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Processing of Mengovirus Precursor Polypeptides in the Presence of Zinc Ions and Sulfhydryl Compounds

KENJI NAKAI[1] AND JEAN LUCAS-LENARD*

Biochemistry and Biophysics Section, University of Connecticut, Storrs, Connecticut 06268

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The effect of zinc ions on the post-translational cleavage of mengovirus polypeptides has been examined. The cleavage of the "A" precursor, which gives rise to the capsid proteins, was the most sensitive at concentrations of zinc chloride from 0.1 to 1.0 mM. Beta-mercaptoethanol and dithiothreitol antagonized the zinc-promoted inhibition of cleavage. Our results indicate that zinc ions interfere with the proper folding of the nascent polypeptide precursor rather than inhibit the proteases responsible for the cleavages. Thus, proper folding of mengovirus polypeptide "A" appears to be necessary for subsequent processing by proteases.

It is well known that amino acid analogues (6) and certain proteolytic enzyme inhibitors (7, 10, 12) block the cleavages of the precursor polypeptides in picornaviruses. Recently, it has been found (3, 8, 9) that zinc chloride also inhibits the cleavages of the viral polypeptide precursors of rhinoviruses, poliovirus, and encephalomyocarditis (EMC) virus. The mechanism by which zinc ions inhibit cleavage is not known.

In this report we have examined the effect of zinc ions on the cleavages of mengovirus precursor polypeptides. We find that zinc ions inhibit primarily the cleavage of the precursor termed "A" (10), which is cleaved to the structural proteins of the virion. In contrast to its effect on EMC virus, zinc at concentrations from 0.1 to 1 mM has no effect on the cleavage of C → D → E in mengovirus-infected cells.

We have also observed that reducing agents such as β-mercaptoethanol and dithiothreitol antagonize the zinc-mediated inhibition. Our results suggest that zinc ions affect the configuration of the nascent precursor polypeptides, thus rendering them unsuitable as substrates for proteolytic enzymes, and do not interfere with the proteases involved in the processing of the nascent precursors.

MATERIALS AND METHODS

Infection with mengovirus. L cells, strain 929, were grown in suspension culture in minimal essential medium for suspension culture (spinner medium) supplemented with 9% fetal calf serum (complete medium). The cells were washed with phosphate-buffered saline, suspended at a concentration of 1×10^7/ml, and infected with mengovirus at a multiplicity of infection of 20 PFU per cell. After an adsorption period of 60 min at 37 C, complete medium containing 3 μg of actinomycin D per ml was added, giving a final cell concentration of 1×10^6 cells/ml. The cultures were further incubated for 5 h.

Labeling of infected cells. Five hours after infection, the cells were harvested by centrifugation at 1,000 × *g* for 10 min, washed once with phosphate-buffered saline, and then suspended at a concentration of 1.6×10^7 cells/ml in 2 ml of spinner medium lacking amino acids. The cell suspension was incubated for 5 min at 37 C (preincubation period) and labeled for 25 min with 50 μCi of a ^{3}H-labeled amino acid mixture (New England Nuclear Corp., NET 250) and those unlabeled amino acids absent from the radioactive mixture, including methionine, tryptophan, cysteine, and asparagine, each at 0.25 μM. After the labeling period, the cells were centrifuged to remove the labeled amino acids and incubated further for 60 min with 2 ml of spinner medium and 0.2 ml of fetal calf serum (chase period).

At the end of the labeling or chase period, 0.2 ml of a solubilizing solution containing 10% sodium dodecyl sulfate (SDS), 1% β-mercaptoethanol, and 5 M urea was added.

Where indicated zinc chloride, dissolved in 1 mM HCl, was added at various concentrations during the preincubation, labeling, or chase period as indicated in the figure legends. Dithiothreitol or β-mercaptoethanol was added to the cell suspension as noted and at the concentrations designated in the figure legends.

Acrylamide gel electrophoresis of viral proteins. Urea-SDS polyacrylamide gels (7.5%) were prepared by the methods of Summers et al. (11) and Lucas-Lenard (10). Gel columns used were 0.6 by 20 cm. Disc gel electrophoresis was carried out at 7 mA/gel until the tracking dye had migrated to near the

[1] Permanent address: Department of Biochemistry, Osaka City University, School of Medicine, Osaka, Japan.

bottom of the gel (approximately 20 h). The samples to be analyzed were prepared as described above. After the addition of the solubilizing solution, the samples were dialyzed overnight against 0.5 M urea, 0.1% SDS, 0.1% β-mercaptoethanol, and 0.01 sodium phosphate buffer, pH 7.2 (2). The conditions for electrophoresis and counting of labeled viral proteins have been described (10).

^{14}C-labeled mengovirus capsid proteins. Cells were infected with mengovirus at a multiplicity of infection of 30 PFU/cell as described above. Five hours after infection, the cells were harvested by centrifugation and suspended at 1×10^6 cells/ml in complete medium lacking amino acids and containing 1 μCi of a ^{14}C-labeled amino acid mixture per ml (New England Nuclear Corp., NEC-445) and the unlabeled amino acids mentioned above used to supplement the ^{3}H-labeled amino acids. The virus was purified by the method of Abreu and Lucas-Lenard (1). The capsid proteins were prepared from the purified virus by using solubilizing solution and subjected to gel electrophoresis as described above.

RESULTS

Effect of zinc ions on the cleavage of mengovirus precursor proteins. The normal cleavage pattern of mengovirus precursor polypeptides is shown in Fig. 1. Five hours after infection, the cells were labeled for 25 min with radioactive amino acids as described in Materials and Methods. When the reaction was stopped at the end of the labeling period (Fig. 1a), the high-molecular-weight precursor polypeptides A, B, C, and D were observed in relatively large amounts. If a chase period followed the labeling period (Fig. 1b, c), most of the high-molecular-weight precursors disappeared and there was an increase in the stable proteins E, α, β, and γ. With few exceptions, the cleavage pattern of mengovirus proteins is thought to be similar to that of EMC virus (4, 10).

The effect of zinc ions on the processing of mengovirus precursors is shown in Fig. 2. Zinc chloride in 1 mM HCl was added to cells 5 min before the labeling period at the concentrations indicated in the figure legend. The preincubation was carried out to allow time for the zinc chloride to enter the cells before the start of the pulse period. The same concentration of zinc ions was maintained during the 25-min pulse period and the 60-min chase period. In the control sample containing 0.2 mM HCl without zinc chloride, only small amounts of the A, B, C, and D precursor polypeptides were present after the pulse and chase periods (Fig. 2a). These data also indicate that 0.2 mM HCl did not interfere with cleavage since the pattern was the same as in Fig. 1b, which did not contain HCl.

Zinc ions effectively inhibited the cleavages of the polypeptide A, but did not affect the C → D → E cleavages, since little C or D accumulated in its presence and E was present in large amounts (Fig. 2b–d). This is in contrast to the effect of zinc chloride on the cleavages of EMC virus polypeptide precursors (3), in which case both the A protein cleavages and the C protein cleavages were stopped. With increasing zinc ion concentrations (Fig. 2), the amount of polypeptide A increased and the amount of α, one of its cleavage products, decreased. It is not clear from the data whether zinc ions also inhibit the cleavages of a precursor longer than polypeptide A (5, 10), since such a precursor was present in variable amounts throughout these studies.

Effect of β-mercaptoethanol on the zinc chloride-promoted inhibition of cleavage. The mechanism of inhibition of cleavage in the presence of zinc ions is not clear. Butterworth and Korant (3) have proposed two possibilities to explain the zinc ion effect. The first is that the proteolytic enzymes involved in the processing of the precursors are inhibited by zinc ions. The other is that zinc ions cause an improper folding of the nascent polypeptides, thus rendering them inactive as substrates for proteolysis. Since the data in the literature do not allow one to distinguish between these possibilities, an attempt was made to determine the nature of the zinc effect.

Our argument was based on the premise that if zinc ions do affect the folding of the precursor polypeptides, then they might involve the sulfhydryl groups of the nascent precursors as they are being synthesized on ribosomes. Thus, both zinc ions and β-mercaptoethanol were added together to the infected cells. Both were present throughout the preincubation, labeling, and chase periods. Beta-mercaptoethanol, particularly at 10 mM, greatly reduced the inhibition of cleavage caused by 0.1 mM zinc ions (cf. Fig. 2d and 3a, b). The amount of precursor polypeptide A remaining was small, and the amount of α capsid protein was increased. The ratio of zinc ions to β-mercaptoethanol is apparently important, for 2.5 mM β-mercaptoethanol had little effect on preventing the inhibition of cleavage by 0.5 mM zinc, but 10 mM (ratio of zinc to β-mercaptoethanol of 1:20) was very effective (Fig. 3c, d).

These results suggested that sulfhydryl compounds could be useful in ascertaining whether the zinc was interfering with the folding of the nascent precursors or inhibiting the proteases involved in the cleavages of the precursors. It was necessary, however, to rule out the possibility that the mercaptoethanol was somehow

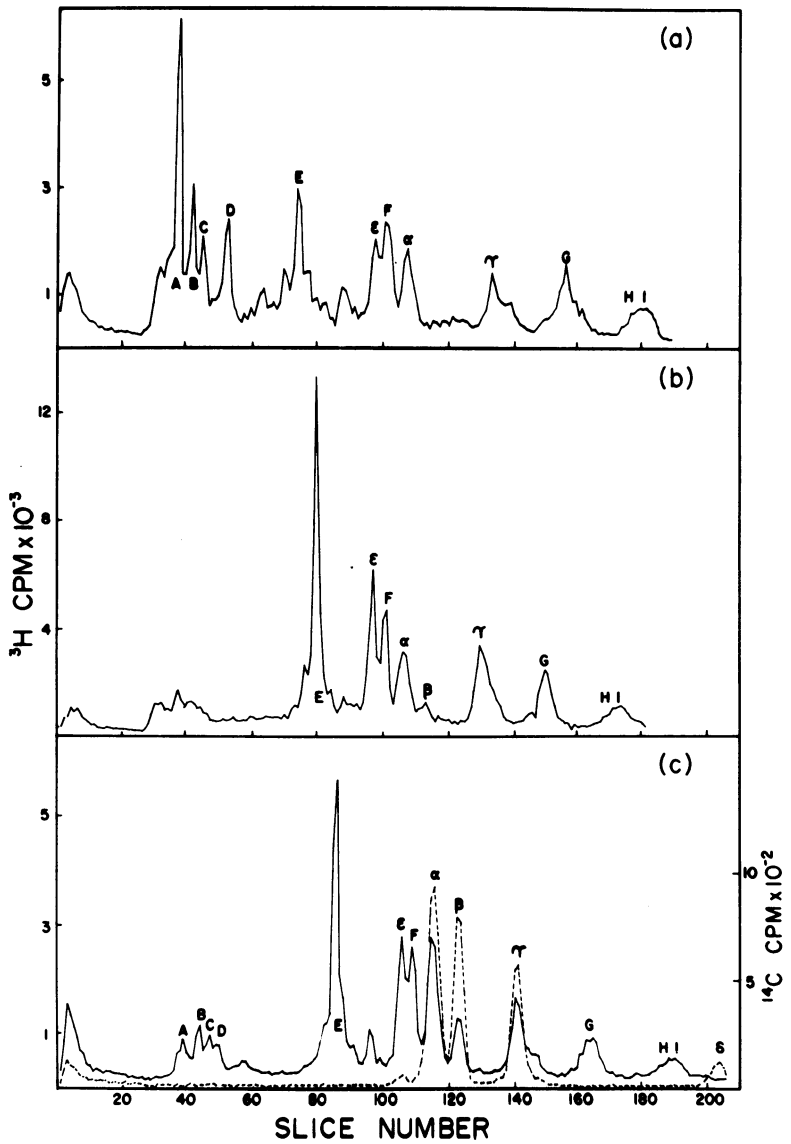

FIG. 1. *Normal cleavage pattern of mengovirus precursor polypeptides. (a) At 5 h after infection, L cells were labeled for 25 min with a ^{3}H-labeled amino acid mixture; (b) the labeled cells were chased with unlabeled amino acids for 60 min; (c) the cells were chased for 120 min. At the end of the labeling or chase period, the reaction was stopped by the addition of solubilizing solution as described in Materials and Methods. Approximately 100 to 200 μl of each sample was applied to a disc gel and subjected to electrophoresis. Panel c also shows the electrophoresis pattern (dashed line) of ^{14}C-labeled capsid proteins isolated from purified mengovirus. Migration is from left to right in all the figures.*

altering the entry of the zinc ions into the cell. Mercaptoethanol alone at 2.5 or 10 mM did not affect the normal cleavage pattern of mengovirus precursors, nor did ethanol at 10 mM in the presence of zinc ions prevent the inhibitory effect of the zinc ions (data not shown).

Dithiothreitol at 10 mM was also able to suppress the effect of 0.1 mM zinc (compare Fig. 2d and 4), indicating that the sulfhydryl group is the important feature of the molecule responsible for the antagonism.

Effect of adding zinc ions and/or β-mercaptoethanol at different points in the pulse-chase period. In an attempt to determine whether the zinc inhibits cleavage because it inactivates the proteolytic enzymes involved in

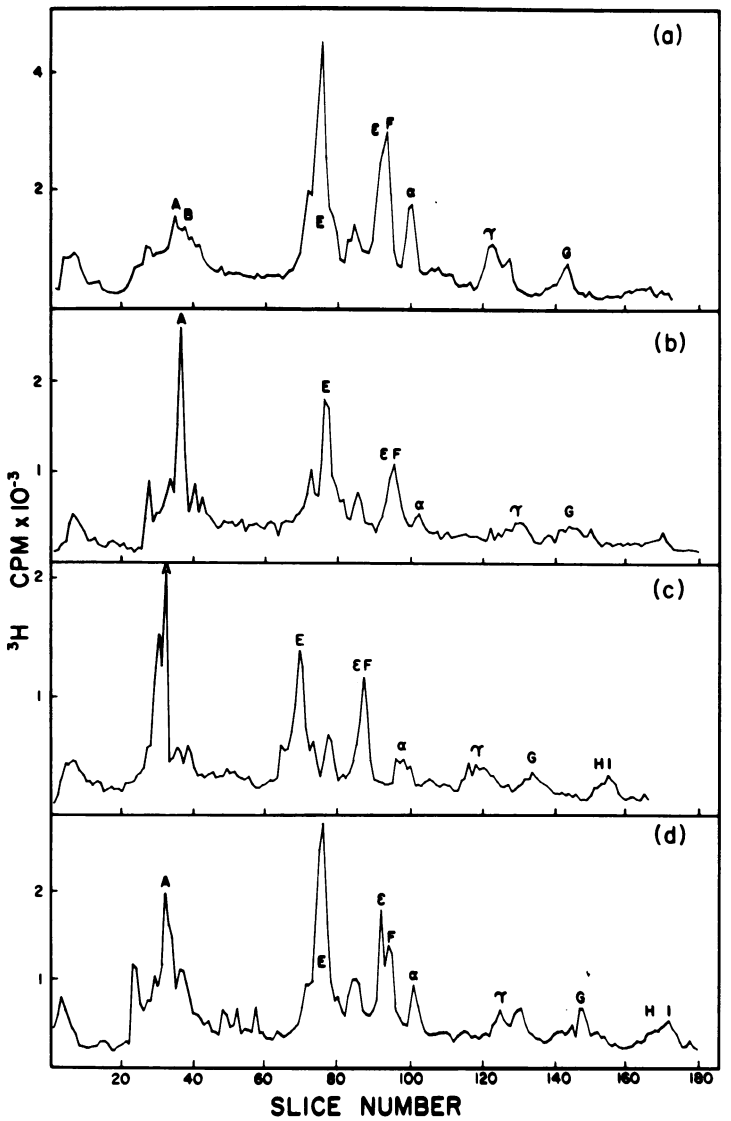

FIG. 2. *Effect of zinc chloride on the cleavage of mengovirus precursor polypeptides. At 5 h after infection, the cell suspension was incubated with or without zinc chloride from the start of the preincubation period to the end of the chase period. After the chase period the reaction was stopped and analyzed by acrylamide gel electrophoresis as described in Materials and Methods. (a) No $Zn(Cl)_2$, plus 0.2 mM HCl [the final HCl concentration when $Zn(Cl)_2$ is used]; (b) 1.0 mM $Zn(Cl)_2$; (c) 0.5 mM $Zn(Cl)_2$, and (d) 0.1 mM $Zn(Cl)_2$.*

cleavage or because it alters the configuration of the nascent precursor polypeptide A, the zinc was added at different times during the pulse-chase periods. In Fig. 5a, 0.1 mM zinc ions were present during the 5-min preincubation and 25-min labeling periods. At the end of this time the cells were centrifuged suspended in medium free of zinc, and incubated for 60 min (chase period) without zinc. If Fig. 2d and 5a are compared, it is evident that cleavage of precursor A did not take place. Furthermore, when 10 mM β-mercaptoethanol was added to the chase medium without zinc chloride, the β-mercaptoethanol was unable to counteract the effect of the zinc (Fig. 5b). These results suggested that the zinc ions were affecting the folding of the nascent precursor polypeptides rather than inactivating the proteolytic enzymes. If the latter were true, cleavage should have taken place when the β-mercapetoethanol was present during the chase period.

Further evidence that the zinc is affecting

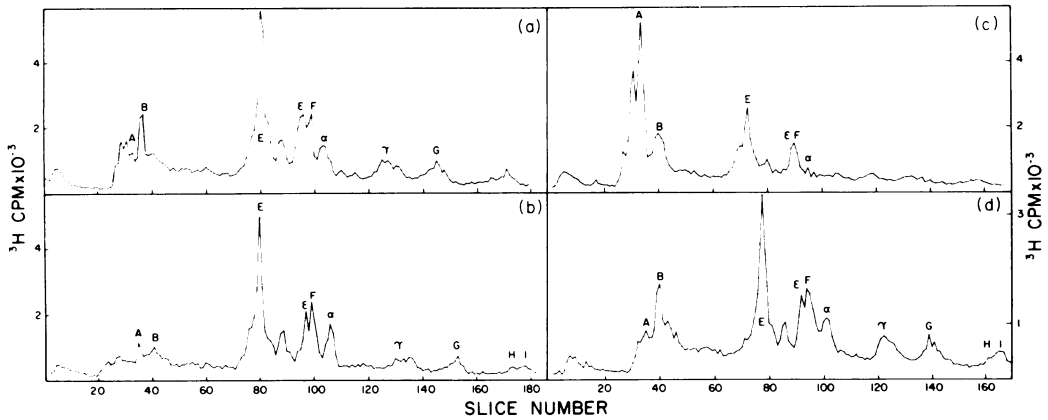

FIG. 3. *Effect of β-mercaptoethanol on the zinc chloride-promoted inhibition of cleavage. A mengovirus-infected cell suspension was incubated with zinc chloride and β-mercaptoethanol at various concentrations from the start of the preincubation period to the end of the chase period. The reaction was stopped and analyzed by polyacrylamide gel electrophoresis as described in Materials and Methods. (a) 0.1 mM $Zn(Cl)_2$ and 2.5 mM β-mercaptoethanol; (b) 0.1 mM $Zn(Cl)_2$ and 10 mM β-mercaptoethanol; (c) 0.5 mM $Zn(Cl)_2$ and 2.5 mM β-mercaptoethanol; (d) 0.5 mM $Zn(Cl)_2$ and 10 mM β-mercaptoethanol.*

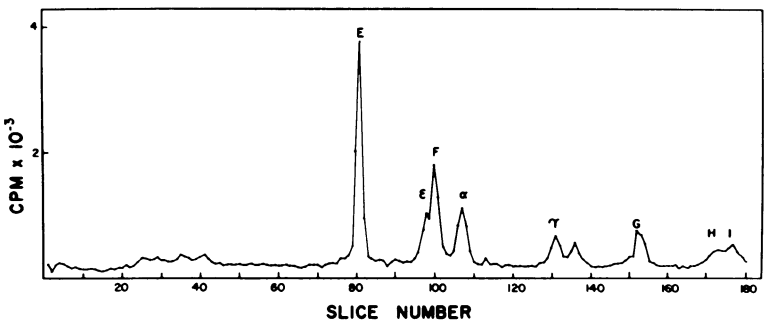

FIG. 4. *Effect of dithiothreitol on the zinc chloride-promoted inhibition of cleavage. A mengovirus-infected cell suspension was incubated with 0.1 mM zinc chloride and 10 mM dithiothreitol from the start of the preincubation period to the end of the chase period. The reaction was stopped and analyzed by acrylamide gel electrophoresis as described in Materials and Methods.*

the folding of the nascent polyprotein substrate comes from the experiment depicted in Fig. 5c. Here, zinc was absent during the preincubation and labeling periods and was present only during the chase period. As shown, practically all of the A precursor was cleaved, indicating that once the polypeptide is synthesized, zinc ions have no effect on its cleavage.

If zinc ions interfere with the folding of the nascent precursor polypeptides as they are being synthesized on ribosomes, then the addition of β-mercaptoethanol during the labeling period should prevent the inhibitory effect of the zinc. Zinc ions were preincubated with the infected cell suspension for 20 min before the labeling period, and the excess zinc was removed by pelleting the cells and resuspending them in zinc-free medium for the pulse and chase periods. Even though zinc ions were absent from the labeling solution, there was little cleavage of the A precursor when the cells were preincubated with 0.1 or 0.5 mM zinc chloride (Fig. 6a, c), indicating that once the zinc has entered the cell, it is not easily washed out. On the other hand, if β-mercaptoethanol was present during the labeling and chase periods, good cleavage of the A precursor occurred (Fig. 6b, d). These results suggest that when zinc and β-mercaptoethanol are present together in the cell, the β-mercaptoethanol is able to protect the nascent precursor polyeptide from zinc ions. The proper folding of the nascent precursor is retained and thus cleavage occurs normally. The data also suggest that after zinc ions have interfered with the folding of the nascent chain, its effect is not reversible. Finally, the results

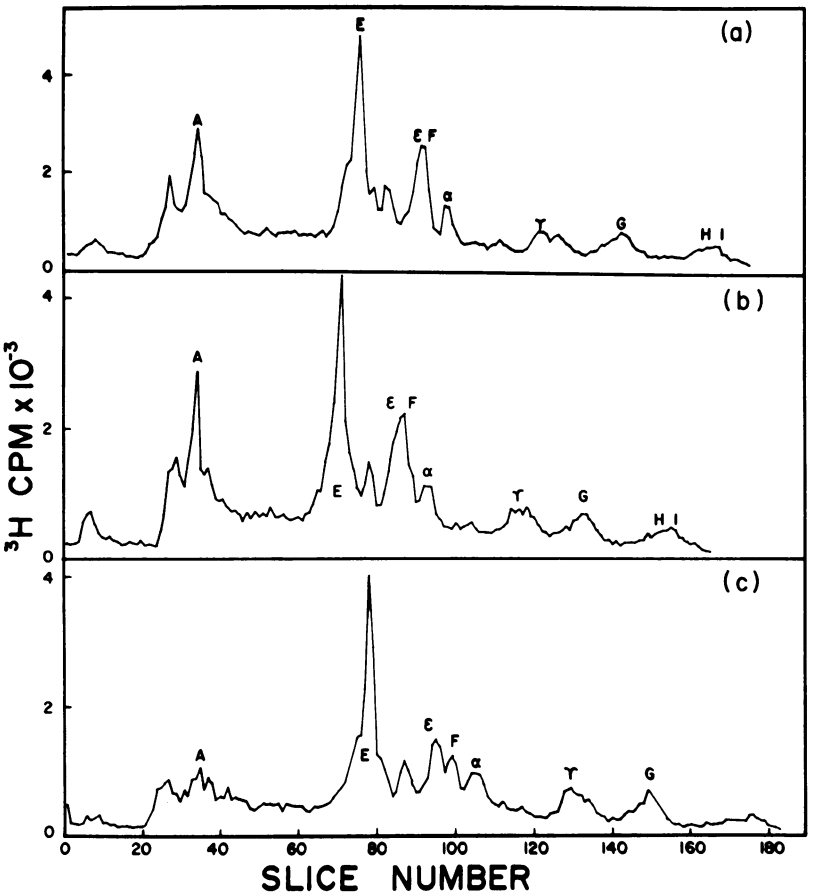

FIG. 5. *Effect of adding zinc ions and/or β-mercaptoethanol at different points in the pulse-chase period. (a and b) The cell suspension was incubated with 0.1 mM zinc chloride during the 5-min preincubation and the 25-min labeling periods. At the end of this time the cells were centrifuged, suspended in medium free of zinc (a) without β-mercaptoethanol and (b) with 10 mM β-mercaptoethanol, and incubated for 60 min (chase period). (c) The cells were labeled in the absence of $Zn(Cl)_2$, and 0.1 mM zinc chloride was added only during the 60-min chase period. The reactions were stopped and subjected to gel electrophoresis as described in Materials and Methods.*

rule out the possibility that β-mercaptoethanol acts by preventing the entry of the zinc into the cell, since it had the same effect even when the zinc was already within the cell.

DISCUSSION

To study the precursor-product relationships in human rhinovirus-1A, poliovirus, and EMC virus, Butterworth and Korant (3) used zinc chloride as an inhibitor of post-translational cleavages. However, they did not demonstrate clearly the mechanism of action of zinc ions. In this report we have studied the effect of zinc ions on post-translational cleavage in mengovirus-infected L cells. The primary target of zinc ions at concentrations from 0.1 to 1.0 mM is polypeptide A, though a precursor slightly larger than A also sometimes accumulates. This result is in contrast to the effect of zinc ions on human rhinovirus-1A, which was considerably more sensitive (8). In the latter case, not only was there an inhibition of cleavage of polypeptides A and C, but also precursors larger than the capsid protein precursor accumulated (3, 8). Poliovirus and EMC virus were also sensitive to zinc ions, but less so than human rhinovirus-1A. In the case of EMC virus, which like mengovirus is a cardiovirus, the capsid precursor A, polypeptide C, and two polypeptides of molecular weight 120,000 and 130,000 accumulated at 1.5 mM zinc chloride. Thus, by comparison to these other picornaviruses, mengovirus seems to be the least sensitive.

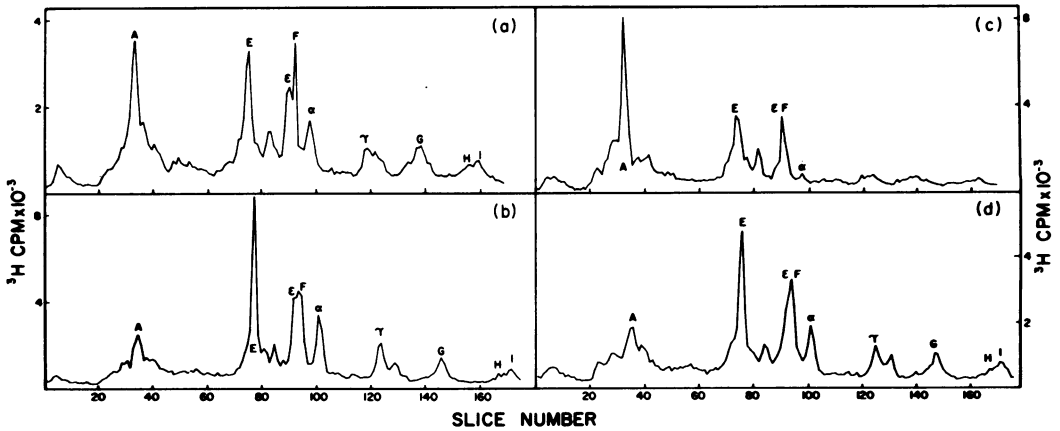

FIG. 6. *Effect of β-mercaptoethanol on the inhibition of cleavage by zinc chloride. Mengovirus-infected cells were preincubated in the presence of zinc for 20 min. The cells were centrifuged, suspended in medium free of zinc with or without 10 mM β-mercaptoethanol, labeled for 25 min, and chased for 60 min. The reactions were stopped and analyzed by gel electrophoresis as described in Materials and Methods. (a) 0.1 mM $Zn(Cl)_2$ was present during the preincubation period; (b) as in (a), but 10 mM β-mercaptoethanol was present from the start of the labeling period until the end of the chase period; (c) 0.5 mM $Zn(Cl)_2$ was present during the preincubation period; (d) as in (c), but 10 mM β-mercaptoethanol was present from the start of the labeling period to the end of the chase period.*

Interesting observations were also obtained from the experiment using β-mercaptoethanol. As shown in Fig. 3, zinc ions have no effect on the cleavage of polypeptide A in the presence of β-mercaptoethanol. This result prompted us to examine the detailed mechanism by which zinc inhibits post-translational cleavage. From the use of amino acid analogues, it is known that the proper folding of the nascent polypeptide is essential for cleavage to occur (6). Our results suggest that the inhibition of cleavage by zinc ions results from the improper folding of the nascent polypeptide. This conclusion is based on the results of several experiments. For example, when zinc ions are added after precursor A is formed but before it is cleaved, the zinc has no effect (Fig. 5c). Secondly, $Zn(Cl)_2$ only inhibits cleavage if it is present during the labeling period, when the nascent polypeptide precursor is being synthesized and presumably folded into its three-dimensional structure (Fig. 5a). Furthermore, when β-mercaptoethanol is present during the labeling period (Fig. 6b, d), it effectively protects the nascent polypeptide from the action of the zinc. Finally, once the nascent polypeptide has been improperly folded due to the presence of the zinc, its cleavage does not occur even in the presence of —SH compounds (Fig. 5b).

The mechanism by which sulfhydryl compounds protect the processing of mengovirus precursor polypeptide from inhibition by zinc ions is not known. It is possible that they react with the biologically active form of zinc, forming zinc-thiol complexes and thereby effectively remove the zinc ions from solution. The zinc is thus unavailable for interaction with the thiol and/or other reactive groups of the nascent precursor, and cleavage occurs normally.

ACKNOWLEDGMENTS

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