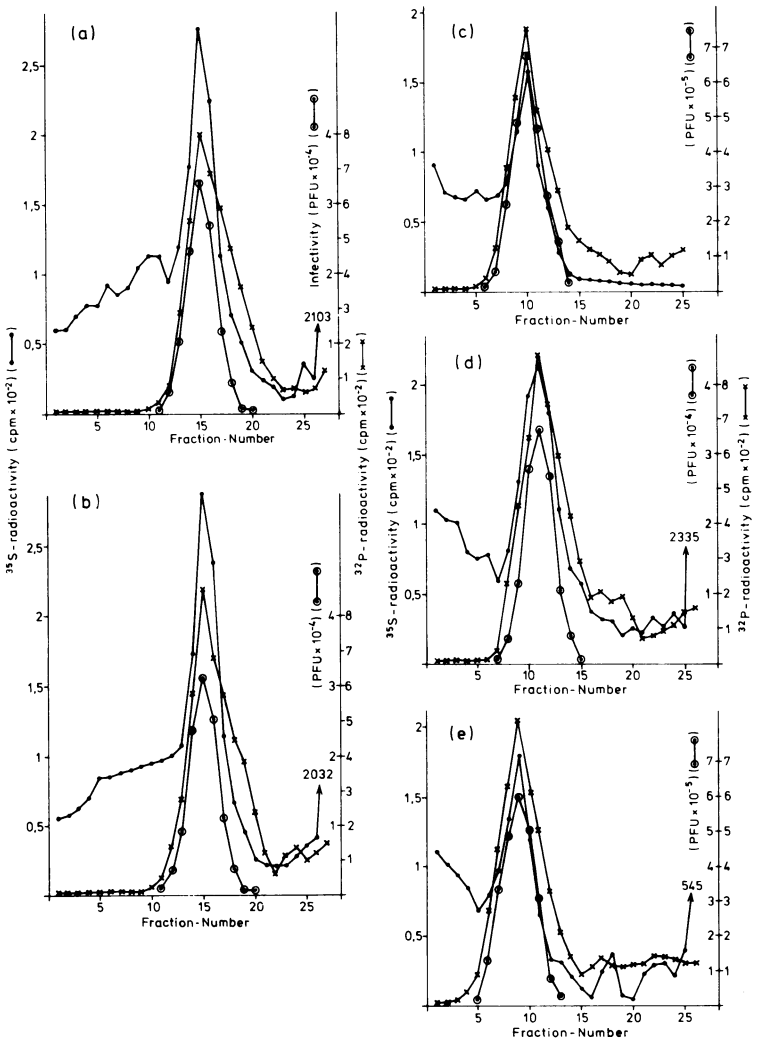

FIG. 3. *Sedimentation of the RNA-A protein complex in sucrose gradients after incubation with spermidine or Mg^{2+}. An acetic acid pellet, containing the RNA-A protein complex, was prepared from a mixture of 7.5×10^{11} infectious ^{35}S-and ^{32}P-labeled phage MS2 (1.8×10^{13} physical particles) as described in Materials and Methods. The pellet was dissolved in 0.33 ml of buffer A for 5 min at 37°C and then chilled to 0°C. From this solution, five 45-μl aliquots were taken. To two aliquots, 5 μl of buffer A was added. These solutions represent the controls and were allowed to stand for 30 min at 0 (a) and 37°C (b). To three further aliquots, 5 μl of buffer A with appropriate concentrations of spermidine and Mg^{2+}, respectively, was added, so that the solutions became 3×10^{-4} M with respect to spermidine (c) and 3×10^{-4} (d) and 5×10^{-3} (e) M with respect to Mg^{2+} in excess of the EDTA concentration. These three aliquots were incubated for 30 min at 37°C. After incubation, 45 μl of each was layered onto a 5 to 20% sucrose gradient, where each gradient contained the same buffer as the sample. Centrifugation was carried out in an SW56 rotor for 4 h at 45,000 rpm and 0°C. After centrifugation, the gradients were fractionated into ice-cold vials. From each fraction, infectivity on intact E. coli cells and ^{32}P-labeled RNA and ^{35}S-labeled A protein were measured as described previously (11). Centrifugation was from right to left.*

the gradient as highly aggregated molecules. This may be concluded from the increased ^{35}S radioactivity at the bottom of the gradients after spermidine or Mg^{2+} incubation (Fig. 3c, e).

DISCUSSION

In this paper I studied the effect of the polyamine spermidine on the RNA-A protein complex isolated from phage MS2.

When the infectivity of the complex was

tested after incubation with spermidine, it was observed to be increased depending on the spermidine concentration and the time of incubation at 37°C. Maximal, 20-fold-enhanced infectivity of the complex was detected at 3×10^{-4} M spermidine after 20 to 30 min at 37°C. The resulting infectivity corresponded to a yield of about 2×10^{-5} PFU per original infectious phage used for acetic acid precipitation. At spermidine concentrations higher than 3×10^{-4} M, the infectivity of the complex was stimulated to a lesser extent and was finally reduced to approximately 10% that of the control solution at 5×10^{-3} M spermidine.

As might be expected from the reported effect of spermidine on the structure of various nucleic acids, the ^{35}S-,^{32}P-labeled RNA-A protein complex sedimented considerably faster in sucrose gradients after incubation with 3×10^{-4} M spermidine than after incubation with the low-ionic-strength buffer A without spermidine. At this concentration of spermidine, a markedly more compact structure was obviously conferred on the RNA-A protein complex molecules. Under these conditions, some of the complex molecules were furthermore dissociated. Since only the complex but not the RNA alone was infectious for intact *E. coli* cells, the increase in the infectivity of the complex after spermidine incubation may therefore be higher than the measured 20-fold enhancement if one assumes that some infectious complex molecules have been dissociated. The lowered stimulation of complex infectivity at higher spermidine concentrations and longer incubation times shown in Fig. 1 may then be explained by increased complex dissociation.

Since it is well known that in many cases Mg^{2+} fulfills roles comparable to those of spermidine, I also studied the effect of this cation on the complex. At concentrations at which optimal stimulation of infectivity could be achieved with spermidine (3×10^{-4} M), no increase in infectivity of the complex was observed with Mg^{2+}, but its considerably faster sedimentation in sucrose gradients was similar to that caused by spermidine incubation. This indicates clearly that the stimulatory effect of spermidine on the infectivity of the complex cannot be replaced by Mg^{2+} at the same concentration, whereas the capacity of both ions to make the structure of the complex more compact seems to be similar. The same maximal 20-fold-enhanced infectivity of the complex could only be achieved if a Mg^{2+} concentration (5×10^{-3} M) higher by more than one order of magnitude than that of spermidine was used. This much greater capacity of the trivalent cation spermidine compared with that of the divalent Mg^{2+} ion in stimulating the infectivity of the RNA-A protein complex cannot be explained by its different valence alone.

At 5×10^{-3} M Mg^{2+}, the complex sedimented slightly faster than at 3×10^{-4} M Mg^{2+} or spermidine. Furthermore, some complex molecules were dissociated as by incubation with 3×10^{-4} M spermidine at 37°C for 30 min.

The infectivity of RNA alone, tested on *E. coli* spheroplasts, was not increased by incubation with those spermidine or Mg^{2+} concentrations that maximally enhanced the infectivity of the RNA-A protein complex on intact *E. coli* cells. These results were obtained with the RNA from both the virus and the acetic acid pellet. It has been reported (6) that approximately 100 molecules of spermidine are still associated with RNA phenol extracted from the virus. Thus the possibility arises that addition of external spermidine to the phenol-extracted phage RNA in my assay cannot further stimulate the infectivity of RNA if the internal spermidine content already represents the saturation level. However, this possibility can probably be excluded, because no increased infectivity after spermidine incubation was detectable with RNA extracted from the acetic acid pellet. This RNA should not contain significant amounts of internal spermidine, since the strongly basic polyamine should have been removed from the RNA during acetic acid treatment. Considering all these data, it may be concluded that the more compact structure of phenol-extracted RNA induced by the spermidine or Mg^{2+} concentrations tested does not play a role in its infection of spheroplasts.

From my results it remains unclear what is the mechanism of spermidine or Mg^{2+} action that leads to the considerable increase in infectivity of the complex. It may be that enhanced infectivity is achieved by the change of conformation of complex RNA, especially by stabilization of double-stranded regions and loops as has been reported for other nucleic acids. Direct electron microscopy of the stabilizing effect of spermidine on the secondary structure of RNA alone shows a considerably higher percentage of RNA molecules with well-characterized loops and hairpin structures when the molecules are spread in the presence of spermidine (3×10^{-4} M). Even in the presence of 5×10^{-3} M Mg^{2+}, less secondary structure of RNA is observed than with 3×10^{-4} M spermidine (unpublished data). These results, however, show only the stronger capacity of spermidine compared with Mg^{2+} to stabilize the secondary structure of the RNA, but provide no evidence as to whether this effect of spermidine and Mg^{2+} may also be responsible for the increase in complex infectiv-

ity. Therefore, it is also possible that the nonmeasurable conformation of the A protein in the complex is affected by the spermidine or Mg^{2+} concentration and leads to a more active protein and therefore a complex molecule.

Furthermore, the increased infectivity of the complex may arise only if both the RNA and the A protein conformation have been affected.

The RNA of turnip yellow mosaic virus has been reported to be more stable to RNase in the presence of polyamine (12). Therefore, the increase in infectivity of the RNA-A protein complex by spermidine could, at least partially, also be due to the higher stability of the infecting complex molecules to cell-associated RNases.

By whatever mechanism the spermidine molecules stimulate the complex infectivity, I believe that my in vitro results suggest that spermidine associated with the RNA in the phage also acts during in vivo infection of the phage. It could function by maintaining a stable conformation of the infecting RNA-A protein complex molecules and protecting them from cell-associated RNases.

ACKNOWLEDGMENTS

I thank P. H. Hofschneider for helpful comments and B. Martin for reading this manuscript. I also wish to thank R. Rimmelspacher for preparing the figures.

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