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DNA stimulates ATP-dependent proteolysis and protein-dependent ATPase activity of protease La from *Escherichia coli*

(*lon* gene/*capR*/protein degradation/DNA-binding protein)

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ABSTRACT **The product of the *lon* gene in *Escherichia coli* is an ATP-dependent protease, protease La, that also binds strongly to DNA. Addition of double-stranded or single-stranded DNA to the protease in the presence of ATP was found to stimulate the hydrolysis of casein or globin 2- to 7-fold, depending on the DNA concentration. Native DNA from several sources (plasmid pBR322, phage T7, or calf thymus) had similar effects, but after denaturation the DNA was 20–100% more effective than the native form. Although poly(rA), globin mRNA, and various tRNAs did not stimulate proteolysis, poly(rC) and poly(rU) were effective. Poly(dT) was stimulatory but $(dT)_{10}$ was not. In the presence of DNA as in its absence, proteolysis required concomitant ATP hydrolysis, and the addition of DNA also enhanced ATP hydrolysis by protease La 2-fold, but only in the presence of casein. At much higher concentrations, DNA inhibited proteolysis as well as ATP cleavage. Thus, association of this enzyme with DNA may regulate the degradation of cell proteins *in vivo*.**

The product of the *lon* gene (also called *capR* or *deg*) in *Escherichia coli* (1) has recently been shown to be the ATP-dependent protease (2, 3), protease La (2, 4–6). This novel enzyme is an ATPase (2–4) as well as a serine protease (7, 8), and inhibition of ATP hydrolysis also prevents proteolysis (2–6). This coupling of protein and ATP hydrolysis can account for the energy requirement for protein breakdown in intact cells (9–14). Furthermore, in *lon*$^-$ strains, the rates of degradation of abnormal proteins (14–17) and certain normal proteins (17, 18) are decreased. Thus, this protease appears to catalyze the rate-limiting steps in degradation of cell proteins *in vivo* (2, 4, 14).

One additional important property of the *lon* gene product is that it binds to DNA with high affinity (1). The precise biochemical reasons why these multiple functions (i.e., protein hydrolysis, ATP hydrolysis, and DNA binding) reside in the same protein are unclear. The present studies were undertaken to determine whether binding of the protease to DNA may alter its proteolytic function. One precedent for DNA affecting a proteolytic process is in the cleavage of bacteriophage λ repressor and the lexA protein by the *recA* gene product (19, 20). These specific cleavages which are essential for phage induction require the presence of polynucleotides and also ATP or an ATP analog (19, 20). An influence of DNA binding on the activity of protease La could have profound physiological implications and may help explain why the *lon* gene product affects a wide variety of cellular processes (21), including the rapid degradation of certain DNA-binding proteins (18), the expression of certain genes (21), sensitivity to UV and other DNA-damaging agents (22, 23), and even cell division (24, 25).

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MATERIALS AND METHODS

Bacterial Strains. *E. coli* K-12 strain RGC121/pJMC40 was provided by A. Markovitz. This strain (1) carries the wild-type *lon*$^+$ (*capR*$^+$) allele on the chromosome and on the plasmid pSC101. These cells were grown in LB medium supplemented with tetracycline at 5 μg/ml, harvested at stationary phase, and kept frozen at −70°C. *E. coli* strain SA1026/pAA101 was obtained from S. Adhya. This strain carries *his*$^-$, *cya*$^-$, and *gal*$^\nabla$ alleles on the chromosome and the complete *gal* operon on the plasmid pBR313.

Preparation of *lon* Gene Product. Initial experiments used lon protein provided by A. Markovitz, which was purified according to the method of Zehnbauer *et al.* (1). For most experiments, we purified the enzyme from frozen cells by a modification (7) of that method. The cells (100 g) were resuspended in 0.1 M KH_2PO_4/K_2HPO_4, pH 6.5/10 mM 2-mercaptoethanol/1 mM Na_2EDTA/20% (vol/vol) glycerol, and disrupted by French press at 13,000 psi (1 psi = 6.9×10^3 Pa). After centrifugation at 30,000 × *g* for 1 hr, the resulting supernatant was spun at 110,000 × *g* for 2 hr to remove ribosomes. The extracts were then fractionated by chromatography on phosphocellulose and then on DEAE-cellulose as described by Zehnbauer *et al.* (1). The fractions with high specific activity were pooled, concentrated by ultrafiltration with an Amicon XM 100 membrane, and loaded onto a Sephacryl S-300 column (1.5 × 55 cm) equilibrated with 50 mM Tris·HCl, pH 7.8/5 mM $MgCl_2$/100 mM NaCl/20% (vol/vol) glycerol. Fractions (1.5 ml) were collected at a flow rate of 20 ml/hr. The lon protein eluted from this step was >97% pure when analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis as described by Laemmli (26).

Assays. Degradation of [*methyl*-^{3}H]casein or [*methyl*-^{14}C]-apohemoglobin was measured in the presence and absence of 1 mM ATP (2, 7). All assays were carried out in 25 mM Tris·HCl, pH 7.8/5 mM $MgCl_2$ with and without DNA. The incubations were for 30 min at 37°C with the purified *lon* gene product in a final volume of 0.5 ml. Rates of protein degradation were expressed as the percentage of [^{3}H]casein hydrolyzed to trichloroacetic acid-soluble material in 30 min in a 0.5-ml reaction mixture.

ATPase activity was assayed by using purine-labeled [^{3}H]ATP in the same reaction conditions used for the protease assay but in a final volume of 50 μl. The reaction products were separated on polyethyleneimine-cellulose plates as described by Arai *et al.* (27), and the conversion of [^{3}H]ATP to [^{3}H]ADP was determined. For convenience, ATPase activity was routinely measured in much smaller volumes than the protease assay. Control experiments showed that rates of ATP cleavage and proteolysis were similar by the two approaches.

Kinetic values (K_m and V_{max}) were determined graphically by the method of Lineweaver and Burk.

Materials. Poly(dT) and poly(rU) were obtained from PL Biochemicals. According to the manufacturer, these polynucleotides contained an average of 1000 bases (approximately 8S). Poly(rA), poly(rC), calf thymus DNA, and yeast tRNA were purchased from Sigma; phosphocellulose (P11) and DEAE-cellulose (DE-52) were from Whatman; Sephacryl S-300 was from Pharmacia; *Eco*RI was from Bethesda Research Laboratories; [^{3}H]ATP was from New England Nuclear; polyethyleneimine-cellulose plates were from Brinkman. Globin mRNA was a gift from Eric Rosenthal, M13 DNA and T7 DNA were kindly provided by Charles Richardson, and liver rRNA was a gift from Paul Wassarman. Plasmid pBR322 DNA and pAA101 DNA were prepared by standard methods (28). To denature pBR322 DNA, the circular plasmid was cut with *Eco*RI and boiled for 5 min followed by rapid cooling on ice. Polynucleotide concentrations were determined by measuring their A_{260}. [^{3}H]Casein and [^{14}C]globin were prepared as described by Rice and Means (29). ^{125}I-Labeled insulin was purchased from Cambridge Nuclear.

RESULTS

Effects of DNA on Protease La. Plasmid pAA101 DNA markedly stimulated [^{3}H]casein degradation by purified protease La in the presence of ATP (Fig. 1). No hydrolysis was seen in the absence of ATP, whether DNA was added or not. An even larger stimulation by DNA was observed with [^{14}C]globin than with [^{3}H]casein (Table 1). However, casein was used routinely as a substrate because it was hydrolyzed more rapidly and thus was more sensitive (2, 7, 8). ^{125}I-Labeled insulin was not degraded by this enzyme either in the presence or in the absence of the DNA. In similar experiments, we found no effect of DNA on the activity of the various ATP-independent proteases purified from *E. coli* including proteases Do, Re, Mi, Fa, So, Pi, and Ci (8) whether ATP was added or not (data not shown). Thus, the effect of DNA was specific to protease La and was not due to some DNA-induced modification of the substrates.

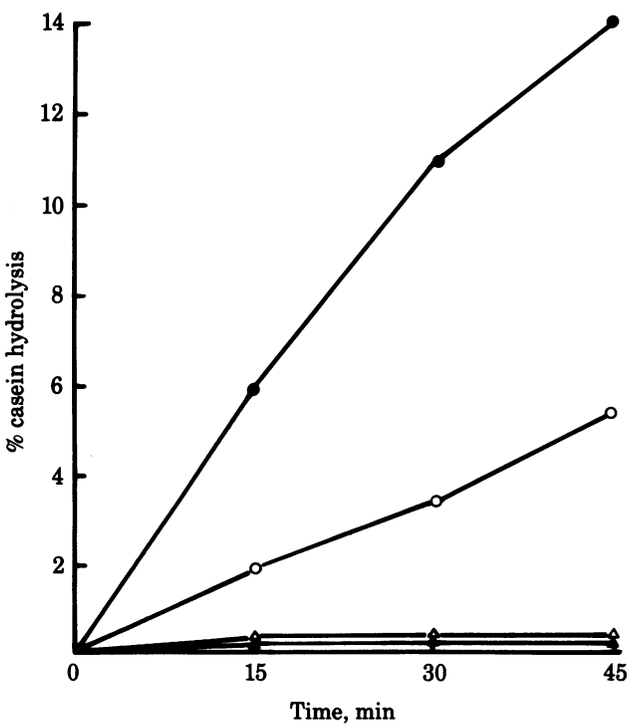

FIG. 1. Effect of plasmid pAA101 DNA on protease La activity. Casein hydrolysis by purified protease La was measured with no addition (△), with 1 mM ATP (○), with DNA (▲) at 5 μg/ml, and with both ATP and DNA (●). Incubations were at 37°C with reaction mixtures (0.5 ml) containing 0.68 μg of protease La and 23.5 μg of [^{3}H]casein. The reaction was terminated by addition of 40 μl of bovine serum albumin (30 mg/ml) as carrier and then 60 μl of 100% (wt/vol) trichloroacetic acid.

Table 1. DNA stimulation of ATP-dependent proteolysis by protease La

Substrate	Addition	% substrate hydrolyzed: No DNA	% substrate hydrolyzed: With DNA	DNA stimulation, % of control
A. Effects on degradation of different proteins				
[^{3}H]Casein	ATP	3.4	11.0	320
[^{14}C]Globin	ATP	0.31	3.0	968
^{125}I-Labeled insulin	ATP	0.0	0.0	—
B. Requirement for ATP hydrolysis				
[^{3}H]Casein	ATP	2.5	13.3	530
[^{3}H]Casein	CTP	1.9	5.6	295
[^{3}H]Casein	UTP	1.7	5.1	300
[^{3}H]Casein	dATP	2.1	10.6	505
[^{3}H]Casein	ATP + vanadate	0.07	0.28	—
[^{3}H]Casein	[β,γ-methylene]ATP	0.01	0.02	—

Experiment A used plasmid pAA101 DNA at 5 μg/ml. In Experiment B, denatured calf thymus DNA was used at the same concentration; vanadate, an inhibitor of ATP hydrolysis, was at 0.1 mM, and other nucleotides were at 1 mM. Assays were performed for 30 min as described in Fig. 1.

Effects of Various Polynucleotides. A number of other DNA species, both double-stranded and single-stranded, also stimulated the ATP-dependent degradation of [^{3}H]casein 2- to 7-fold (Table 2). Native double-stranded DNA, including intact plasmid pBR322 DNA, phage T7 DNA, and calf thymus DNA, was active but after denaturation the stimulatory effect was about 20–100% greater. Because *lon*$^-$ cells are particularly sensitive to UV (22, 23), we tested whether UV-damaged DNA affected proteolysis by this *lon* gene product. After UV-irradiation, plasmid pAA101 DNA was also about 30% more effective than the untreated material. Although these effects were relatively small, UV treatment and denaturation repeatedly caused increased stimulatory effect.

None of the natural RNAs tested—including globin mRNA, yeast tRNA, *E. coli* W tRNA, and liver rRNA—promoted casein degradation as did DNA (although a very small increase in protease La activity was observed with liver rRNA). Poly(rA), which permits maximal ATP-dependent cleavage of phage λ repressor by recA (19), had no significant effect at any concentrtion tested (Table 2; Fig. 2*A*). However, poly(rU) and poly(rC), as well as poly(dT), were effective in enhancing proteolysis. Unlike poly(dT), oligonucleotides composed of 10 thymidylic acids had no stimulatory effect.

The increase in the rate of proteolysis also depended on the concentration of DNA (Fig. 2*A*). As the DNA concentration increased, the stimulation of casein hydrolysis by phage M13 DNA increased sharply and reached a maximum at about 1 μg of the DNA in the 0.5-ml reaction mixture. Poly(dT), M13 DNA, and plasmid pBR322 DNA had similar dose–response curves but poly(dT) consistently caused a greater stimulation. When denatured calf thymus DNA was added at much higher concentrations (i.e., >20 μg per 0.5-ml reaction mixture) the

Table 2. Effect of different polynucleotides on casein hydrolysis by protease La in presence of ATP

Polynucleotide	Form	% casein hydrolyzed	Relative activity, % of control
None	—	2.3	100
pAA101 DNA	Native	6.5	283
	Denatured	8.4	365
	UV-irradiated	7.9	343
pBR322 DNA	Native	5.3	230
	Denatured	6.4	278
T7 DNA	Native	5.4	235
	Denatured	7.1	309
M13 DNA	Native	6.8	296
Calf Thymus DNA	Native	7.8	339
	Denatured	14.7	639
Liver rRNA	Native	3.1	130
Globin mRNA	Native	2.5	109
Yeast tRNA	Native	2.4	104
E. coli W tRNA	Native	2.5	109
Poly(rA)		2.5	109
Poly(rC)		5.1	222
Poly(rU)		6.2	270
Poly(dT)		9.7	422
$(dT)_{10}$		2.2	96

The plasmid pAA101 DNA (250 μg/ml) was treated with UV for 60 sec at a distance of 25 cm from one germicidal lamp. Assays were performed as in Table 1. Polynucleotides were present at 5 μg/ml. These data are the average of at least three independent experiments, each of which showed similar effects on proteolysis. In control experiments, no degradation of casein was observed in the presence of polynucleotide alone.

proteolytic reaction gradually decreased until no casein hydrolysis was detectable (Fig. 2*B*). The inhibitory effect by very high DNA concentrations was also observed with other DNA species (data not shown).

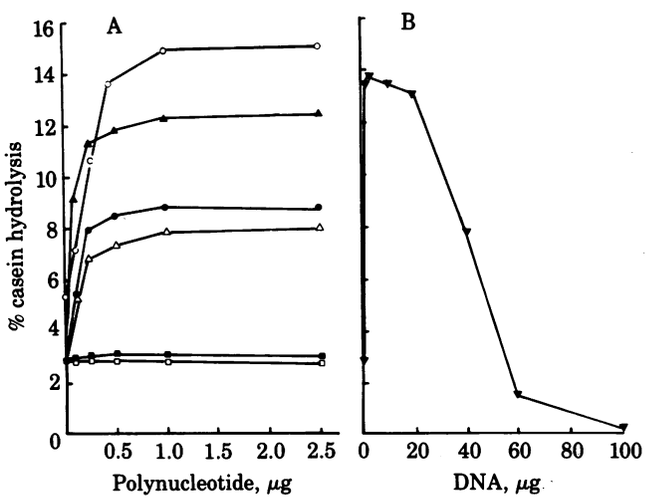

FIG. 2. Effects of polynucleotide concentration on proteolytic activity of protease La. Casein hydrolysis by protease La was measured as described in Fig. 1 in the presence of various polynucleotides and 1 mM ATP. (*A*) Effects of poly(dT) (▲), M13 DNA (●), pBR322 DNA (△), poly(rA) (■), and $(dT)_{10}$ (□). Also shown is the stimulatory effect of M13 DNA (○) when twice the amount of protease La (1.36 μg) was present in the reaction mixture. (*B*) Denatured calf thymus DNA tested at both low (stimulatory) and high (inhibitory) concentrations.

The DNA concentrations showing half-maximal stimulation depended on the concentration of protease La in the reaction mixture. With 0.68 and 1.36 μg of protease La, the M13 DNA concentrations showing half-maximal stimulation were about 0.12 and 0.22 μg (Fig. 2*A*). Thus, the DNA concentration providing half-maximal stimulation appeared approximately proportional to protease La concentration.

In the presence of DNA, ATP-dependent proteolysis occurred more rapidly but still showed the same K_m (8 μM) for ATP as seen in the absence of DNA (data not shown). The relative increase in proteolytic rate was dependent on the concentration of [^{3}H]casein in a complex fashion. In the presence of poly(dT) (Fig. 3*A*) or calf thymus DNA (data not shown), the apparent V_{max} for casein increased 3- to 4-fold in different experiments (from 49 to 159 pmol/hr for the data in Fig. 3*A*). Because protease La may cleave [^{3}H]casein at many sites to yield acid-soluble radioactivity, these stimulatory effects of DNA may be explained either by more rapid hydrolysis of the same peptide bonds in the substrate or by the enzyme cleaving the casein molecules at a larger number of sites. In addition, DNA also consistently increased the K_m for casein almost 2-fold (from 0.74 to 1.3 μM for the data in Fig. 3*A*), and at very low casein concentration (<0.3 μM), poly(dT) or calf thymus DNA actually decreased the rate of proteolysis (Fig. 3*B* and data not shown). Thus, the change in the dependency on substrate concentration caused by addition of DNA from nearly hyperbolic to sigmoidal suggest some cooperative interaction between the subunits of protease La in the presence of DNA.

ATP Hydrolysis. Larimore *et al.* (4) found that casein hydrolysis by protease La requires concomitant cleavage of ATP,

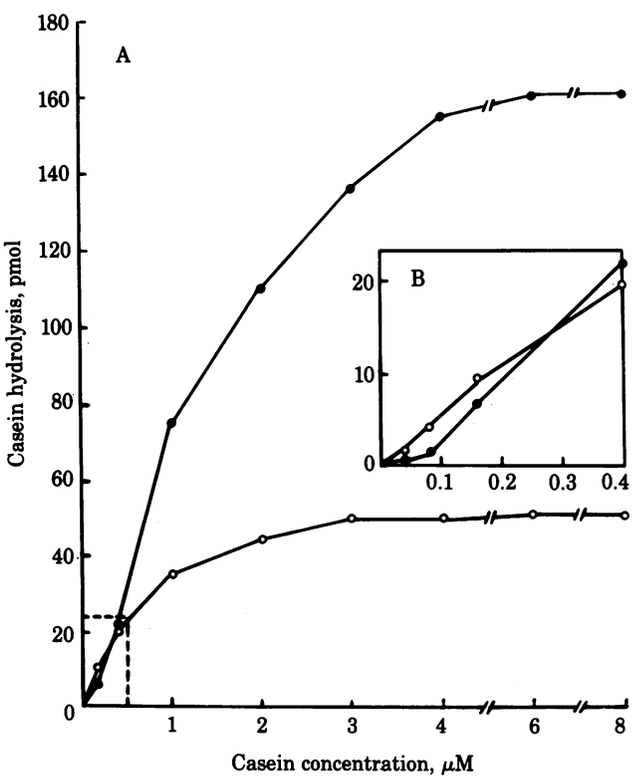

FIG. 3. Effect of poly(dT) on protease La activity at different concentrations of [^{3}H]casein. (*A*) Casein degradation was measured for 30 min without (○) and with (●) poly(dT) at 5 μg/ml as described in Fig. 1. The rate of proteolysis was expressed as pmol of [^{3}H]casein hydrolyzed to acid-soluble form per hour. Graphical analysis of these data indicates a 3.3-fold increase in V_{max} and 1.8-fold increase in K_m. (*B*) Effects of low casein concentrations are shown in a greater detail.

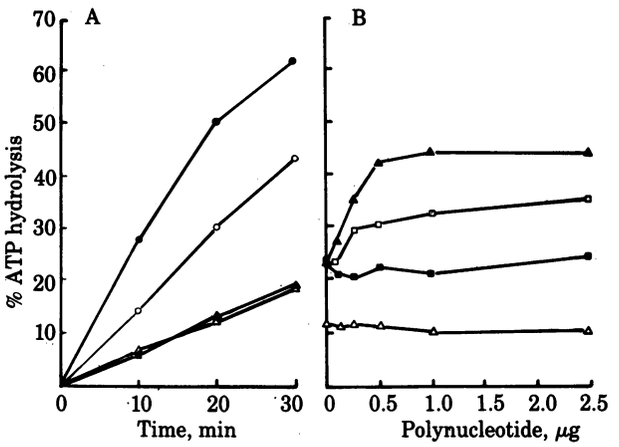

FIG. 4. Effect of DNA on ATPase activity of protease La with and without casein. (*A*) ATPase activity of protease La was measured with no addition (△), with casein (○), with denatured calf thymus DNA (▲), and with both casein and DNA (●). DNA was added at a final concentration of 2.5 μg/50-μl reaction mixture and casein was at 25 μg/50 μl. Incubations were at 37°C with 50-μl reaction mixtures containing 0.68 μg of purified protease La and 0.5 mM ATP including 60 pmol of [^{3}H]ATP (29.5 Ci/mmol). Thus, the ratio of DNA to enzyme was the same as that in Figs. 1, 2, and 3. The reaction was stopped at different times by addition of 5 μl of 20% (wt/vol) $NaDodSO_4$ and the ATP hydrolysis was determined as described by Arai *et al.* (27). ATPase activity was expressed as the percentage of [^{3}H]ATP converted to [^{3}H]ADP in a 50-μl reaction mixture during the incubation period indicated. (*B*) The ATPase was assayed as described above except that reaction mixtures contained various concentrations of polynucleotides and were incubated for 15 min. Denatured calf thymus DNA was tested both with (▲) and without casein (△); poly(dT) (□) and poly(rA) (■) were tested in the presence of casein only. Preparations of casein and DNA by themselves did not show any ATP hydrolysis in control experiments.

in accord with earlier suggestions (13). DNA-stimulated proteolysis also seemed to require ATP hydrolysis because addition of vanadate (0.1 mM), a potent inhibitor of many ATPases, abolished casein degradation in the presence of DNA as in its absence (Table 1). Furthermore, when ATP was replaced with a non-hydrolyzable ATP analog, [β, γ-methylene]ATP, little or no proteolysis by protease La was observed in the presence or absence of DNA (Table 1; refs. 2–6). Other nucleoside triphosphates (CTP, UTP, and dATP) also can support proteolysis by this enzyme but not as well as ATP (2–4). With each of these nucleotides, denatured calf thymus DNA enhanced [^{3}H]casein degradation 3- to 5-fold (Table 1).

Recently, protease La was shown to have an inherent ATPase activity that is stimulated by casein (3, 5, 6). Denatured calf thymus DNA as well as native plasmid pBR322 DNA also enhanced approximately 2-fold the ATP hydrolysis that accompanied casein degradation (Fig. 4*A*; data not shown). However, DNA did not affect the ATPase activity in the absence of the protein substrate (Fig. 4*A*). In similar experiments, poly(dT) also stimulated the casein-dependent ATPase activity but poly(rA) did not (Fig. 4*B*) as was seen with proteolysis (Fig. 2*A*). Furthermore, at high concentrations of calf thymus DNA, at which the stimulation of proteolysis decreased (Fig. 2*B*), the rate of ATP hydrolysis decreased to levels seen in the absence of the DNA (data not shown). Thus, DNA enhances the ATPase activity in a fashion similar to the stimulation of proteolysis, and presumably these two effects are causally related.

DISCUSSION

A number of different DNA species, both native and denatured, increased the rate of protein degradation by protease La (Table 2). Because poly(rC), poly(rU), and poly(dT) were also stimulatory, no specific sequence of deoxynucleotides appears to be essential for this effect. Because the lon protein has been suggested to be a specific repressor of the *gal* operon (21), we systematically investigated the effects of plasmid pAA101 DNA carrying the *gal* operon on proteolysis (Fig. 1). However, this plasmid DNA had a stimulatory effect on proteolysis similar to that of other DNA species (Fig. 1; Table 2). Nevertheless, this effect of DNA seems to be specific because it was not seen with the seven other known *E. coli* proteases (all of which are independent of ATP) (8).

Because the DNA concentration giving half-maximal stimulation of proteolysis appeared to be proportional to the concentration of protease La (Fig. 2*A*), a stoichiometric relationship may exist between DNA and protease molecules for this effect. In the absence of DNA, protease La is a tetramer (2) comprised of 94,000-dalton subunits. In the presence of the single-stranded DNA of phage M13, the half-maximal rate of cleavage occurred when approximately 180 nucleotides were present per tetramer molecule of protease La (or 45 nucleotides per monomer). Although maximal proteolysis seems to require a definite relationship between the amount of DNA and protein, it is unclear at present what is the nature of the DNA binding site, how binding to the polynucleotides activates proteolysis, or why DNA at higher concentrations can completely inhibit this process (Fig. 2*B*).

Unlike other known proteolytic enzymes, protein degradation by protease La requires ATP hydrolysis (2–6). The enzyme has an ATPase activity that is stimulated by protein substrates (3, 5, 6) and that is sensitive to inhibition by vanadate (Table 1; refs. 2 and 5). A similar requirement for ATP cleavage was observed in the presence and absence of DNA. Furthermore, DNA enhanced the ATPase activity of protease La seen in the presence of casein (Fig. 4*A*). Thus, the effects on ATP cleavage and proteolysis appear to be closely linked. Poly(rA) had no effect on either process; poly(rC), poly(rU), and poly(dT) stimulated both ATP and casein hydrolysis (Fig. 4*B* and other data not shown). These results provide further evidence for an essential role of ATP cleavage in proteolysis and even suggest a stoichiometric relationship between the hydrolysis of peptide bonds and ATP.

In contrast to the present observations, Charette *et al.* (30, 31) recently reported that double-stranded DNA inhibits the ATP-dependent hydrolysis of casein by protease La and that, in the absence of casein, DNA and certain RNA species stimulate the ATPase activity of protease La. The explanation for these findings is not clear. Possibly, their use of only very low concentration of casein (as in Fig. 3*B*) could have given the impression that DNA only inhibits proteolysis. In addition, their finding of polynucleotide-stimulated ATPase activity in the absence of casein may reflect contamination of their protease La preparation by one of the several DNA-associated ATPases found in *E. coli* (19, 20, 27, 32).

The effect of polynucleotides and the ATP requirement of protease La indicate striking similarities to the *recA* gene product (19). During induction of phage λ, the recA protein hydrolyzes specifically the λ repressor (19) and the *lexA* gene product (20). However, the mechanism of the ATP-dependent processes appears to be quite different for the lon and recA proteins. Craig and Roberts (19) have shown that hydrolysis of ATP is not necessary for repressor cleavage, but a polynucleotide is absolutely required. On the other hand, casein degradation by protease La requires concomitant ATP hydrolysis (Table 1) and DNA stimulates but is not essential for this process (Table 2). The specific polynucleotides affecting the two enzymes differ also

(Table 2, ref. 19). Finally, the recA protein shows a DNA-dependent ATPase activity, unlike the ATPase of the lon protein, whose activity is stimulated by casein, especially in the presence of DNA (Fig. 4A).

The affinity of the lon protein for DNA (1) and this novel activation of proteolysis by DNA suggest that, *in vivo*, this enzyme may act as a protease while bound to the *E. coli* chromosome. Possibly this association with DNA may be involved in regulation of protein breakdown under different growth conditions (12, 13, 33, 34). In addition to degradation of abnormal and certain normal proteins (14–18), the *lon* mutation affects various cellular processes such as recovery from UV irradiation and other DNA-damaging agents (22, 23), septum formation during cell division (24, 25), and expression of genes for synthesis of capsular polysaccharides (21). It has been suggested that these processes involve the lon protein acting as a DNA-binding protein to regulate gene expression (21) or, alternatively, that these processes involve short-lived polypeptides (12, 17) that are degraded by the lon protein [e.g., as probably occurs for the *N* gene product of phage λ (18)]. These two alternatives, however, may not be independent mechanisms because the interaction of protease La with DNA can promote the degradation of certain proteins and perhaps differentially affect the breakdown of proteins that are also bound to DNA. An important related question for investigation is whether the specificity of protease La for polypeptides is altered by DNA binding. In fact, DNA enhanced hydrolysis of globin differentially (Table 1). In addition, the effects of DNA on casein hydrolysis were also most pronounced at high substrate concentrations (Fig. 3); thus, in the presence of DNA, the enzyme may attack a wider variety of polypeptide sequences, which could have important physiological consequences.

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