

A Specific Complex between a Restriction Endonuclease and Its DNA Substrate*

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Abstract. In the presence of Mg^{++} , ATP, and *S*-adenosylmethionine, the DNA restriction endonuclease R·K forms a specific complex with its DNA substrate. The complex can be detected by its retention on nitrocellulose membranes.

We have previously reported the purification and characterization of an endonuclease from *E. coli* strain K that cleaves double-stranded DNA synthesized in other *E. coli* strains but is without effect on DNA synthesized in strain K itself.¹ This enzyme has been called endonuclease R·K. Its cofactor requirements are most unusual: in addition to Mg^{++} , it requires ATP and *S*-adenosylmethionine. Several closely similar nucleases with different specificities but the same cofactor requirements have now been described. Thus, an enzyme from *E. coli* lysogenic for phage P1 attacks DNA from *E. coli* lacking P1,^{1, 2} and an enzyme from *E. coli* strain B attacks DNA from strains other than B.^{2, 3} These enzymes are involved in the phenomenon of host-controlled modification and restriction, whereby bacterial cells of one strain are able to destroy DNA from cells of foreign strains.⁴ A current working hypothesis is that resistance to a particular restriction endonuclease is conferred by specific methylation at nucleotide sequences which would otherwise be vulnerable to that enzyme.

We have been interested in the mechanism by which restriction endonuclease recognizes its DNA substrate, and the role that *S*-adenosylmethionine and ATP play in this process. Jones and Berg have shown that complexes of RNA polymerase with DNA bind strongly to nitrocellulose membrane filters.⁵ Under the conditions employed, free nucleic acid is not retained by the membrane; however, any nucleic acid bound to the enzyme is retained, providing evidence for the formation of a nucleic acid-enzyme complex. This technique has been employed to demonstrate the formation of aminoacyl-tRNA-synthetase-tRNA complexes⁶ and of complexes of the lactose repressor with its operator.⁷ By a modification of this general procedure we have detected a specific complex between restriction endonuclease R·K and DNA.

Materials and Methods. Commercial *S*-adenosylmethionine was purified by elution from "Biorex" 70 with 4 *N* acetic acid. ATP and other nucleotides were obtained from Sigma Chemicals. ³H-thymidine-labeled DNA from phage λ grown on *E. coli* strain K, designated λ·K DNA, was prepared from strain CR34(λ) as described previously.¹ ³²P-labeled λ DNA sensitive to endonuclease R·K was prepared from strain C600.4(λ) and will be referred to as λ·C DNA. The glycerol gradient fraction of endonuclease R·K prepared from *E. coli* K as previously described was employed in all experiments.¹

The reaction mixtures for binding contained per 0.25 ml: 25 μ moles TES, 0.07 μ mole ethylenediaminetetraacetate (EDTA), 1.6 μ moles $MgCl_2$, 3 μ moles mercaptoethanol, 0.1 μ mole adenosine triphosphate (ATP), 0.02 μ mole *S*-adenosylmethionine, approximately 10^{10} phage equivalents of ^{32}P λ -C DNA and of 3H λ -K DNA, and 5 μ l of enzyme. Incubation was carried out at 30°C for 2 min, and 0.03 ml of 0.1 *M* EDTA, pH 8.0, was then added. The pH of the reaction mixture is 7.7. The membrane filters used were Schleicher and Schuell B6, 24 mm diameter. In all experiments except the first one, the following procedure was used: The filters were treated with boiling distilled water for 5 min, washed, and stored in 0.1 *M* Tris, pH 8.0, with 2.6×10^{-4} *M* EDTA and 1.2×10^{-2} *M* mercaptoethanol (TEM). Each filter was then washed with 5 ml TEM prior to application of the sample. The sample was filtered slowly (approximately 1.0 ml/min) with gentle suction. The filter was then washed with 5 ml TEM at the same flow rate. Filtration was done at room temperature. The filters were dried and counted by liquid scintillation.

Results.—Retention of λ -C DNA on membrane filters in the presence of restriction endonuclease R·K: We have previously found that endonuclease R·K specifically degrades λ -C DNA without attacking λ -K DNA. Figure 1 shows that when a reaction mixture containing the enzyme and both DNA's is passed through a membrane, most of the λ -C DNA is retained, but the λ -K DNA is not.

The amount of λ -C DNA retained on the membrane increases with the amount of enzyme added, reaching a saturation level of approximately 70 per cent of the input. The proportion of λ -K DNA retained remains fairly constant at less than 15 per cent. Thus, under the conditions employed, the enzyme appears to form a specific complex with λ -C DNA.

When heat inactivated enzyme is used, DNA retention is low and nonspecific. Large amounts of DNA remain on the membrane when enzyme preparations less pure than the glycerol gradient fraction are employed—but the retention is not specific.

Requirements of the reaction: Maximum specific retention requires the addition of ATP, *S*-adenosylmethionine, and Mg^{++} , these being the require-

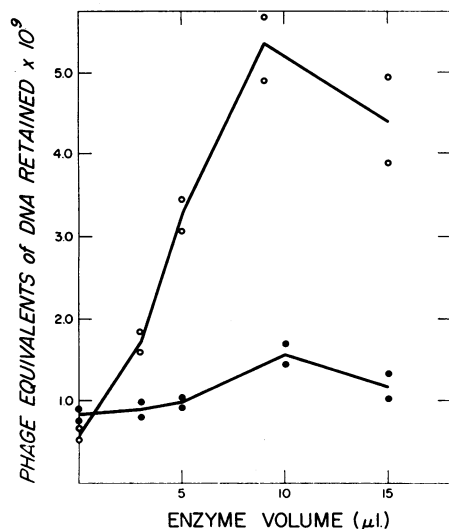


FIG. 1.—Endonuclease-dependent retention of λ -C DNA on membrane filters. Reaction mixtures of 0.25 ml with the composition described in the text, and containing 4.8×10^9 phage units of 3H λ -K DNA (39,400 cpm) and 7.2×10^9 phage units of ^{32}P λ -C DNA (2600 cpm) were incubated for 2 min at 37° with differing amounts of endonuclease R·K. They were filtered as described in the text except boiled filters were not used and no EDTA was added after incubation. —○—, ^{32}P λ -C DNA; —●—, 3H λ -K DNA. Each assay was done in duplicate.

TABLE 1. *Requirements for retention of λ -C DNA: Phage equivalents of DNA retained ($\times 10^8$).**

Components omitted	λ -K DNA	λ -C DNA	C/K
Enzyme	0.42	0.1	0.2
	0.79	0.5	0.6
ATP and <i>S</i> -adenosylmethionine	0.42	0.2	0.4
	0.50	0.3	0.5
ATP	0.76	2.7	3.5
	0.95	2.1	2.2
<i>S</i> -adenosylmethionine	0.71	0.3	0.4
	0.50	0.2	0.4
Mg ⁺⁺	0.68	1.0	1.4
	0.70	1.2	1.7
None (complete system)	0.97	7.7	7.9
	0.44	4.7	10.7

* The input of each DNA was 1.2×10^9 equivalents per reaction.

ments for nuclease activity as well. Table 1 shows the amount of retention observed when different components of the reaction mixture are omitted. In this series of experiments the amount of λ -C DNA retained following incubation with the complete system corresponds to approximately 60 per cent of the input. It may be noted that specific retention is reduced but not abolished if Mg⁺⁺ or ATP are not added.

The ATP requirement for maximum binding can be replaced by dATP but not by the other three nucleoside triphosphates or by ADP, AMP, adenine, or pyrophosphate. No specific retention is observed if *S*-adenosylmethionine is omitted, and the requirement for it is not satisfied by *S*-adenosylethionine, 5'-thiomethyl adenosine, or *S*-adenosylhomocysteine. For an incubation time of two minutes, the temperature optimum is approximately 30°C.

When heat denatured λ -C DNA is substituted for the native material in the reaction mixture, no specific retention is observed.

Effects of various reagents on binding: Several reagents were tested for their effect on the specific retention of λ -C DNA to membranes. The results may be seen in Table 2. After two minutes incubation at 37°, EDTA, sodium dodecyl

TABLE 2. *Effects of various reagents on retention: Phage equivalents of DNA retained ($\times 10^8$).**

Sample	λ -K DNA	λ -C DNA	C/K
No enzyme	0.15	0.01	0.1
	0.26	0.09	0.3
Complete system	1.20	4.6	3.8
	0.64	4.5	7.0
Complete system; EDTA addition	0.67	7.2	10.7
	0.80	7.3	9.1
Complete system; SDS addition	0.10	0.01	0.1
	0.09	0.01	0.1
Complete system; NaCl addition	2.00	8.4	4.2
	1.80	7.3	4.1
Complete system; boiled filters and EDTA addition	0.43	10.0	23.2
	0.41	10.0	24.4

* The input of each DNA was 1.1×10^9 equivalents per reaction.

sulfate, or NaCl were added to the reaction mixture. The addition of EDTA to a concentration of 10^{-2} *M* leads to an almost twofold increase in the amount of λ -C DNA retained. In the presence of 0.4 per cent SDS, all the DNA becomes filterable. Retention is not reduced by the addition of NaCl to a concentration of 0.5 *M* or even, as shown in a separate experiment, 1.0 *M*. The use of membranes treated with boiling water combined with the addition of EDTA after two minutes of incubation gives marked enhancement in specific binding, 95 per cent of the input λ -C DNA being retained in this case.

Effect of cofactor concentration: The dependence of specific retention upon the concentration of *S*-adenosylmethionine or ATP is shown in Figure 2. Retention decreases rather sharply as the concentration of *S*-adenosylmethionine passes below that previously found to be required for endonuclease action.¹ This is not the case for ATP. Even at concentrations as low as 4×10^{-8} *M*, nearly half of the λ -C DNA is retained. This concentration is several hundred times lower than that required for endonuclease activity.

Kinetics of complex formation: The kinetics of complex formation at 30° are shown in Figure 3. Aliquots were removed at intervals from the reaction mixture and EDTA was added. The maximum specific retention occurred at 1.5–2 minutes with 85 per cent of the λ -C DNA retained. By 20 minutes, the amount decreased to 25 per cent of the input. This was followed by a further but slower decrease in the amount of DNA retained. If EDTA is added after two minutes but incubation at 30° is continued, there is an initial decrease in retention during the next minute but the amount of DNA bound thereafter remains essentially constant for at least four hours. The addition of EDTA apparently stabilizes the complex.

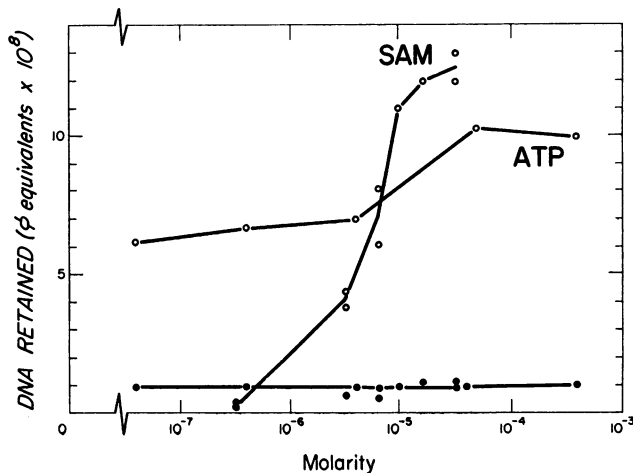
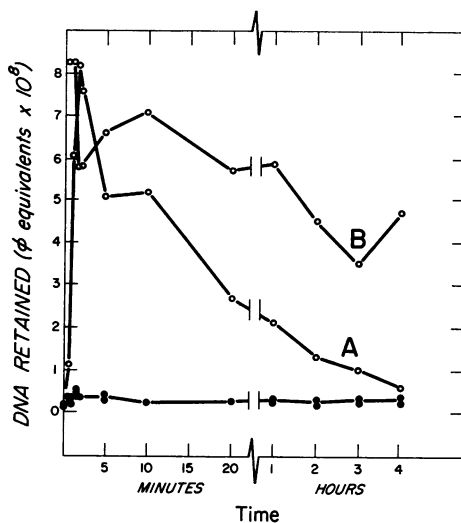


FIG. 2.—Dependence of retention on cofactor concentration. In one set of incubations, the ATP concentration was held constant while the *S*-adenosylmethionine (SAM) concentration was varied; in the other, the *S*-adenosylmethionine concentration was held constant, and the ATP concentration was varied. Each assay contained 1.3×10^9 phage units of ^{32}P λ -C DNA (2170 cpm) and 1.3×10^9 phage units of ^3H λ -K DNA (4580 cpm). Incubation and filtration were done as described in the text. —○—, ^{32}P λ -C DNA; —●—, ^3H λ -K DNA

FIG. 3.—Kinetics of complex formation. Two reaction mixtures (2.5 ml each) were incubated at 30°. (A) Aliquots of 0.25 ml were removed at intervals, and 0.03 ml 0.1 *M* EDTA, pH 8.0, was added before filtering. (B) 0.3 ml 0.1 *M* EDTA, pH 8.0, was added to the entire mixture after 2 min at 30°. Incubation was then continued. Aliquots were removed at intervals. Each aliquot contained 1×10^9 phage units of ^{32}P λ -C DNA (1075 cpm), and 1×10^9 phage units of ^3H λ -K DNA (4640 cpm). —○—, ^{32}P λ -C DNA; —●—, ^3H λ -K DNA.



Discussion. When λ -C DNA and λ -K DNA are incubated with endonuclease R·K in the presence of *S*-adenosylmethionine, ATP, and Mg^{++} , and passed through a membrane filter, the λ -C DNA is specifically retained. Heat denatured λ -C DNA is not retained. Following incubation, the addition of 0.4 per cent SDS or phenol abolishes specific retention. Molar NaCl has no effect. The most likely explanation of these observations is that λ -C DNA forms a specific complex with endonuclease R·K which binds to the membrane.

The cofactor requirements are the same as those for restriction. The *S*-adenosylmethionine requirement for retention is in the same concentration range as observed for restriction. The specific retention seen at an ATP concentration of 4×10^{-8} *M*, where nucleolytic action has not been observed, suggests that ATP may be involved in at least two distinct steps: (1) formation of a non-hydrolytic complex at low concentration levels, and (2) formation of more stable or more numerous complexes and nucleolytic action at higher concentrations. (A reservation to this suggestion is that very low levels of nucleolytic action at 4×10^{-8} *M* ATP may have gone undetected in our previous experiments.)

The apparent loss of complex following incubation for longer than two minutes may reflect the release of the enzyme following cleavage of its substrate. This is consistent with the stabilizing effect of EDTA, for EDTA also inhibits endonuclease action. Thus, even at high ATP concentration, complex formation and hydrolysis appear to be two distinct and separable stages in the over-all action of restriction endonuclease.

Development of this system and the possibility it provides for isolating the DNA-enzyme complex should enable us to investigate the following two questions: (1) Are *S*-adenosylmethionine and ATP or derivatives of either of these compounds actually present in the DNA-enzyme complex? (2) What chemical reactions, if any, are undergone by *S*-adenosylmethionine and ATP during the hydrolytic step itself?

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¹ Meselson, M., and R. Yuan, *Nature*, **217**, 1110 (1968).

² Linn, S., and W. Arber, these PROCEEDINGS, **59**, 1300 (1968).

³ Roulland-Dussoix, D., and H. W. Boyer, *Biochim. Biophys. Acta.*, **195**, 219 (1969).

⁴ Arber, W., and S. Linn, *Ann. Rev. Biochem.*, **38**, 467 (1969).

⁵ Jones, O. W., and P. Berg, *J. Mol. Biol.*, **22**, 199 (1966).

⁶ Yarus, M., and P. Berg, *J. Mol. Biol.*, **28**, 479 (1967).

⁷ Riggs, A. D., S. Bourgeois, R. F. Newby, and M. Cohn, *J. Mol. Biol.*, **34**, 365 (1968).