Specific Stimulation of the T7 Gene 6 Exonuclease by the Phage T7 Coded Deoxyribonucleic Acid Binding Protein†

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ABSTRACT: Bacteriophage T7 codes for a single-stranded DNA binding protein. This protein is the product of gene 2.5 and has been found previously to stimulate specifically the activity of the phage-coded DNA polymerase. We report here that the T7 DNA binding protein also stimulates the activity of the phage-coded exonuclease. The gene 6 exonuclease is a double-stranded DNA specific 5′-exonuclease that has been implicated in destruction of bacterial DNA, removal of RNA primers during DNA replication, genetic recombination, and DNA maturation. The enzyme is markedly inhibited by physiological concentrations of NaCl. This inhibition, which is due to a marked reduction in the V_{max} of the enzyme, can be largely overcome by the phage-coded DNA binding protein. This stimulation is specific since the *Escherichia coli* DNA binding protein is without effect. The stimulation by the binding protein is apparently not due to its coating of the 3′ single-stranded tails generated during the digestion. Kinetic studies show that the stimulation is due to a combined effect on both the K_m and V_{max} of the exonuclease. These studies are consistent with a loose binding of the binding protein to either the DNA or the exonuclease.

The single-stranded DNA binding protein of bacteriophage T7 is an abundant class II protein made after T7 infection (Studier, 1972). This protein, which has a subunit molecular weight of 25 000, is the product of gene 2.5 (Dunn & Studier, 1981). Because of its map position within a region of the T7 genome coding for functions involved in phage DNA metabolism, it has been assumed that this protein has an important role in phage DNA replication and recombination. This assumption is strengthened by the analogy with a similar T4-coded protein, the gene 32 product, which has been implicated in T4 DNA replication and recombination (Alberts & Frey, 1970).

The T7 DNA binding protein was purified by Reuben & Gefter (1973, 1974) and Scherzinger et al. (1973) on the basis of its high affinity for single-stranded DNA. These workers showed that the protein specifically stimulated the activity of the T7 phage coded DNA polymerase in vitro. The protein also forms an important component of systems of T7 proteins that carry out replication of DNA in vitro (Romano & Richardson, 1979). Thus, there is good reason to believe that the protein plays an important role in phage T7 DNA replication in vivo.

In our pursuit of an understanding of the mechanisms of recombination of T7 DNA promoted by phage-infected extracts in vitro, we have discovered two additional properties of the T7 DNA binding protein (Sadowski et al., 1980). First, the protein promotes the reassociation of complementary single strands of T7 DNA into a duplex form. This reaction requires physiological concentrations of salt but does not consume ATP. By analogy with the well-studied *rec*A protein of *Escherichia coli* (Weinstock et al., 1979), this ability of the T7 DNA binding protein to renature DNA could have an important role in facilitating pairing of complementary single strands during genetic recombination.

A second novel feature of this protein is its ability to stimulate the exonuclease activity of the T7 gene 6 protein (Sadowski et al., 1980). This activity is specific to the gene 6 exonuclease as the T7 binding protein has no such effect on *E. coli* exonuclease III or λ exonuclease. Furthermore, the activity is peculiar to the T7 DNA binding protein as the gene 32 protein of phage T4 had a much less marked effect.

In view of the importance of the gene 6 exonuclease in destruction of host cell DNA (Sadowski & Kerr, 1970), genetic recombination (Powling & Knippers, 1974; Kerr & Sadowski, 1975; Lee & Sadowski, 1981), and DNA maturation (Miller et al., 1976), we have studied the possible mechanisms by which the T7 DNA binding protein stimulates the activity of the gene 6 exonuclease. The results of these studies are reported here.

Materials and Methods

Bacterial and Phage Strains. *E. coli* 011′ (*sup*E) and BBW/1 (*sup*F) were used as the permissive hosts for T7 amber mutants and *E. coli* B (*sup*°) was the nonpermissive host. T7 phage amber mutants in gene 1.3 (T7 DNA ligase, *am* HA13), gene 3 (T7 endonuclease I, *am*29), gene 4 (DNA primase, *am*64), gene 5 (DNA polymerase, *am*28), and gene 6 (T7 exonuclease, *am*147) have been described previously (Sadowski, 1977). Multiple mutants were constructed by standard phage crosses and used to make phage-infected cells.

DNA Substrates. Phage T7 DNA labeled with either [^{14}C]thymine or [^{3}H]thymidine was prepared as described previously (Sadowski et al., 1974). The preparation of ^{3}H-labeled *E. coli* DNA has also been described (Sadowski, 1971).

*Hae*III-cut T7 DNA was labeled with ^{32}P at the 5′ termini as follows. Sixty micrograms of mature T7 DNA was digested with *Hae*III restriction enzyme (10 units) in a 0.2-mL reaction containing 6 mM Tris[1]-HCl buffer, pH 7.4, 6 mM $MgCl_2$, and 6 mM NaCl. After 3 h at 37 °C, the reaction was heated to 65 °C for 5 min, and the DNA was ethanol precipitated after addition of $^{1}/_{10}$th volume of 3 M sodium acetate and 3 volumes of ethanol. The pellet was dried and dissolved in 100 μL of a solution containing 0.05 M Tris-HCl buffer, pH 8, 0.01 M $ZnSO_4$, and 0.1% $NaDodSO_4$. After addition of 315 units of

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[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

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bacterial alkaline phosphatase the reaction was incubated at 30 °C for 30 min. An additional aliquot of enzyme (555 units) was added and the incubation was continued for 30 min at 60 °C. The reaction was terminated by addition of 0.5 μmol of EDTA and phenol extraction, ether extraction, and ethanol precipitation (Maxam & Gilbert, 1980). The 5′ termini were labeled in a 0.1-mL reaction containing 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine, 10 μm [γ-^{32}P]ATP (2×10^4 cpm/pmol), and 18 units of polynucleotide kinase. After 30 min at 37 °C, the DNA was freed of ATP by Sephadex G-100 chromatography. The DNA was precipitated twice with ethanol and redissolved in 50 μL of water (OD_{260} = 9.2). The final specific activity was about 2.7×10^4 cpm/pmol of DNA termini.

Phage and Bacterial Proteins. The T7 gene 6 exonuclease was prepared as described previously by Sadowski et al. (1974) from T7 1.3*am*3*am*4*am*5*am*-infected *E. coli* 1200 cells. The enzyme was free of detectable DNA endonuclease, gyrase, nicking–closing activity, and T7 DNA binding protein and was apparently homogeneous by $NaDodSO_4$–polyacrylamide gel analysis.

The T7 DNA binding protein was prepared from phage T7 1.3*am*3*am*4*am*5*am*6*am*-infected *E. coli* B cells. The cells were grown in 4 L of SLBH medium to a density of about 1×10^{10}/mL in a Labline high-density rotary fermentor with forced oxygenation of 8–16 L/min at 30 °C (Greene et al., 1974). The cells were infected at a multiplicity of 10 phages per cell, and growth was continued for 17.5 min, after which time the culture was poured over frozen blocks of 0.15 M NaCl and centrifuged at 11000*g* for 15 min. The typical yield of infected cells was 160 g wet weight.

The purification procedure developed was modeled after that of Reuben & Gefter (1974) with the following modifications. The poly(ethylene glycol) precipitation was omitted, and the sonic extract was treated with 50 μg/mL of pancreatic DNase for 1 h at 10 °C. NaCl was added to a concentration of 1 M, and the extract was centrifuged at 45 000 rpm for 3 h. After overnight dialysis the extract was chromatographed on double-stranded DNA–cellulose followed by chromatography on single-stranded DNA–cellulose. The magnesium precipitation step was omitted, and the eluate from the single-stranded DNA–cellulose column was chromatographed on DEAE-Sephacel instead of DEAE-cellulose in 0.02 M Tris-HCl, pH 6.8, 1 mM 2-mercaptoethanol, 50 mM NaCl, and 10% glycerol and eluted with a 0.05–0.5 M NaCl gradient (buffer B). The fractions containing binding protein were pooled, dialyzed, applied to a 0.5-mL DEAE-Sephacel column, and eluted with 1.0 M NaCl in buffer B. The fractions containing the binding protein were dialyzed against 0.02 M Tris-HCl buffer, pH 7.4, 1 mM EDTA, and 50% glycerol and stored at −20 °C. The yield of binding protein was typically about 2 mg from 15 g of cells. Routinely, the purification of the protein was monitored by $NaDodSO_4$–polyacrylamide gel electrophoresis. The purified preparations invariably showed a doublet by Coomassie staining, a phenomenon which has also been found for the T3 binding protein (Yamagishi et al., 1981). The purified T7 protein had no detectable endonuclease activity on supercoiled DNA and likewise demonstrated no gyrase or nicking–closing activity. As shown below (Table I) the protein preparation liberates a small amount of acid-soluble material from duplex DNA. *E. coli* exonuclease I was purified from uninfected *E. coli* B cells (grown in the rotary fermentor to a density of 5×10^{10}/mL) by the procedures of Yajko et al. (1974) up to the DEAE-cellulose step. The active fractions were then chromatographed on double-stranded and single-stranded DNA–cellulose, DEAE-cellulose, and phosphocellulose as described by Ray et al. (1974). The active fractions were concentrated on a small DEAE-cellulose column and stored at 0 °C.

Table I: Effect of T7 DNA Binding Protein on Gene 6 Exonuclease Activity in the Presence and Absence of NaCl[a]

additions	nmol of DNA made acid soluble
(1) gene 6 exonuclease only	1.86
(2) gene 6 exonuclease + T7 DNA binding protein	3.45
(3) T7 DNA binding protein only	0.011
(4) gene 6 exonuclease + NaCl	0.12
(5) gene 6 exonuclease + NaCl + T7 DNA binding protein	1.6
(6) T7 DNA binding protein + NaCl	0.001

[a] Reaction mixtures (0.1 mL) contained 50 mM Tris-HCl buffer, pH 8.0, 5 mM $MgCl_2$, 1 mM dithiothreitol, 20 mM KCl, and 6.5 nmol of ^{3}H-labeled T7 DNA (31 000 cpm/nmol). Where indicated, the following additions were made: T7 gene 6 exonuclease, 1.86 units; NaCl, 0.1 M; T7 DNA binding protein, 11 μg. Acid-soluble radioactivity was measured after 15 min at 37 °C.

E. coli alkaline phosphatase was from BRL, and T4 polynucleotide kinase was purchased from Boehringer. The restriction enzyme *Hae*III was obtained from New England BioLabs. The DNA binding protein of *E. coli* was obtained from J. Hurwitz and J. Chase.

Results

T7 DNA Binding Protein Overcomes the Inhibitory Effect of NaCl on T7 Exonuclease Activity. In the course of studying the effect of purified T7 proteins in an in vitro T7 recombination system, we observed that the T7 DNA binding protein markedly stimulated in vitro recombination promoted by the gene 6 exonuclease (Sadowski et al., 1980). Upon examining this phenomenon further, we found that the binding protein stimulates the exonuclease activity. Since these experiments were done in the presence of 0.1 M NaCl, we studied the effect of the DNA binding protein on the exonuclease activity in the absence and the presence of NaCl. An example of such an experiment is shown in Table I.

It can be seen that the presence of 0.1 M NaCl markedly inhibits the exonuclease activity (Table I, lines 1 and 4) whereas the addition of the T7 DNA binding protein to a reaction containing NaCl stimulates the exonuclease activity around 13-fold (Table I, lines 4 and 5). Addition of DNA binding protein to the exonuclease in the absence of salt produces nearly a 2-fold stimulation of activity (Table I, line 1 vs. 2).

T7 DNA Binding Protein Overcomes the Inhibitory Effects of Several Salts. Several salts were examined for their inhibitory effects, and it was found that KCl, LiCl, and CsCl all inhibited the exonuclease activity to an extent comparable to that caused by NaCl. As previously reported (Kerr & Sadowski, 1972a), 20 mM KCl has a stimulatory effect (data not shown). Furthermore, the T7 DNA binding protein overcame the inhibitory effects of all the salts tested, producing stimulations that ranged from 8- to 20-fold. Since the intracellular concentration of K^+ ions in *E. coli* may be as high as 500 mM depending on the medium (Epstein & Schultz, 1965), it is possible that the ability of the binding protein to overcome the inhibitory effects of salt is physiologically important.

E. coli DNA Binding Protein Does Not Stimulate the Gene 6 Exonuclease. Both the single-strand DNA binding protein

Table II: Effect of *E. coli* DNA Binding Protein on T7 Gene 6 Exonuclease[a]

additions	nmol of DNA made acid soluble
(1) gene 6 exonuclease	0.023
(2) gene 6 exonuclease + *E. coli ssb* (4 μg)	0.011
(3) gene 6 exonuclease + *E. coli ssb* (20 μg)	0.029
(4) gene 6 exonuclease + T7 DNA binding protein	0.312

[a] Reaction mixtures (0.1 mL) contained 50 mM Tris HCl buffer, pH 8.0 5 mM $MgCl_2$, 1 mM dithiothreitol, 20 mM KCl, 100 mM NaCl, 10 nmol of ^{3}H-labeled T7 DNA (31 000 cpm/nmol), and 0.8 unit of gene 6 exonuclease.

(*ssb*) of *E. coli* and the T7 DNA binding protein have been shown to stimulate the activity of the T7 DNA polymerase on a gapped template (Reuben & Gefter, 1974) and to stimulate the ability of the T7 DNA polymerase and T7 DNA primase to replicate duplex DNA (Romano & Richardson, 1979). It was therefore of interest to determine whether the *E. coli* protein would stimulate the activity of the gene 6 exonuclease. From the data in Table II it is apparent that while the T7 binding protein stimulated the exonuclease activity markedly, even very high levels of the *E. coli* protein had no effect. The protein was nevertheless fully active when assayed with T7 DNA polymerase and DNA primase according to Romano & Richardson (1979) (data not shown).

Stoichiometry of the Reaction. In an attempt to gain some insight into the mechanism of the effect of binding protein on exonuclease activity, we systematically varied the substrate concentration, exonuclease concentration, and the amount of DNA binding protein and measured the exonuclease activity.

Examination of data resulting from experiments of this type shows that the stimulatory effect produced by the binding protein seemed to saturate near a constant concentration of binding protein irrespective of whether the enzyme or substrate concentration was varied (see Figure 1). This may mean that the binding protein must achieve a certain critical concentration before it exerts its stimulatory effect. As we will argue below, the shape of the curves in Figure 1 can be interpreted as indicating a loose binding of the DNA binding protein to either the DNA substrate or the exonuclease.

Hydrolysis of the Initial Phosphodiester Bond Is Not Salt Sensitive. In order to determine whether the effects of salt and DNA binding protein were occurring at the initial stage of exonucleolytic digestion, we examined the action of the exonuclease upon a DNA substrate labeled at its 5′ termini with ^{32}P by polynucleotide kinase. Since the exonuclease acts from 5′ termini (Kerr & Sadowski, 1972b), the liberation of ^{32}P radioactivity reflects the initiation of the reaction and the first phosphodiester bond cleavage. Since this reaction is extremely rapid, the experiments were done at 0 °C and with an 8-fold excess of DNA termini over enzyme molecules.

The results in Figure 2 showed that the presence of NaCl had little discernible effect on the initiation of the exonuclease digestion. The failure of NaCl to inhibit hydrolysis of the initial phosphodiester bond was not due to the fact that this experiment was done at 0 °C. When the hydrolysis of uniformly labeled T7 DNA was measured at 0 °C under conditions identical with those shown in Figure 2, the rate of hydrolysis was still markedly inhibited by NaCl, and this inhibition could be overcome by the presence of the DNA binding protein (data not shown).

Since the hydrolysis of the first phosphodiester bond was not inhibited by NaCl, the binding protein did not exert a

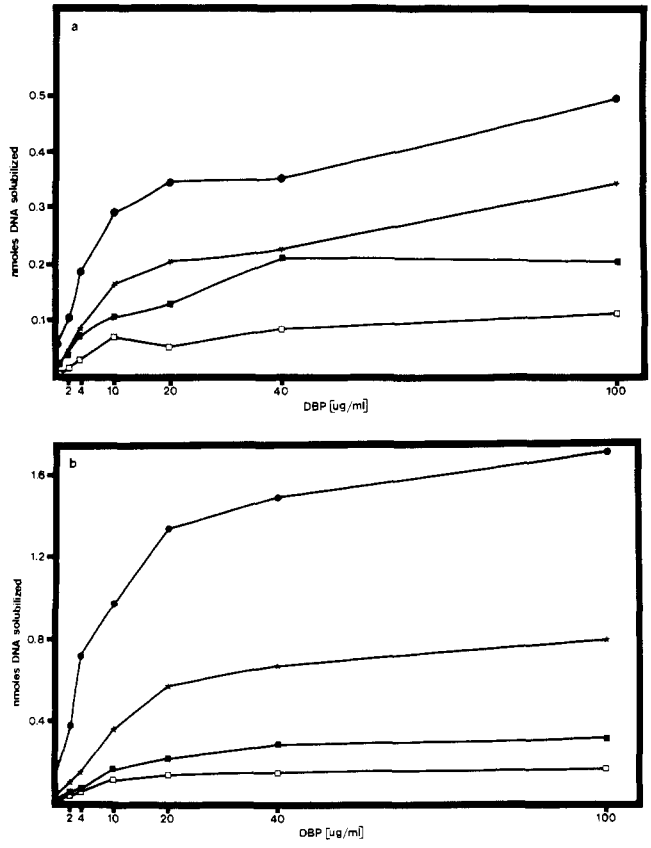


FIGURE 1: (a) Effect of varying substrate concentrations on the stimulatory effect of T7 DNA binding protein on gene 6 exonuclease activity. Reaction mixtures (0.05 mL) contained 50 mM Tris-HCl buffer, pH 8.0, 5 mM $MgCl_2$, 20 mM KCl, 1 mM dithiothreitol, 0.1 M NaCl, 0.5 unit of T7 gene 6 exonuclease, and increasing amounts of T7 DNA binding protein. The substrate concentration of ^{3}H-labeled T7 DNA (48 000 cpm/nmol) was varied as follows: (□—□) 13 μM; (■—■) 26 μM; (★—★) 52 μM; (●—●) 104 μM. The nanomoles of added DNA made acid soluble after 15 min at 37 °C were plotted against the concentration of T7 DNA binding protein added (micrograms per milliliter). (b) The reactions had the same composition as in (a) except that the DNA concentration was constant (104 μM) and the amount of exonuclease added per assay was varied as follows: (□—□) 0.25 unit; (■—■) 0.5 unit; (★—★) 1 unit; (●—●) 2 units.

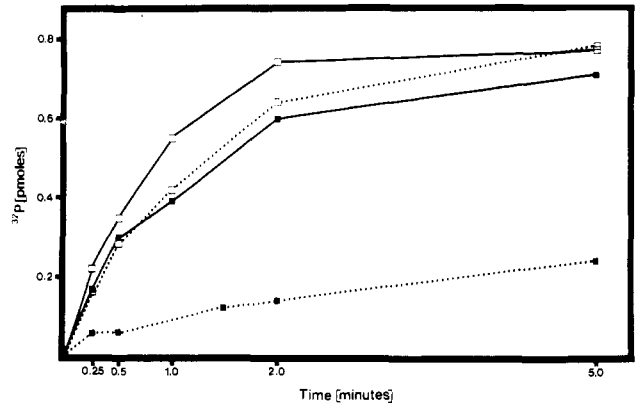


FIGURE 2: Effect of T7 DNA binding protein on the hydrolysis of 5′-^{32}P-labeled DNA by the gene 6 exonuclease. Reaction mixtures (0.4 mL) contained 50 mM Tris-HCl buffer, pH 7.4, 5 mM $MgCl_2$, 20 mM KCl, 1 mM dithiothreitol, and either no NaCl or 0.1 M NaCl. Each reaction contained 3.2 nmol (total nucleotide) of *HaeIII*-digested, 5′-^{32}P-labeled T7 DNA (about 6 pmol of termini, 20 000 cpm/pmol), 1.5 units of gene 6 exonuclease, and 3 μg of T7 DNA binding protein where indicated. After the indicated time at 0 °C, 50-μL aliquots were removed for determination of acid-soluble ^{32}P radioactivity. (□···□) −NaCl, −binding protein. (□—□) +NaCl, −binding protein. (■—■) +NaCl, +binding protein. (■···■) −NaCl, +binding protein.

Table III: Effect of NaCl and T7 DNA Binding Protein on K_m and V_{max} of T7 Gene 6 Exonuclease[a]

additions	kinetic constants	
	K_m (mM)	V_{max} (nmol of nucleotide hydrolyzed/min)
(1) no added NaCl	0.97	4.82
(2) 0.1 M NaCl	0.26	0.11
(3) 0.1 M NaCl + T7 DNA binding protein	0.097[b]	0.34[b]

[a] Reactions (0.2 mL) contained 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 20 units of gene 6 exonuclease. The substrate concentrations (^{3}H-labeled T7 DNA) were 20 μM, 40 μM, 100 μM, 200 μM, and 1 mM (total nucleotide). After 5, 10, and 20 min at 37 °C, 50-μL aliquots were removed for determination of acid-soluble material. Initial velocities were determined by computer fitting of a polynomial to the plots of enzymatic activity vs. time. The experimental data were fitted by computer to the Michaelis–Menten equation according to the median method of Cornish-Bowden & Eisenthal (1974, 1978). [b] Means of three separate experiments.

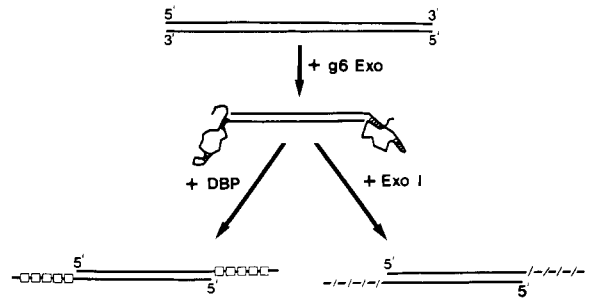


FIGURE 3: Schematic diagram of possible mechanism of inhibition of gene 6 exonuclease by NaCl. A duplex DNA molecule is digested by the gene 6 exonuclease to yield a molecule with 3′ single-stranded tails. These tails then undergo intrastrand base pairing that somehow inhibits access of the exonuclease to the 5′ end. The DNA binding protein could stimulate the exonuclease by coating the single-stranded tails and melting the intrastrand base pairs. The stimulatory effect of binding protein should be mimicked by exonuclease I, which digests the 3′ single-stranded tails.

Table IV: Effect of *E. coli* Exonuclease I on the T7 Exonuclease[a]

additions	activity (nmol of acid-soluble nucleotide)
(1) gene 6 exonuclease alone	0.12
(2) gene 6 exonuclease + *E. coli* exonuclease I	0.35
(3) gene 6 exonuclease + T7 DNA binding protein	4.3
(4) T7 DNA binding protein alone	0.05
(5) *E. coli* exonuclease I alone	0.05
(6) denatured DNA + *E. coli* exonuclease I	1.12

[a] Reaction mixtures (0.1 mL) contained 50 mM Tris-HCl buffer, pH 8.0, 5 mM $MgCl_2$, 1 mM dithiothreitol, 20 mM KCl, 0.1 M NaCl, and 10 nmol of native ^{14}C-labeled T7 DNA (lines 1–5, 2850 cpm/nmol) or denatured ^{14}C-labeled T7 DNA (line 6). Where indicated, the following amounts of proteins were added: T7 exonuclease, 4 units; T7 DNA binding protein, 5 μg; *E. coli* exonuclease I, 0.225 unit. Acid-soluble material was determined after 15 min at 37 °C.

stimulatory influence on this reaction and may have even caused a slight inhibition of the rate of cleavage in the presence of salt. Most surprising, however, was the finding that in the absence of NaCl, the binding protein markedly retarded the rate of cleavage of the 5′-terminal mononucleotide. Since these reactions were done with an excess of termini over enzyme molecules, one possible interpretation of this result is that the binding protein has the ability to change the mode of action of the exonuclease from a random one to a processive one. That is, the exonuclease stays bound to a DNA molecule for a longer time before coming off to begin hydrolysis of another molecule.

Effect of Binding Protein on K_m and V_{max} of Gene 6 Exonuclease. In order to determine whether the binding protein might affect the affinity of the exonuclease for its DNA substrate, we determined the K_m and the V_{max} for the exonuclease in the presence and absence of salt and the effect of binding protein on these parameters. Initial velocities were measured in the presence of varying concentrations of T7 DNA and the K_m and V_{max} determined by the method of Cornish-Bowden & Eisenthal (1974, 1978).

From the data in Table III, it is apparent that NaCl causes a dramatic decrease in the V_{max} of the exonuclease (4.82 to 0.11 nmol/min) while causing a decrease in the K_m from 0.97 to 0.26 mM. The stimulatory effect of binding protein on the exonuclease activity in the presence of NaCl is apparently caused by a combined decrease in the K_m (0.26 to 0.097 mM) and an increase in the V_{max} (0.11 to 0.34 nmol/min).

Inhibition of Exonuclease by NaCl Is Not Due to the 3′ Single-Stranded Terminus. The 5′-exonuclease activity of the gene 6 product generates a DNA molecule that has protruding 3′ single-stranded tails (Kerr & Sadowski, 1972b). Since the presence of salt inhibits the gene 6 exonuclease, it might be thought that the salt might facilitate intrastrand base pairing in this tail that in turn might loop back on itself and sterically hinder access of exonuclease molecules to the recessed 5′ terminus (see Figure 3). The DNA binding protein might overcome this inhibition by coating the 3′ single-stranded tail and melting the intrastrand paired bases.

If this model were correct, then removal of the 3′ single-stranded tail during the reaction should abolish the hypothetical inhibitory effect. To test this hypothesis we used *E. coli* exonuclease I, a 3′-exonuclease that is specific for single-stranded DNA, to remove the single-stranded tails. If the hypothesis were correct, we might expect that exonuclease I should mimic DNA binding protein in stimulating the gene 6 exonuclease.

The results of such an experiment are shown in Table IV, where it can be seen that the exonuclease I stimulated the gene 6 exonuclease only marginally. The gene 6 exonuclease alone hydrolyzed 0.12 nmol of DNA (Table IV, line 1) whereas addition of exonuclease I caused the hydrolysis of 0.35 nmol (Table IV, line 2). Since the exonuclease I would be expected to hydrolyze an amount of DNA equal to that hydrolyzed by the gene 6 exonuclease, the actual degree of stimulation by the exonuclease I was estimated to be only about 0.05 nmol of DNA hydrolyzed. In contrast, the addition of DNA binding protein caused a marked stimulation of the gene 6 exonuclease (Table IV, line 3). The amount of exonuclease I added would have been sufficient to hydrolyze in excess of 1 nmol of single-stranded DNA (Table IV, line 6).

Thus, we conclude that the inhibitory effect of NaCl on the gene 6 exonuclease is not attributable to the 3′ single-stranded tail generated during the reaction. Furthermore, the stimulatory effect of the DNA binding protein does not appear to be due to its binding to this single-stranded tail.

Failure To Detect a Protein–Protein Interaction between the Gene 6 Exonuclease and the T7 DNA Binding Protein. Numerous attempts to detect a complex between the exonuclease and the DNA binding protein were unsuccessful. The methods used included sucrose gradient centrifugation in the

absence and in the presence of DNA, gel filtration chromatography, and affinity chromatography using purified DNA binding protein as the affinity ligand and passing labeled T7-infected cell extracts over the affinity column.

Discussion

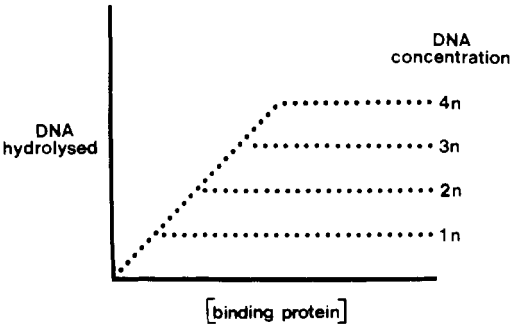
The purified T7 DNA binding protein has a large repertoire of functions that include its ability to bind cooperatively to single-stranded DNA (Banks et al., 1981), to stimulate the T7 DNA polymerase (Scherzinger et al., 1973; Reuben & Gefter, 1973), and to promote renaturation of DNA (Sadowski et al., 1980). We have focused in this paper on its ability to stimulate specifically the activity of the gene 6 exonuclease in the presence of moderate concentrations of salt. We have extended our previous studies of the specificity of this stimulation (Sadowski et al., 1980) to include the *E. coli* single-stranded DNA binding protein. Since this protein had previously been shown to stimulate the T7 DNA polymerase with and without added T7 gene 4 priming protein, it was perhaps surprising that the *E. coli* protein had no effect on the gene 6 exonuclease activity. This points out a functional difference between the DNA binding proteins of T7 and *E. coli* and suggests that the T7 protein may have functions in vivo apart from a possible role in DNA replication.

The stimulatory effect of the T7 binding protein is most marked in the presence of moderate concentrations of NaCl (0.1 M). Such concentrations of salt inhibit the exonuclease markedly, and this inhibitory effect can be largely reversed by the addition of T7 binding protein. The inhibition can be produced by several monovalent cations including potassium. Since the intracellular concentration of potassium ions may be as high as 0.5 M (Epstein & Schultz, 1965), it is likely that the gene 6 exonuclease would be inactive were it not for the presence of the T7 DNA binding protein.

The inhibitory effect of salt is on the V_{max} of the exonuclease: salt actually decreased the K_m of the enzyme for DNA. It is not clear why salt has such a dramatic effect on the V_{max} of the enzyme.

The effect could be due to a change in the conformation of the enzyme or the substrate or both. The salt might cause a local change of conformation of the DNA that would obstruct the progress of the exonuclease. The binding protein might in turn prevent or reverse this change, thus stimulating the progress of the enzyme. From the experiments reported here, we can say that the salt is not affecting the ability of the enzyme to carry out its initial hydrolytic event at a 5′ terminus (Figure 2). Likewise, it appears that the inhibitory effect of salt and its reversal by the binding protein are not due to the creation of a 3′ tail of single-stranded DNA during the exonucleolytic reaction (Table IV).

The kinetic results (Table III) and the effects of varying the concentrations of exonuclease, binding protein, and DNA when coupled with our inability to detect complexes between the exonuclease and the binding protein in the presence or absence of DNA suggest a loose binding model to account for effects of the binding protein reported here. If the binding protein were forming a tight complex with DNA, one would expect that for a given concentration of DNA, the substrate would be activated to its maximum extent by a saturating amount of binding protein and then the activity would level off. Increasing the DNA concentration would result in a curve in which the slope would remain the same but the activity would saturate at a higher level with more added binding protein. Thus, plotting several results in which the DNA concentration and the binding protein concentration are varied should generate the following family of curves:



This is clearly different from the observed results (Figure 1a) where the slope of the curves increased with increasing DNA concentration. By analogy, tight binding between the exonuclease and the binding protein would generate a family of curves with the same slope when the enzyme concentration and the binding protein concentration were varied. In fact, the slopes of the curves increased with increasing enzyme concentration as well (Figure 1b). Thus, there may be some loose interaction between the binding protein and the exonuclease and/or the DNA substrate.

While the present studies do not allow us to define precisely the mechanism of stimulation of the T7 gene 6 exonuclease by the homologous single-stranded DNA binding protein, they nevertheless point to an important role for this protein in T7 DNA metabolism in vivo.

Acknowledgments

We thank Janice Reid for her help in typing the manuscript.

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Purification of Human Neutrophil Collagenase and Production of a Monospecific Antiserum†

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ABSTRACT: Although there is good evidence for the presence of human neutrophil (PMN) collagenase, only moderate purification has been reported. The probable explanation for this fact is that most assays used to specifically measure collagenase activity are not reliable if high levels of several different proteases are also present in the assay mixture. The PMN granule is just such a concentrated mixture. Therefore, polyacrylamide gel electrophoresis was used to identify and quantitate the α1 3/4 and α2 3/4 cleavage products diagnostic for mammalian collagenase. White cells (85% PMN's) were lysed in 0.34 M sucrose and the granules were obtained. The granules were lysed by sonication, and the lysate was chromatographed on a Sephadex G-200 column followed by a Trasylol–Sepharose 4B column. This procedure resulted in a 1350-fold purification and a yield of 75 μg of enzyme/unit of blood. The collagenase was inhibited by ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid but not by sulfhydryl or serine protease inhibitors. The preparation was free of elastase, which has been shown to cleave type III collagen into α1 3/4 and α1 1/4 pieces. The p*I* of collagenase was shown to be 4.7 by isoelectric focusing, and the enzyme lost activity below a pH of 6.5 if collagen was absent. Antiserum was produced by 100-μg injections of the purified collagenase into rabbits. Titers were measured by the enzyme-linked immunosorbent assay. For determination of the specificity, collagenase and PMN extract were isoelectrically focused and blotted onto nitrocellulose. The antibody recognized only one band of protein in the PMN extract, which comigrated with the purified collagenase.

Several authors have reported the presence of human PMN[1] collagenase (Lazarus et al., 1968a,b; Turto et al., 1977; Horwitz et al., 1977; Uitto et al., 1979; Macartney & Tschesche, 1980). These enzymes are similar in action to other collagenases isolated from human skin (Stricklin et al., 1977), rheumatoid synovium (Evanson et al.; 1967; Wooley et al., 1975; Harris, 1972), periodontium (Christner, 1980), macrophages (Senior et al., 1972), and platelets (Chesney et al., 1974), as well as collagenase from other vertebrate sources (Barrett, 1979). Each enzyme has been reported to degrade collagen in its native triple-helical conformation into two fragments a 3/4 and 1/4 piece. The exact site of this cleavage has been determined and is between residues 772 and 773, a glycine–isoleucine bond in the α1 chain and a glycine–leucine bond in the α2 chain (Fietzek et al., 1973; Highberger et al., 1975; Gross et al., 1974).

The fate of the 3/4 and 1/4 fragments depends on the temperature in which the collagenase performs its cleavage. At body temperature (37 °C) both the 3/4 and 1/4 fragments are thermally unstable as triple helices and unwind to form separate α-chain fragments. These fragments are susceptible to general proteolytic digestion because it is the triple helix of collagen that confers unique relative resistance to all known neutral general proteases (McCroskery et al., 1973). When collagenase is assayed at a temperature lower than the melting temperature for the 3/4 helix (32 °C) or 1/4 helix (28 °C), then these fragments each remain as a triple helix (Sakai & Gross, 1967). They are not susceptible to general proteases, can be isolated, and are diagnostic for a true collagenase (Turto et al., 1977; Christner, 1980).

Recently, there has been some confusion about what is a true collagenase. There have been reports in the literature that PMN elastase can degrade certain type III (Gadek et al., 1980) and IV (Mainardi et al., 1980) collagens. Adding to the confusion is the fact that if the fibrillar gel dissolution assay

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[1] Abbreviations: DFP, diisopropyl fluorophosphate; PMSF, phenylmethanesulfonyl fluoride; NEM, *N*-ethylmaleimide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMN, polymorphonuclear (leucocyte); ELISA, enzyme-linked immunosorbent assay; SAPNA, succinyltrialanine *p*-nitroanilide; Temed, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; ACD, acid-citrated dextrose; PBS, phosphate-buffered saline.

0006-2960/82/0421-6005$01.25/0