

ATP-induced conformational changes in the restriction endonuclease from *Escherichia coli* K-12

(DNA recognition/enzyme-DNA complexes/electron microscopy)

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ABSTRACT ATP induces a conformational change in the *Escherichia coli* K-12 restriction enzyme that allows it to discriminate between unmodified and modified DNA recognition sequences. This conformational change does not require ATP hydrolysis. However, ATP hydrolysis is a requirement for DNA cleavage.

Under the appropriate conditions, the restriction enzyme from *Escherichia coli* K-12 (*EcoK*) will catalyze three different reactions. It cleaves unmodified DNA in the presence of S-adenosylmethionine (Ado-Met), ATP and Mg²⁺ (1). This endonucleolytic reaction is accompanied by an ATP-hydrolyzing activity that continues after DNA cleavage has gone to completion (2). *EcoK* can also act as a DNA-methyl transferase, which modifies its recognition sites in unmodified DNA (3).

The restriction reaction has been studied *in vitro* in detail in both *EcoK* and the related enzyme *EcoB* (4), and several distinctive steps have been characterized (Fig. 1). *EcoK* binds Ado-Met rapidly and undergoes a slow transition to an activated form, *EcoK**-Ado-Met (steps 1 and 2). *EcoK**-Ado-Met forms an initial complex (step 3) with any DNA molecule regardless of the presence or absence of host specificity sites (sK sites), the sites on the DNA that confer susceptibility to the restriction and modification system. If sK sites are present on the DNA, a more stable recognition complex is formed (step 4). It is this interaction of *EcoK**-Ado-Met with the sK sites that determines which of the enzymatic activities will be expressed. The sK sites can exist in three different forms: fully modified (methylated on both strands), fully unmodified (unmethylated on both strands), and heteroduplex (one strand methylated and one unmodified). Modified DNA is neither cleaved nor further modified (step 5). Heteroduplex DNA is not a substrate for restriction (1) but has been shown to be the best substrate for methylation (5). This methylation is stimulated by ATP (step 6). It is not known whether the enzyme-bound Ado-Met is sufficient to allow methylation to take place or whether additional Ado-Met is required. Unmodified DNA is cleaved endonucleolytically after the addition of ATP to a recognition complex. However, DNA cleavage is preceded by the formation of a new complex that has the characteristic of being retained on nitrocellulose filters (step 7), a property that distinguishes it from the initial and recognition complexes. This indicates a further conformational change from *EcoK**-Ado-Met to a new enzyme species which has been designated *EcoK*[†]. After DNA cleavage, extensive ATP hydrolysis takes place, but no turnover in the endonucleolytic activity is observed (step 8). Methylation takes place at the recognition sites (6). DNA cleavage, on the other hand, occurs elsewhere on the DNA after the interaction

of the enzyme with the recognition sequences (7). Mapping studies with PM2 DNA have shown that the cleavage sites are not random, suggesting that they may be related to the readily denaturable regions of the genome (unpublished data).

In this paper we show that *EcoK**-Ado-Met is able to bind specifically to sK sites, regardless of whether these are modified or not. ATP induces an alteration of the enzyme that permits it to remain at an unmodified sK site or releases it from a modified one. Whereas ATP hydrolysis was not needed for this conformational change, it was an absolute requirement for DNA cleavage.

MATERIALS AND METHODS

Chemicals. Ado-Met, ATP, and heparin were obtained from Sigma. The commercial Ado-Met was further purified by elution from Bio-Rex 70 with 4 M acetic acid (1). Adenosine 5'-(β,γ -imido)-triphosphate was purchased from Boehringer Mannheim, and [³H]Ado-Met (6S Ci/mmol) was obtained from New England Nuclear.

DNA and Enzyme Preparations. ³²P-Labeled unmodified λ DNA and modified λ DNA were prepared as described (1). The restriction endonuclease *EcoK* was purified to homogeneity by a modification of the procedure published elsewhere (1).

Methods. All reactions were carried out in 100 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate (Hepes), pH 8.0/0.26 mM EDTA/6 mM MgCl₂/12 mM 2-mercaptoethanol. *EcoK* was assayed by measuring binding of labeled unmodified DNA to filters (8). Electrophoresis on agarose gels was carried out essentially as described by Hayward and Smith (9). The gels were dried and autoradiographed.

Electron Microscopy. Complexes between *EcoK* and DNA were fixed with 0.1% glutaraldehyde and mounted for electron microscopy by the protein-free method (10).

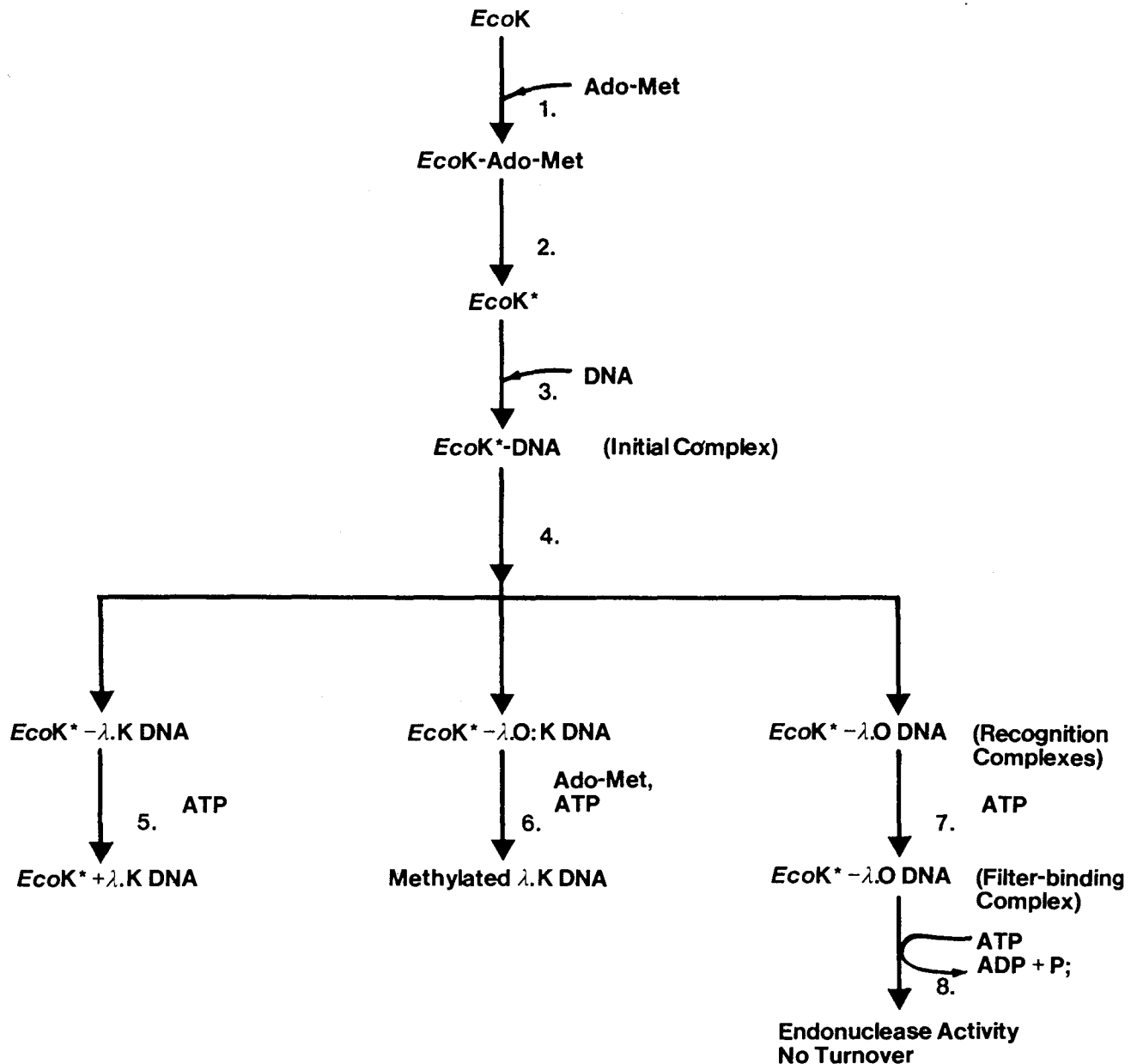
RESULTS

ATP Releases Enzyme-Bound Ado-Met as the Recognition Complex Formed with Unmodified DNA Is Transformed into One that Is Retained on Filters. Earlier (4) we had shown that the first step in the reaction mechanism of *EcoK* involved the binding of Ado-Met and the transition of the enzyme to *EcoK**-Ado-Met (Fig. 1). Since then, we have found that the binding of radioactive Ado-Met by *EcoK* is strong enough to allow its detection by gel filtration chromatography. This allowed us to do experiments of the kind shown in Fig. 2. A recognition complex was formed by incubating a mixture of ³²P-labeled unmodified λ DNA, [³H]Ado-Met, and *EcoK*.

Abbreviations: *EcoK*, restriction enzyme from *E. coli* K-12; sK sites, host specificity sites.

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FIG. 1. Reaction mechanism of endonuclease *EcoK*.

Heparin was added to the incubation to eliminate nonspecific binding to DNA without affecting the specific binding to the *sK* sites (4). The mixture was then analyzed by chromatography on Bio-Gel A-15m. Three peaks of [³H]Ado-Met were found (Fig. 2A): one cochromatographing with the DNA, one at the position of the free enzyme, and one of free Ado-Met. Thus, the enzyme in the recognition complex still has Ado-Met bound to it. Enzymatic activity (measured as the ability to bind unmodified DNA to nitrocellulose filters) was found at the position of the DNA and also in the position of the free enzyme, although in this case most of the activity was inhibited by the heparin. Addition of ATP alone to the DNA-containing fractions converted them to a form that was quantitatively retained by nitrocellulose filters. Interestingly, when these fractions were analyzed in the presence of additional [³²P]DNA, some of the added DNA was also bound to the filters, indicating that a fraction of the enzyme had redistributed from the DNA initially present to that added later.

In a second experiment (Fig. 2B), the filter-binding enzyme-DNA complex was analyzed in a similar way. A recognition complex was formed as in the previous experiment: heparin was added for 1 min, then ATP for 20 sec, and the reaction was stopped with EDTA (7). The short incubation with ATP allowed the transition to the filter-binding complex to take place without permitting significant DNA cleavage. In this case, only two peaks of [³H]Ado-Met were found, corresponding to the positions of the free enzyme and of unbound Ado-Met. No Ado-Met cochromatographed with the DNA, even though these fractions contained enzyme as demonstrated by quantitative retention of the DNA on nitrocellulose filters. These enzyme molecules were now firmly bound to the DNA; when the fractions were assayed for their ability to bind added DNA, no reaction was observed. Similar results were obtained when the time of incubation with ATP was increased to 30 min to allow extensive DNA cleavage (results not shown) although in this case the proportion of the DNA that could be bound to

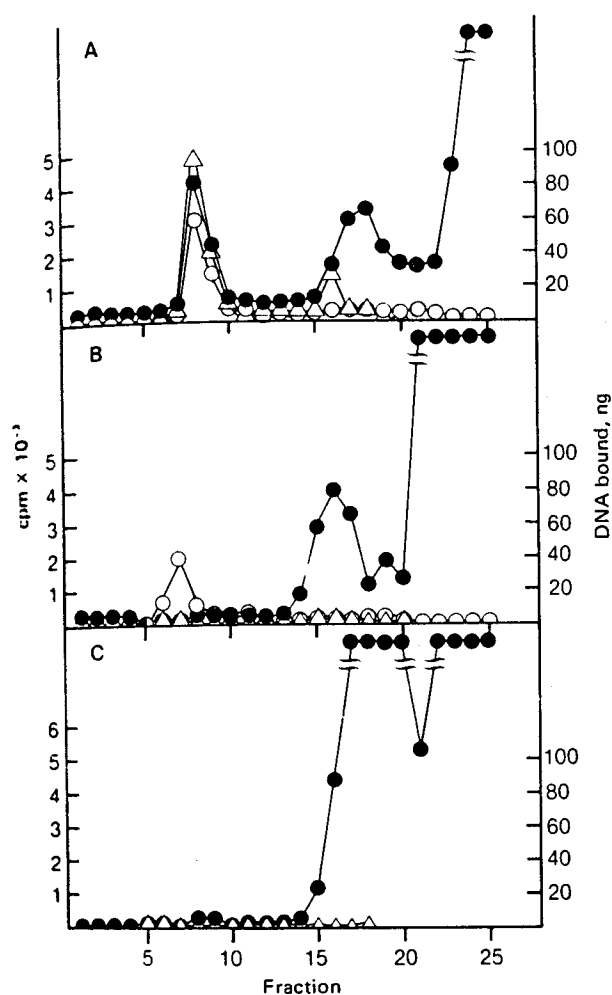


FIG. 2. Effect of ATP and Ado-Met bound to recognition complexes formed with unmodified and modified DNAs. (A) Recognition complex with unmodified $\lambda.O$ [^{32}P]DNA. (B) Filter binding complex with unmodified $\lambda.O$ [^{32}P]DNA. (C) Recognition complex with modified $\lambda.K$ DNA after incubation with ATP. Three 125- μ l reaction mixtures were set up containing 0.15 nmol of [3H]Ado-Met (68 Ci/mmol); 1.75 μ g of $\lambda.O$ [^{32}P]DNA was added to reactions A and B, and 1.65 μ g of $\lambda.K$ DNA was added to reaction C. *EcoK* (50 μ l) was added to each reaction and they were incubated for 3 min at 30°. Heparin (10 μ g) was added and incubation was continued for 1 min. Reaction A was terminated by the addition of 50 μ l of 0.5 M EDTA (pH 8.0). In reactions B and C, 0.1 μ mol of ATP was added, incubation was continued for 20 sec and 3 min, respectively, and EDTA was added. All three samples were applied to columns (0.6 \times 40 cm) containing 10 ml of Bio-Gel A-15m that had been equilibrated against 100 mM Tris, pH 8.0/0.26 mM EDTA/12 mM 2-mercaptoethanol and were eluted with the same buffer at 4°. The flow rate was 12 ml/hr. Fractions (0.25 ml) were collected and aliquots (50 μ l) were assayed for radioactivity with Instagel. Aliquots (20 μ l) were assayed for binding of $\lambda.O$ [^{32}P]DNA to filters. Finally, 100- μ l aliquots of the DNA-containing fractions of reaction B were filtered directly through nitrocellulose filters. \bullet , [3H]Ado-Met; \circ , $\lambda.O$ [^{32}P]DNA; Δ , *EcoK* activity.

filters decreased to almost half and the DNA-containing fractions showed ATPase activity in the absence of added DNA. *EcoK* in the recognition complex still had Ado-Met bound to it, and ATP caused this Ado-Met to be released. The enzyme, in whole or in part, remained attached to the unmodified DNA after Ado-Met had been released, because the DNA-containing fractions were retained on nitrocellulose filters and continued incubation led to DNA cleavage and ATP hydrolysis.

Visualization of the ATP-Induced Alteration of Recognition Complexes Formed with Unmodified DNA. The complexes formed between *EcoK**-Ado-Met and DNA can be visualized by electron microscopy. Previous studies had shown that *EcoK**-Ado-Met bound specifically and quantitatively to the sK sites on restriction fragments of PM2 and λ DNAs (10). A low yield of complexes with *EcoK**-Ado-Met bound at random positions was also observed with fragments lacking sK sites.

We have used electron microscopy to examine the effect of ATP on a recognition complex formed with fragment 6 from *EcoRI* digestion of $\lambda.O$ DNA. This fragment contains the sK1 site of λ DNA (10). Because the enzyme cleaves the DNA at a considerable distance from the recognition sites, it was expected that ATP would cause the enzyme to translocate from the recognition site to the cleavage sites. This was not the case. Filter-binding complexes were prepared under conditions that allowed little DNA cleavage. Examination of the filter-binding complex formed in the presence of ATP showed that the enzyme was still located at the sK1 site. However, ATP appeared to have decreased the size of the enzyme. To ascertain that this apparent change in size was not due to variation between different electron microscopic preparations, we prepared, fixed, mixed, and spread recognition and filter-binding complexes on the same grid. The diameter of the proteins was measured perpendicular to the axis of the DNA and the results were plotted as a histogram (Fig. 3). It is clear that molecules of two different apparent sizes are present: the "large" ones correspond to the recognition complexes and the "small" ones to the filter-binding complexes. Even after DNA cleavage the small enzyme molecules were detected bound to the sK sites in the same number as before ATP addition (Table 1). Unfortunately,

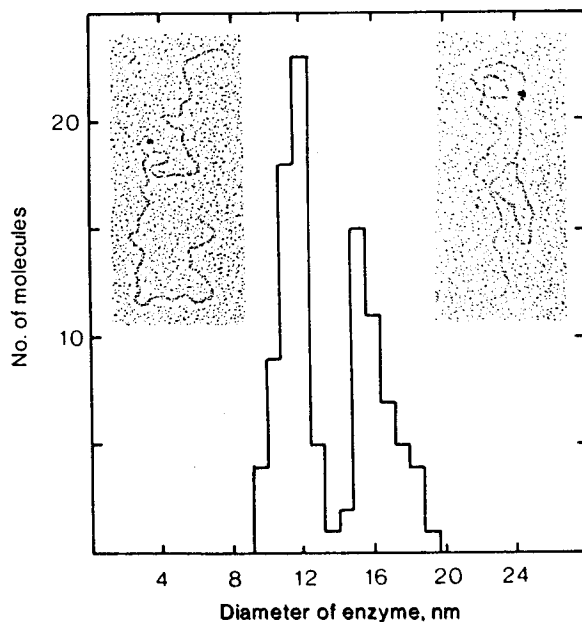


FIG. 3. Diameter of *EcoK** on recognition and filter-binding complexes. Complexes were formed by incubation of *EcoK* with Ado-Met and the *EcoRI* f6 fragment of unmodified $\lambda.O$ DNA in the absence or presence of ATP. The conditions were similar to those outlined above so that no DNA cleavage took place. The complexes were mixed and fixed before mounting for electron microscopy. The diameter of the enzyme molecules bound to the specific sK site was measured perpendicular to the DNA axis and the data are shown as a histogram. (Insets) Typical complexes; with (to the left) and without (to the right) ATP.

Table 1. Effect of ATP on recognition complexes

DNA	ATP	No. of <i>EcoK</i> -DNA complexes with		Complexes, % total DNA
		Single enzyme	Several enzymes	
f6/ λ .O	-	69	11	79
f6/ λ .O	+	64	1	65
f6/ λ .K	-	44	6	50
f6/ λ .K	+	5	0	5

Enzyme-DNA complexes were formed and prepared for electron microscopy as described for Fig. 3 except that the samples were incubated for 30 min to allow DNA cleavage. The number of DNA fragments carrying no, one, or several enzyme molecules was counted. The *EcoRI* fragment 6 in the first two reactions was isolated from unmodified λ .O DNA; that in the second two was from modified λ .K DNA.

the nature of the evidence does not allow us to distinguish between a rearrangement of the subunits or the loss of one or more subunits from the enzyme.

ATP Releases Enzyme-Bound Ado-Met from a Recognition Complex Formed with Modified DNA. Evidence for the existence of stable complexes between *EcoK**-Ado-Met and modified DNA had previously been obtained by glycerol gradient centrifugation (4). Gel filtration of complexes formed with modified λ DNA and [3 H]Ado-Met confirmed these results and yielded elution patterns similar to those shown in Fig. 2A. When a similar complex was further incubated with ATP before analysis, neither 3 H counts nor enzyme activity was found at the DNA position (Fig. 2C).

The complexes formed between *EcoK**-Ado-Met and modified DNA in the absence of ATP are stable. That this interaction is dependent on the presence of sK sites (albeit modified ones) was evident from similar experiments with the *EcoRI* fragment 5 from λ DNA, which contains no recognition sites for the enzyme. Neither [3 H]Ado-Met nor enzymatic activity comigrated with this fragment (unpublished data). As in the case of complexes with unmodified DNA, ATP releases Ado-Met from the modified DNA complexes. However, in sharp contrast to the former, *EcoK**-Ado-Met does not bind modified DNA to filters (10). This suggests that ATP either releases *EcoK**-Ado-Met from modified DNA or alters it to a form that is unable to bind DNA to filters.

Visualization of the ATP-Induced Release of *EcoK-Ado-Met from Recognition Complexes Formed with Modified DNA.** Electron microscopy experiments were also carried out with modified restriction fragments and fragments lacking sK sites. ATP eliminated the small number of randomly located complexes formed with the latter (less than 1% complexes were detected). On the other hand, K-modified *EcoRI* f6 fragments formed complexes in the absence of ATP at the same high frequency as with unmodified fragments (Table 1). Rather surprisingly, the enzyme was located at the sK site in most of these complexes. In the presence of ATP, most of the *EcoK**-Ado-Met molecules were eliminated from these DNA fragments (Table 1, last line). This was in sharp contrast to the complexes formed with unmodified DNA fragments, with which ATP stabilized rather than destabilized the interaction with the recognition site (Table 1, lines 1 and 2).

Thus, modification of the sK site does not prevent its recognition by *EcoK**-Ado-Met. However, ATP then changes the structure of the enzyme in such a way that it dissociates from the modified sites and binds more tightly to the nonmodified sites. Therefore, it is ATP that allows the enzyme to discriminate between modified and nonmodified DNA.

Alteration of *EcoK-Ado-Met Can Be Induced by a Nonhydrolyzable ATP Analogue.** One remaining question was whether the ATP-induced transformation of *EcoK**-Ado-Met required ATP hydrolysis. Adenosine 5'-(β,γ -imido)-triphosphate is a nonhydrolyzable ATP analogue in which the oxygen between the β and γ phosphates has been replaced by an imido group (11). This analogue inhibited the enzyme (in assays of the ATPase activity) and the inhibition was relieved by increasing the ATP concentration. The inhibition is competitive with ATP; the apparent K_i is about 10^{-4} M, or 5 times the K_m for ATP (1).

The formation of filter-binding complexes with 32 P-labeled unmodified DNA can be readily detected if this analogue is used in place of ATP. These complexes were analyzed by electron microscopy. The same transition from "large" enzymes (Table 2, line 1) to "small" enzymes (line 3) was observed with the analogue as with ATP (line 2). The analogue also released *EcoK**-Ado-Met from modified DNA fragments.

The analogue was then tested for its ability to sustain endonucleolytic activity by using electrophoresis on agarose gels as the assay. Whereas ATP allowed extensive DNA cleavage (Fig. 4), no activity was observed with the analogue; on incubation with ATP, the DNA was cleaved even when heparin was added together with the ATP. Because heparin inactivated free enzyme, only those *EcoK**-Ado-Met molecules already present on the DNA could be triggered to cleavage. Electron microscopic experiments with phage PM2 supercoiled DNA showed that neither double-stranded cleavage nor single-strand nicking took place in the presence of the analogue.

It is clear then that discrimination between modified and unmodified recognition sites, the conformational shift from "large" to "small" enzyme forms, and the ability to bind DNA to filters are all effects of ATP that occur in the absence of ATP hydrolysis. On the other hand, concomitant or prior ATP hydrolysis is necessary for endonucleolytic activity. Because the addition of ATP to a filter-binding complex formed with the analogue allows DNA cleavage, it is likely that the interaction of the analogue (and by implication ATP) with *EcoK** is readily reversible. This is supported by our failure to detect stable complexes between radioactive ATP and *EcoK**-Ado-Met under any of the conditions that we have used.

Role for ATP in Enzyme Discrimination of DNA Sequences. It has been often proposed, although seldom demonstrated, that certain DNA enzymes require ATP because its hydrolysis provides the energy necessary to separate the polynucleotide chains. An alternate explanation is that the energy is used to move the enzyme along the DNA. In this study we

Table 2. Alteration of *EcoK* by ATP and adenosine 5'-(β,γ -imido)-triphosphate

	% of DNA fragments with		
	"Large" enzyme	"Small" enzyme	No enzyme
f6/ λ .O + Ado-Met + <i>EcoK</i>	68.8	6.6	24.6
f6/ λ .O + Ado-Met + ATP + <i>EcoK</i>	4.7	72.6	22.7*
f6/ λ .O + Ado-Met + β,γ -imido ATP + <i>EcoK</i>	5.7	70.2	24.1

EcoK was complexed to the *EcoRI* fragment 6 from λ .O DNA and prepared for electron microscopy as described in Fig. 3. The DNA fragments were then classified according to whether they had *EcoK** bound at the sK1 site. The enzyme molecules bound to DNA were further classified into large or small.

* Contains cleaved fragments.

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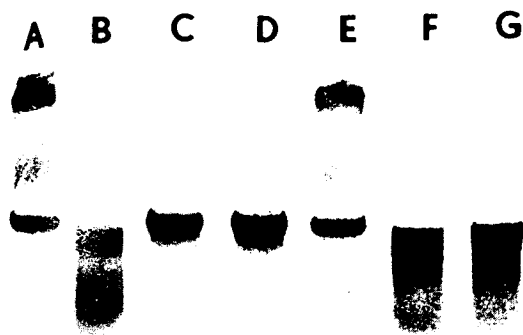


FIG. 4. DNA cleavage in the presence of β,γ -imido ATP. All reactions contained 20 μ M Ado-Met and 0.3 μ g of 32 P-labeled λ O DNA (45,000 cpm) in a final volume of 50 μ l. The samples were incubated at 30° for 2 min (first incubation), the indicated additions were made, and the incubation was continued for a further 60 min (second incubation). The incubations were stopped by the addition of sodium dodecyl sulfate to a concentration of 1%. The samples were electrophoresed on a 0.7% agarose slab gel. Lanes: A, control incubation without enzyme; B, ATP added to 0.2 mM, no further additions; C, ATP (0.2 mM) and heparin (50 μ g/ml) added before the enzyme; D, ATP (0.2 mM) added for the first incubation, heparin (50 μ g/ml) added before the second incubation; E, β,γ -imido-ATP (0.2 mM) was added before the enzyme, no further additions were made; F, as E except that ATP (0.2 mM) was added during the second incubation; G, as F except that heparin (50 μ g/ml) was added together with the ATP in the second incubation.

have shown that ATP is required in two distinct steps of the *EcoK* restriction reaction. The first ATP effect is novel in that it allows *EcoK* to discriminate between a methylated and an unmethylated recognition site. Without ATP, *EcoK**-Ado-Met is able to recognize and form stable complexes at both kinds of sites. It is the ATP that induces a major conformational change in *EcoK**-Ado-Met that accompanies the acquisition of a new recognition mechanism. No ATP hydrolysis is required and the reaction clearly differs from the models mentioned above.

The release of enzyme-bound Ado-Met from recognition complexes and the apparent change in size of *EcoK*[†] do not tell us whether an Ado-Met binding subunit has been released or *EcoK*[†] has undergone a subunit rearrangement. It is possible the *EcoK**-Ado-Met dissociates, releasing the modification subunit but leaving the site recognition and nuclease subunits on the DNA. The modification subunit has been shown to be the one required for Ado-Met binding (unpublished data). Enzyme dissociation would also account for the lack of turnover in the restriction reaction.

In a second step, ATP hydrolysis is necessary for DNA cleavage to occur. This fact is probably related to the question of how the enzyme can cleave at sites that may be as far as 7000 base pairs from the nearest sK site. A hypothesis in which ATP dissociates *EcoK**-Ado-Met and the dissociated moiety is responsible for endonuclease activity can be easily disposed of if the released subunits diffuse through the medium to their

cleavage sites, one would expect to see cleavage of modified DNA present in the reaction. Alternatively, if the dissociated moiety moves along the DNA to a cleavage site, one would expect to detect it by electron microscopy, particularly in the complexes prepared with the nonhydrolyzable ATP analogue. The analogue-induced complex would not undergo DNA cleavage, and part of *EcoK* should be present at the cleavage site. Neither event is observed. Modified DNA is not cleaved even when present at a high concentration, and no protein is detected at sites other than the recognition sites in complexes formed with adenosine 5'-(β,γ -imido)-triphosphate.

The second possibility is one in which ATP dissociates the *EcoK**-Ado-Met bound to the recognition site. *EcoK*[†] would remain fixed to the sK site through its original active site while a second site, made available by the conformational change, would move the DNA past the enzyme until a cleavage site was found. This intermediate loop structure in which *EcoK*[†] binds to both sK and cleavage sites would be the one that is retained on filters. Two experiments lend some support to this view. (i) Fragment f2 from *EcoRI* digestion of λ O DNA and a smaller *Hin* F fragment from the same region contain the sK site but few or no cleavage sites. Neither of them formed a filter-binding complex with *EcoK* although the conformational change to the small enzyme was observed. Therefore, the filter-binding complex requires the presence of *EcoK*[†], an unmodified sK site, and a cleavage site(s). (ii) Under conditions such that heparin inhibits DNA cleavage completely, the number of recognition complexes as seen in the electron microscope is only decreased to half the control value, indicating the existence of two separate heparin-binding sites on the enzyme. Unfortunately, there is no electron microscopic evidence for the loop structure although this can be explained by the fact that the glutaraldehyde used to prepare the complexes from electron microscopy results in the loss of filter-binding complexes (unpublished data). Finally, the role of ATP hydrolysis in triggering endonuclease activity in such structures remains unexplained. Nevertheless, it is likely to be as complex and unusual as the preceding steps in the mechanism.

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