Regulation of DNA ligation by the *rec*B protein in *Escherichia coli* cells

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The covalent closing of hydrogen-bonded λDNA circles in unpurified or slightly purified extracts of *Escherichia coli*, which was previously shown to require ATP and *rec*B gene products, was found not to require *rec*C gene product. The closing activity in extracts of the λ lysogen that overproduces DNA ligase about 500-fold over the normal strain was still strictly dependent on ATP, but not on NAD. This suggests that the substrate was not freely available for the action of DNA ligase in *rec*B⁻ extract. ATP could be replaced in the system by a mixture of low concentrations of the 4 dNTPs, but by none of them alone.

DNA ligase regulation *ATP dependence* Rec*BC enzyme* *Bacteriophage λ DNA*

1. INTRODUCTION

The purified DNA ligase enzyme which catalyzes the closing of single-strand cuts in double-stranded DNA is known to use NAD as its energy source in *Escherichia coli* cells, cleaving it to AMP and NMN in the process [1,2]. Several reports have appeared, however, showing that the covalent closing of bacteriophage λ hydrogen-bonded DNA circles is stimulated by ATP but not appreciably by NAD in unpurified or slightly purified extracts of *E. coli* [3–5]. The bacterial ligase that requires NAD is nevertheless also necessary for the ATP-dependent closing of the λDNA circles, as is suggested by the bacterial mutant that produces a temperature-sensitive DNA ligase [5]. The NAD requirement in the unpurified extract seems to be dependent on the nature of the substrate for ligase.

In addition to the ligase enzyme other proteins have also been found to be needed for the ATP-dependent closing of hydrogen-bonded λDNA circles. Extracts of the *rec*B and *pol*A mutants were observed not to catalyze ligation, suggesting some role for the *rec*BC enzyme and DNA polymerase I in the reaction [6].

The purified DNA ligase of *E. coli* has been suggested to be partially in a charged form [4]. In the absence of single-strand cuts, the NAD is cleaved in the cell and the AMP portion of it is attached covalently to the enzyme. In this form the enzyme can catalyze ligation with the energy obtained by releasing AMP [7]. It has been suggested that ligation can occur in unpurified extracts without NAD, because the extract contains enough 'charged ligase' [4].

The aim of this work was to obtain more information on the factors that regulate ligation in unpurified extract of *E. coli*. Evidence has been presented that the *rec*BC enzyme is not required for the ATP-requiring ligation phenomenon, but only its *rec*B subunit. Furthermore, the ATP requirement is also strictly retained in extracts of cells that greatly overproduce the DNA ligase enzyme. Finally, it is shown that low concentrations of all 4 deoxyribonucleoside triphosphates can replace ATP in the reaction, but none of them alone can do so at such concentrations.

2. MATERIALS AND METHODS

2.1. *Bacterial and phage strains and chemicals*

The following *E. coli* K12 strains were used: C600 and 1200, which are deficient in endonuclease I and RNase I activity [8], were ob-

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tained from Dr D. Court, JC6722 *rec*B and JC5489 *rec*C22 were gifts from Dr A. Clark and the DNA ligase-overproducing strain 594 su$^-$ λgt4 lopII lig$^+$S7 was obtained from Dr I. Lehman. The strain W3110 *pol*A and the bacteriophage λc$_1$857 were from Dr H. Echols' collection.

Nucleoside triphosphates were purchased from Boehringer (Mannheim), NAD from Serva (Heidelberg), [*Me*-^{3}H]thymidine from the Radiochemical Centre (Amersham) and polyethylene glycol 6000 from Fluka (Buchs).

2.2. *Preparation of hydrogen-bonded [^{3}H]λDNA circles*

The labelled phage λc$_1$857 was prepared as described [6], except that it was precipitated with polyethylene glycol 6000 and NaCl at concentrations of 10% and 0.5 M, respectively. The final phage band obtained from the CsCl density gradient was dialyzed against 0.01 M Tris-HCl, pH 7.6, 0.1 M NaCl, 1 mM $MgCl_2$. λDNA was released from the phage particles under alkaline conditions [9]. After neutralization and dialysis against 0.01 M Tris-HCl, pH 8.0, 1 mM EDTA, the DNA molecules were circularized by allowing the sample to stand at high ionic strength [10] and then dialyzed against 0.01 M Tris-HCl, pH 8.0, 8 mM $MgSO_4$, 1 mM EDTA.

2.3. *Preparation of the extracts and purification of enzymes*

The preparation of extracts showing ATP-dependent closing activity and the assay of covalent joining activity were performed as described [6]. *E. coli* 594 su$^-$ λgt4 lopII lig$^+$S7 was induced to overproduce DNA ligase according to Panasenko et al. [11].

E. coli DNA ligase, employed to evaluate the quality of the circles, was purified from overproducing λlysogen 594 su$^-$ λgt4 lopII lig$^+$ S7 as described by Panasenko et al. [11] and *rec*BC enzyme from the *E. coli* strain 1200 according to Eichler and Lehman [12] up to stage IV.

3. RESULTS AND DISCUSSION

3.1. *Covalent closing of λDNA circles in extracts of* rec*C mutant*

In [6] we examined ATP-requiring closing activity in extracts of the various mutants of *E. coli*. Since no activity was found with the *rec*B mutant extract, the conclusion was reached that the *rec*BC enzyme must be involved in the event. As shown in fig.1, the *rec*C mutant extract turned out to be fully active for closing activity, which suggests that only the *rec*B subunit of the enzyme was necessary for the reaction. The *rec*BC enzyme could not actually restore the ATP-requiring closing activity of the *rec*B mutant extract when it was added to the extract in a partially purified form. As suggested by the observations of Banfalvi et al. [13], the *rec*BC nuclease activity apparently is the cooperation of at least two dissociable subunits rather than the property of a single multifunctional enzyme and the *rec*B and *rec*C genes may be responsible for different dissociable subunits. Therefore, some of the *rec*B protein could be free in the cell and it might have a function of its own which is different from that of the *rec*BC enzyme. This could also be expected on genetic grounds. *Rec*B and *rec*C proteins are known to be coded from different operons, which are not even next to each other in the sequence, but have an extra operon between them [14].

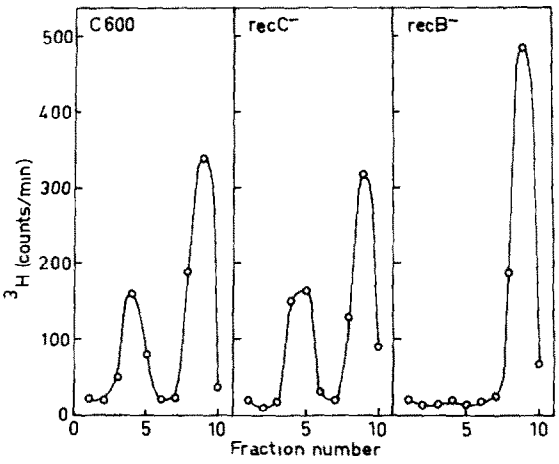

Fig.1. Ligation of hydrogen-bonded λDNA circles in extracts of *rec*B$^-$ and *rec*C$^-$ cells in the presence of ATP. The quantity of closed circles formed was determined by centrifugation in an alkaline sucrose gradient (5–20%). The faster moving peaks of fractions 3–5 represent covalently closed circles. The peak of fractions 8–10 represents hydrogen-bonded circles and linear DNA.

3.2. *ATP dependence of the covalent closing activity in extracts of λ lysogen overproducing DNA ligase*

The amount of DNA ligase in extracts of *E. coli* 594 su⁻ λgt4 lopII lig⁺ S7 can be increased 500-fold over those from wild-type strains [15]. As shown in fig.2, the covalent closing activity in extracts of this strain was observed to be still strictly dependent on ATP, and about the same quantities of closed circles were formed as in extracts of the normal strain. When ATP was replaced by NAD, practically no circles (<1%) were formed. This suggests that there must be some other factor(s) to prevent the ligase from acting on DNA, probably by masking the nicks in the hydrogen-bonded circles. *Rec*B protein apparently functions in this way in the absence of ATP, as is suggested by the evidence presented above. Poly dAT, which has also been observed to function as the substrate for DNA ligase in the unpurified extract [16], is probably such an unnatural DNA that it does not bind any factors that limit ligation. The ligase, which is freely available in the extract, can then act. The reason, why NAD is not required in the ATP-dependent system is not known for certain, although the presence of a sufficient amount of the adenylated form of the DNA ligase has been offered as one explanation [4].

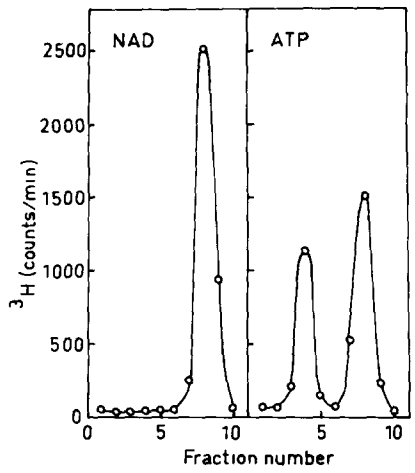

Fig.2. ATP and NAD requirements for ligation in extracts of *E. coli* 594 su⁻ λgt4 lopII lig⁺ S7, which overproduces DNA ligase 500-fold.

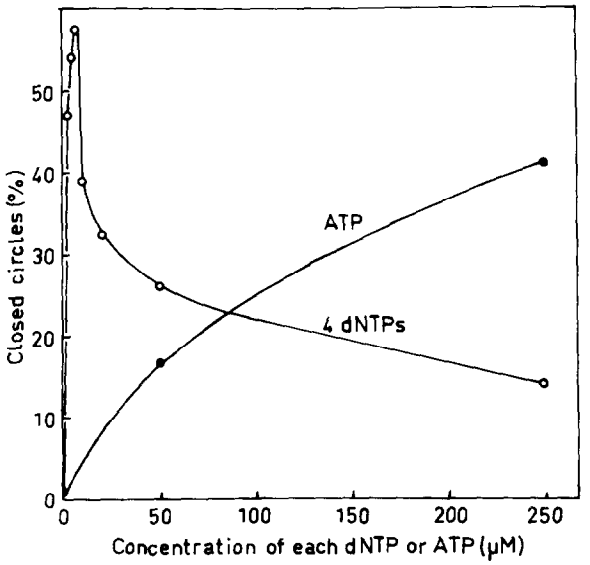

Fig.3. Ligation supported by the 4 dNTPs in extracts of *E. coli* 1200 in the absence of ATP. The ATP dependence is shown for comparison.

3.3. *Covalent closing of the circles in the presence of the 4 dNTPs*

It was found previously that among the nucleoside triphosphates, dATP could replace ATP as a requirement for the covalent closing of the hydrogen-bonded λDNA circles in *E. coli* extracts [6]. As in the case of ATP, the closing activity fell rapidly when the concentration of dATP was increased over the optimal concentration of 300 μM. As shown in fig.3, deoxyribonucleoside triphosphates at much lower concentrations also supported ligation provided that all 4 nucleotides were present, closed circle formation being maximal with 8 μM of each. None of the deoxyribonucleoside triphosphates alone supported the reaction at this concentration, not even dATP.

The closing activity observed in the presence of the 4 dNTPs depended on the same factors as in the presence of ATP. It was present in extracts of the *rec*C mutant, but not of the *pol*A mutant and only little activity was found in those of the *rec*B mutant (table 1). The K^+ requirement was also similar. The system activated by the 4 dNTPs was thus apparently the same as that activated by ATP and dATP.

The role of dNTPs in supporting ligation was probably a regulatory one, and DNA synthesis was

Table 1

Covalent closing of hydrogen-bonded λDNA circles in extracts of various *E. coli* mutant cells in the presence of ATP or the 4 dNTPs

Strain	Closed circles (%)	
	250 μM ATP	8 μM dNTP
C600	33	38
recC$^-$	38	49
recB$^-$	< 1	7
polA	< 1	< 1

not involved, as the hydrogen-bonded DNA circles that were employed as the substrates did not have any gaps, which would have been closed before ligation. These circles also served well as a substrate for purified *E. coli* DNA ligase.

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