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Partial Purification and Properties of a Bacteriophage T7 Inhibitor of the Host Exonuclease V Activity

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Infection of *Escherichia coli* with bacteriophage T7 results in an inhibition of the host exonuclease V (*recB,C* DNase) activity. This inhibition is not observed when cells are infected in the presence of chloramphenicol or with a gene 1 mutant. The protein responsible for the inhibition of exonuclease V has been partially purified from T7-infected cells. The protein which does not possess nuclease or ATPase activity can inhibit all nucleolytic activities associated with exonuclease V. The protein does not, however, inhibit the DNA-dependent ATPase activity associated with exonuclease V. The inhibitory protein has a molecular weight of about 12,000, as determined from sedimentation analysis in glycerol gradients.

Infection of *Escherichia coli* with bacteriophage T7 results in the appearance of several viral-specific proteins which possess nuclease activity (4, 5, 10, 12, 17, 19-21). Studies with these partially purified enzymes have shown that both the exonucleases (10, 12, 17) and endonucleases (4, 5, 19-21) are capable of degrading purified T7 DNA. These results seem to suggest that the development of T7 may be dependent on regulatory mechanisms which function to protect replicating DNA from nucleolytic degradation. It has been shown by Eskin et al. (8) that in vitro T7 DNA can be degraded by the restriction enzyme from *E. coli* B, but in vivo the virus is capable of escaping the restriction process. Recent evidence indicates that the ability of T7 to overcome this restriction is due to a viral function coded for by gene 0.3 (27). The nature of this function is not known at the present time.

Recently we have initiated studies to identify additional regulatory mechanisms that may be involved in protecting intracellular T7 DNA from degradative processes. During these studies we observed that after T7 infection there is a considerable reduction in the host exonuclease V (*recB,C* DNase) activity (2, 3, 18). In the present report evidence is presented that the reduction in exonuclease V activity is the result of a viral-specific inhibitory protein. The protein responsible for this inhibitory effect has been partially purified and characterized. Two additional reports that show that T7 infection results in a reduction in the activity of exonuclease V have also appeared (22, 28).

MATERIALS AND METHODS

Bacteria and phage. *E. coli* ER22 *end I*$^-$, Su$^-$ (ATCC 27213) and *E. coli* JC6720 *recB21 recC22* were obtained from C. C. Richardson and H. Echols, respectively. *E. coli* 011$^-$ (ATCC 27214) was the permissive host for amber mutants. Bacteriophage T7 was obtained from F. W. Studier. The amber mutants used in this study were T7 *am193* (gene 1) and T7 *am29,147* (genes 3 and 6). These mutants will be designated by gene numbers only.

Phage DNA. ^{3}H-labeled T7 DNA was prepared as described previously (5). Phage ϕX174 DNA labeled with ^{3}H or ^{14}C was prepared as described by Sinsheimer (25).

Other materials. [γ-^{32}P]ATP (10 Ci/mmol) was purchased from New England Nuclear Corp. Bovine serum albumin was from Pentex.

Enzymes. T7 endonuclease I was prepared as previously described (5). Alkaline phosphatase was obtained from Worthington Biochemicals Corp. The enzyme was further purified as described by Weiss et al. (29) to remove traces of nuclease activity. Exonuclease V was purified from *E. coli* ER22 according to the procedure described by Wright et al. (31). The properties of the purified enzyme (glycerol gradient fraction) with regard to substrate specificity and ATP dependency were identical to those described by others (9, 16, 31). Exonuclease V activity was measured with native T7 DNA as substrate (31).

Assay for T7 inhibitor protein. The assay system is based on the procedure described by Sakaki et al. (23) for measuring the λ *gam* gene protein inhibition of exonuclease V. In this assay inhibitory protein from T7-infected cells was preincubated with exonuclease V. After the preincubation period DNA was added, and the acid-soluble material resulting from the exonucleolytic degradation of the substrate was measured. Unless indicated otherwise, native T7 DNA

was used as the substrate for exonuclease V. The preincubation mixture in 0.05 ml contained 0.01 M Tris-hydrochloride (pH 8.0), 0.01 M $MgCl_2$, 0.01 M β-mercaptoethanol, 0.5 mM ATP, and 0.5 mg of bovine serum albumin per ml. Exonuclease V (0.025 ml, 0.17 U [23]) was added, followed by the addition of T7 inhibitory protein (2 to 20 μl). The mixture was incubated at 37 C for 10 min, after which time 1 nmol of ^{3}H-labeled native T7 DNA in 10 μl (1.9×10^4 counts/min per nmol) was added. After a 20-min incubation at 37 C, 0.1 ml of salmon sperm DNA (250 μg/ml) and 0.1 ml of 20% trichloroacetic acid were added to the reaction mixture. The solutions were held at 0 C for 10 min and thereafter centrifuged at 10,000 rpm in the Sorvall SE-12 rotor. The acid-soluble radioactivity in the supernatant was measured in a scintillation spectrometer. One activity unit of inhibitor eliminates 1 unit of exonuclease activity in the assay described above.

The effect of purified inhibitory protein on exonuclease V activity with substrates other than native T7 DNA was carried out as described above in the presence of 1 nmol of ^{3}H-labeled single-stranded T7 DNA (1.9×10^4 counts/min per nmol) or 0.5 nmol of ^{3}H-labeled φX174 DNA (8×10^3 counts/min per nmol). The effect of inhibitory protein on the exonuclease V, DNA-dependent ATPase activity was carried out as described above, except that the reaction mixture contained [γ-^{32}P]ATP (10 counts/min per pmol) and unlabeled native T7 DNA. At the end of the incubation period, the ^{32}P released from [γ-^{32}P]ATP, which does not adsorb to norit, was determined according to the procedure described by Goldmark and Linn (9).

Other methods. Protein was determined by the procedure of Lowry et al. (14).

RESULTS

Effect of T7 infection on exonuclease V activity. A culture of *E. coli* ER22 was infected with $T7_{3,6}$, and at various times after infection aliquots were taken, the cells were lysed and centrifuged, and the exonuclease V activity in the supernatant was determined (Fig. 1). A $T7_{3,6}$ mutant was used in these experiments to eliminate the gene 3 endonuclease (5, 20) and gene 6 exonuclease activities (12). Soon after T7 infection (5 min) there is a slight increase in the exonuclease V activity compared to that present in the lysate from uninfected cells. Beginning at about 10 min after infection there is a decline in the exonuclease V activity, and by 25 min after infection the activity is only 16% of that present in the lysate from uninfected cells. Experiments have also been carried out in which portions of the lysate supernatants from $T7_{3,6}$-infected cells were examined for their ability to inhibit exogenous exonuclease V. The results of these studies showed that lysate supernatants prepared from cells at 15, 20, or 25 min after infection produced a 60 to 80% inhibition of exonuclease V activity with native T7 DNA as substrate (data not shown). Exonuclease V is not irreversibly inactivated during T7 infection, since, after DEAE-cellulose column chromatography of the ammonium sulfate fraction obtained during the purification of the inhibitor protein, enzyme activity can be detected in the fractions eluted with 0.3 to 0.4 M NaCl (see Fig. 2). These results suggest that the T7 factor responsible for the inhibitory effect is produced in excess over the endogenous exonuclease V. Additional evidence that a T7-induced function is responsible for the observed inhibition of the host exonuclease V was obtained by measuring this activity in lysates prepared from cells infected in the presence of chloramphenicol or with a T7 gene 1 mutant. Gene 1 codes for a T7-specific RNA polymerase (6), whose function is necessary for the synthesis of most T7 proteins (26). When cells are infected in the presence of chloramphenicol or with a gene 1 mutant, there is no detectable inhibition of the exonuclease V activity (Table 1). Recently similar evidence for a T7-induced inhibition of exonuclease V has been described by Wackernagel and Hermanns (28) and by Sakaki (22). Wackernagel and Hermanns have also obtained preliminary re-

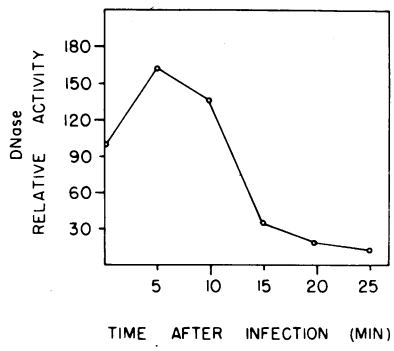

FIG. 1. *Effect of T7 infection on exonuclease V activity. E. coli ER22 was grown in M9 medium supplemented with 0.2% Casamino Acids and infected with $T7_{3,6}$ at a multiplicity of 5.0. Just prior to infection, a 20-ml aliquot was removed from the culture and processed as described below. At various times after infection, a 20-ml aliquot was taken, and the cells were centrifuged and lysed according to the procedure used in purifying the inhibitor protein. The lysates (1.0 ml) were centrifuged at 15,000 rpm for 30 min in the Sorvall SE-12 rotor. The supernatant was collected, and a 5-μl portion was assayed for exonuclease V activity with native T7 DNA as substrate. In the absence of ATP only negligible amounts of DNase activity can be detected. The protein concentration of the lysate supernatants was 2.5 to 2.7 mg/ml.*

TABLE 1. *Exonuclease V activity after infection in the presence of chloramphenicol and with a gene 1 mutant*[a]

Conditions of infection	Exonuclease V activity (U/mg of protein)
Uninfected	50
$T7_{3,6}$	2
$T7_{3,6}$ + chloramphenicol	55
$T7_1$	55

[a] *E. coli* ER22 was infected with $T7_{3,6}$ or $T7_1$, and at 25 min after infection cell lysates were prepared as described in Fig. 1. Cells were also infected with a $T7_{3,6}$ mutant under conditions in which chloramphenicol (100 μg/ml) was added 2 min prior to infection. Cells were collected at 25 min after infection. The lysate supernatants from the uninfected or T7-infected cells were assayed for exonuclease V activity with native T7 DNA as substrate.

sults showing that T7 class II genes 2, 3, 4, and 6 (28) are not responsible for the synthesis of the inhibitory factor. We have obtained similar results.

Purification of T7 inhibitor protein. (i) Growth of bacteria and preparation of cell lysates. *E. coli* ER22 was grown in 2 liters of M9 medium (1) supplemented with 0.2% Casamino Acids at 25 C to a cell density of 3.8×10^8 cells/ml. $T7_{3,6}$ was added at a multiplicity of 5.0, and at 22 min after infection the cells were collected by centrifugation. The pellet was suspended in 200 ml of a 20% sucrose solution containing 0.1 M Tris-hydrochloride (pH 8.0)-0.005 M ethyleneglycol bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA). The suspension was centrifuged, and the pellet was suspended in 60 ml of 0.1 M Tris-hydrochloride (pH 8.0)-0.005 M EGTA. Lysozyme in 0.25 M Tris-hydrochloride (pH 8.0) was added to a final concentration of 50 μg/ml. The cells were kept on ice for 90 min, and Brij 58 was added to 0.5%. After about 20 min lysis of the cells was complete. The lysate was centrifuged for 30 min at 12,000 rpm in the Sorvall SS34 rotor, and the supernatant was collected.

(ii) Streptomycin sulfate precipitation. Streptomycin sulfate (5% solution in 0.01 M Tris-hydrochloride, pH 7.6) was added to the lysate supernatant to a final concentration of 0.4%. The solution was held at 0 C for 30 min and centrifuged. The supernatant was collected, and 25.8 g of ammonium sulfate was added with stirring. After 30 min at 0 C the solution was centrifuged, and the pellet was taken up in 5 ml of a solution containing 0.02 M Tris-hydrochloride (pH 7.6)-0.01 M β-mercaptoethanol-0.001 M EDTA-10% glycerol (buffer A). The ammonium sulfate fraction was dialyzed against buffer A for 5 h at 0 C.

(iii) DEAE-cellulose column chromatography. The dialyzed ammonium sulfate fraction was applied to a column of DEAE-cellulose (1 by 12 cm) previously equilibrated with buffer A. After application of the sample, the column was washed successively with 9 ml of buffer A containing either 0.1, 0.2, 0.3, 0.5, or 0.6 M NaCl. Three-milliliter fractions were collected. The fractions from the DEAE-cellulose column were assayed for exonuclease V and for the T7 inhibitor protein. A peak of inhibitory activity which elutes from the column between 0.5 to 0.6 M NaCl can be detected (Fig. 2b). This

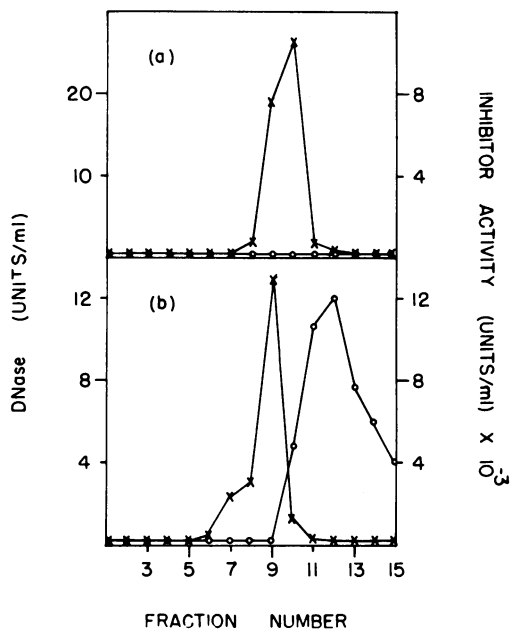

FIG. 2. *Chromatographic profile of exonuclease V and T7 inhibitor protein on DEAE-cellulose. E. coli ER22 was grown in M9 supplemented with Casamino Acids and infected with a $T7_{3,6}$ mutant. At 22 min after infection, the cells were collected and lysed. The lysate supernatant was fractionated with streptomycin sulfate and ammonium sulfate, and after dialysis this fraction was chromatographed on a DEAE-cellulose column as described in Results for the purification of T7 inhibitor protein. An uninfected culture of E. coli ER22 was also processed in an identical manner. Fractions from the DEAE-cellulose column were assayed for exonuclease V activity and inhibitor protein as described in Materials and Methods. (a) Results obtained with uninfected cells; (b) results with infected cells. Symbols: ×, exonuclease V activity with T7 DNA as substrate; O, inhibitor protein activity.*

activity is completely resolved from exonuclease V, which elutes from the column between 0.3 to 0.4 M NaCl. When uninfected cells are processed in an identical manner, there is no detectable inhibitory activity after DEAE-cellulose column chromatography of the dialyzed ammonium sulfate fraction (Fig. 2a). There is, however, a peak of exonuclease V that has a similar chromatographic profile as the enzyme from infected cells (Fig. 2b).

(iv) Phosphocellulose column chromatography. Fractions 12 and 13 from the DEAE-cellulose column were pooled and dialyzed for 10 h at 0 C against 0.02 M potassium phosphate buffer (pH 7.6)-0.01 M β-mercaptoethanol-0.001 M EDTA-10% glycerol (buffer B). The dialyzed material was applied to a phosphocellulose column (1 by 10 cm) previously equilibrated with buffer B. After application of the sample, the column was washed successively with 9 ml of buffer B containing 0.1 M potassium phosphate buffer (pH 7.6) and 9 ml of buffer B containing 0.2 M potassium phosphate buffer (pH 7.6). Fractions of 3 ml were collected. The major portion of the inhibitor protein was obtained in the 0.1 M potassium phosphate buffer eluate. These fractions were pooled, dialyzed overnight against 0.02 M Tris-hydrochloride (pH 8.0)-0.001 M EGTA-0.005 M β-mercaptoethanol-50% glycerol, and stored at −20 C. The inhibitor protein after phosphocellulose column chromatography was purified about 150-fold (Table 2). This estimate of purity is based on the finding that in the crude lysate and ammonium sulfate fractions the inhibitor protein is in considerable excess of exonuclease V. These fractions can therefore inhibit exogenous exonuclease V, thus providing an estimate of inhibitory activity.

(v) Absence of other enzyme activities. The partially purified inhibitor protein does not contain exonuclease activity with either double- or single-stranded T7 DNA in the presence or absence of ATP. Also, there is no detectable endonuclease activity with PM2 DNA as substrate (4) nor is there any ATPase activity in the presence or absence of double-stranded T7 DNA.

TABLE 2. *Purification of the T7 inhibitor protein*

Step	Inhibitor activity[a] (U × 10^{-4})	Protein (mg/ml)	Sp act (U × 10^{-4}/mg of protein)
I. Lysate supernatant	264.0	2.8	1.6
II. Ammonium sulfate	28.0	6.0	0.5
III. DEAE-cellulose	9.0	0.08	17.0
IV. Phosphocellulose	2.5	0.01	240.0

[a] The T7 inhibitor protein activity was determined as described in Materials and Methods using duplex T7 DNA as the substrate for exonuclease V.

Properties of T7 inhibitor protein. (i) Evidence that the inhibitor is a protein. The inhibitor isolated from the phosphocellulose column is heat stable and is not inactivated after heating for 15 min at 90 C (Table 3). Treatment of the inhibitor with trypsin but not RNase or DNase results in complete loss of inhibitory activity (Table 3).

(ii) Inhibition of exonuclease V. Previous studies have shown that highly purified exonuclease V contains four distinct enzymatic activities, which all seem to reside in the same protein complex. These four activities include an ATP-dependent exonuclease activity with linear double- or single-stranded DNA, an endonuclease activity with single-stranded circular DNA which is stimulated sevenfold by ATP, and a DNA-dependent ATPase activity (9, 16, 31). Studies were carried out to test the effect of the T7 inhibitor protein on these four enzyme activities. The inhibitor protein can bring about an extensive reduction in exonuclease V activity when measured with double- or single-stranded T7 DNA (Fig. 3; Table 4). In the presence of ATP exonuclease V is capable of degrading the single-stranded circular DNA of φX174 to acid-soluble material. This reaction is also considerably reduced by the inhibitor protein (Fig. 3). We have also observed that, if exonuclease V and inhibitor protein are incubated with φX174 DNA, inhibition of DNA hydrolysis takes place

TABLE 3. *Inactivation of the inhibitor by various treatments*[a]

Treatment	Inhibition of exonuclease V (%)
None	85
RNase	85
DNase	85
Trypsin	0
Heat (90 C for 15 min)	85

[a] The T7 inhibitor isolated from the phosphocellulose column was incubated with 10 μg of trypsin, pancreatic RNase, or pancreatic DNase for 30 min at 37 C. In the reaction with pancreatic DNase, $MgCl_2$ was present at a final concentration of 0.01 M. At the end of the incubation, EDTA was added to a final concentration of 0.015 M and the solutions were heated at 90 C for 15 min. After heating the inhibitory activity was determined as described in Materials and Methods.

even if *E. coli* exonuclease I (13) is added to the reaction (Table 4). These results suggest that the inhibitor is capable of preventing the exonuclease V endonucleolytic degradation of ϕX174 DNA. In contrast to the ability of the inhibitor protein to reduce exonuclease V activity toward double- and single-stranded DNA, we find that the protein produces only a slight inhibition of the DNA-dependent ATPase activity (Fig. 3; Table 4).

Our studies carried out thus far suggest that the inhibitory protein is specific for exonuclease V. The protein has no effect on the activity of T7 endonuclease I (5, 20) pancreatic DNase, or *E. coli* exonuclease I (13).

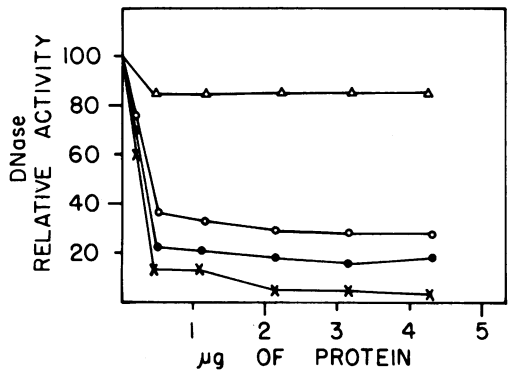

FIG. 3. *Effect of T7 inhibitor protein on exonuclease V activity. The effect of T7 inhibitor protein (phosphocellulose column fraction) on exonuclease V activity was carried out as described in Materials and Methods. Exonuclease V activity was measured with the following substrates: ×, native T7 DNA; ○, single-stranded T7 DNA; ●, ϕX174 DNA; △, ATPase activity with [γ-^{32}P]ATP and native T7 DNA as substrates. Activity obtained in the absence of inhibitor protein is set at 100%.*

(iii) Sedimentation of inhibitor protein in glycerol gradients. Figure 4 shows the sedimentation pattern of the inhibitor protein in 10 to 30% glycerol gradients. When sedimented in glycerol gradients that do not contain KCl (Fig. 4a), the protein cosediments with marker alkaline phosphatase, which has a molecular weight of 86,000 (24). A similar sedimentation rate for the inhibitor protein occurs under the same conditions but in the absence of alkaline phosphatase (data not shown). When the inhibitor protein is centrifuged in glycerol gradients containing 0.15 M KCl a considerable fraction of the protein now sediments as material having a molecular weight of about 12,000 (Fig. 4b).

DISCUSSION

The results of the present study provide evidence that a protein present in cells infected with bacteriophage T7 can mediate a direct inhibition of the host exonuclease V. The protein responsible for the inhibitory effect is not present in cells infected in the presence of chloramphenicol or with a gene 1 mutant. Since the partially purified inhibitor protein does not possess the gene 1 RNA polymerase activity (6), it would be indicated that a T7 class II function (26) is necessary for the exonuclease V inhibition to occur. At the present time, however, no mutant defective in the T7 inhibitory function has been identified. Presumably T7 codes for the inhibitory protein itself, but appropriate mutants will be necessary to establish this point.

In the present study the inhibitory protein was partially purified from *E. coli* ER22 (*rec*$^+$) infected with T7$_{3,6}$. Although exonuclease V is not detected in the infected cell extracts, DEAE-cellulose column chromatography of the

TABLE 4. *Effect of inhibitor protein on various activities associated with exonuclease V*[a]

DNA present in reaction mixture	Activity measured	Exonuclease V activity (nmol of acid-soluble nucleotide)	
		− Inhibitor	+ Inhibitor
Native T7 DNA	Exonuclease	0.56	0.08
Single-stranded T7 DNA	Exonuclease	0.36	0.10
ϕX174 DNA	Endonuclease	0.40	0.07
Native T7 DNA	ATPase	0.60[b]	0.55[b]

[a] Reaction conditions were as described in Materials and Methods. T7 inhibitor protein (2 μg of protein from the phosphocellulose column fraction) was added as indicated. The effect of T7 inhibitor protein on exonuclease V single-strand endonuclease was carried out with the circular DNA from phage ϕX174. In this experiment the inhibitor was incubated under standard conditions, after which time *E. coli* exonuclease I was added to the reaction, and after an additional 20-min incubation period acid-soluble material was determined. In the control reaction exonuclease V was incubated in the absence of inhibitor and exonuclease I. Under the assay conditions used exonuclease I is active towards linear single-stranded DNA.

[b] Nanomoles of norit nonadsorbable ^{32}P.

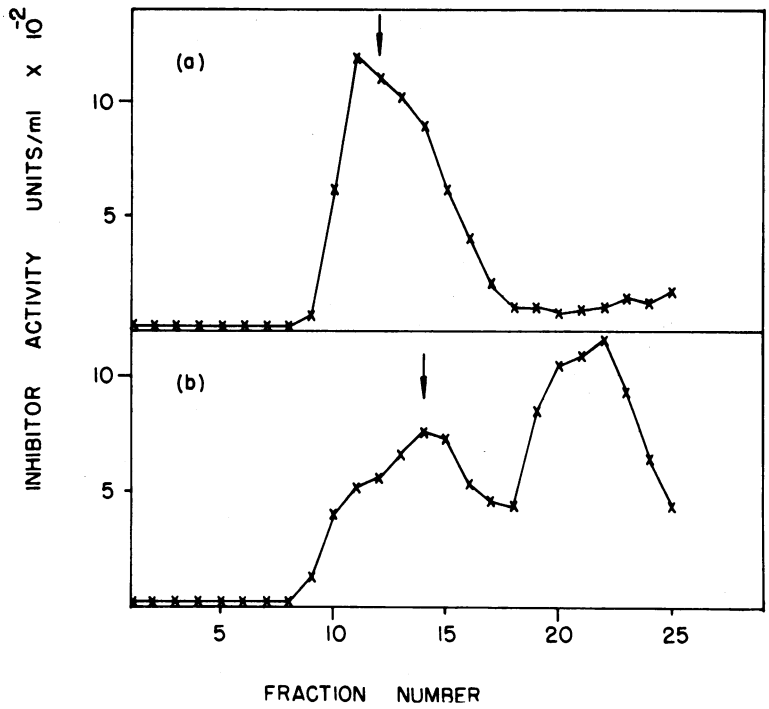

FIG. 4. *Glycerol gradient centrifugation of T7 inhibitor protein. T7 inhibitor protein obtained from the DEAE-cellulose column (Fig. 2) was concentrated in a dialysis bag covered with Sephadex G-200. The concentrated material (0.1 ml) was mixed with 0.1 ml of bacterial alkaline phosphatase and centrifuged in a 10 to 30% glycerol gradient for 27 h at 38,000 rpm and 4 C in the Spinco SW50.1 rotor. The glycerol gradients contained 0.02 M Tris-hydrochloride (pH 8.0)-0.005 M β-mercaptoethanol-0.001 M EDTA. After centrifugation 3-drop fractions were collected from the bottom of the tube, and portions were assayed for inhibitor protein using native T7 DNA as substrate for exonuclease V. The molecular weight of the inhibitor protein was estimated according to the procedure of Martin and Ames (15) based on a molecular weight of 86,000 for alkaline phosphatase (24). The arrow indicates the position to which alkaline phosphatase sediments. (a) The inhibitor protein was centrifuged in glycerol gradients which did not contain KCl. (b) The inhibitor protein was centrifuged in glycerol gradients containing 0.15 M KCl.*

ammonium sulfate fraction results in the appearance of the DNase, which is completely resolved from the inhibitor protein. These results provide evidence that the inhibition is not irreversible, as might be expected if proteolytic degradation of exonuclease V was occurring. Recently we have purified the inhibitor from cells of *E. coli* JC6720 (*recB21, recC22*) infected with T7 wild type. The results of the purification were very similar to those described in Table 2.

The T7 inhibitor protein was purified on the basis of its ability to inhibit exonuclease V activity towards duplex T7 DNA. Studies with the partially purified protein reveal that it is also capable of inhibiting exonuclease V activity when linear single-stranded or single-stranded circular DNAs are used as the substrates. The protein has, however, only marginal inhibitory activity towards the DNA-dependent ATPase activity of exonuclease V. Previous studies have shown that infection with bacteriophage λ results in the formation of a viral-specific protein (*gam* gene product) which selectively inhibits the host exonuclease V (23). The λ *gam* protein, which has been extensively purified, differs somewhat from the T7 activity, since it can inhibit the DNA-dependent ATPase as well as the exo- and endonuclease activities associated with exonuclease V. The T7 and λ exonuclease V inhibitory proteins also appear to differ in size. A major portion of the T7 inhibitory activity sediments in glycerol gradients containing 0.15 M KCl as 12,000-molecular-weight material, whereas the λ *gam* protein has a molecular weight of about 30,000 as determined by Sephadex column chromatography (23). It is interesting to note that when the T7 inhibitory protein is centrifuged in glycerol gradients that do not contain salt all of the activity is re-

covered as material having a molecular weight of about 86,000. This would suggest that in the absence of salt the protein undergoes a conformational change or is capable of extensive aggregation.

The role of the T7 inhibitory protein in the viral life cycle is not known. Previous studies have shown that the pathway of T7 DNA replication occurs in two stages. First, the infecting parental DNA replicates to form linear, monomeric daughter molecules (30). In the second stage (maturation), the monomers are joined together to form linear concatemeric structures (11) which are thereafter cleaved to unit-length T7 DNA, which is packaged in the phage capsid. Presumably at least one function of the inhibitory protein would be to block exonuclease V degradation of T7 replicative intermediates. This inhibition would be particularly important in a T7 infection, since all replicative intermediates are linear structures which are excellent substrates for exonuclease V. Studies with λ mutants defective in the synthesis of the *gam* gene protein also suggest that the inhibitor serves a protective function and its presence is necessary for the formation of late replicating intermediates such as concatemers (7). The λ *gam* gene protein may also have a role in recombination, since in its absence the phage-mediated recombination system (red) is reduced threefold (7). Possibly the T7 inhibitory protein has a similar function, and it may interact directly with certain T7 proteins to produce a more efficient phage recombination system.

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