Short Communication

The Effects of DNA Polymerase I and Nucleotides on Ligation of Hydrogen-Bonded λDNA Circles by *Escherichia coli* DNA Ligase

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The DNA ligase enzyme of *Escherichia coli* (EC 6.5.1.2) catalyzes the closing of single-stranded cuts in double-stranded DNA with the energy obtained from cleavage of NAD to AMP and NMN.[1,2] In our previous studies it was found that efficient covalent closing of hydrogen-bonded λDNA circles in extracts of *E. coli* required ATP or a mixture of the four common deoxyribonucleoside triphosphates (dNTP), as well as potassium ions at an optimum concentration range of 0.15–0.20 M.[3–5] NAD was neither required for, nor improved the efficiency of, ligation.[4,5] The closing of the circles did not occur or was retarded in extracts of *pol*A mutants,[6] indicating that DNA polymerase I (EC 2.7.7.7) was apparently required for efficient ligation.

The role of ATP and DNA polymerase I in ligation in *E. coli* extracts cannot be easily explained. This study was undertaken to determine whether similar effects of ATP on ligation can be observed when purified enzymes are employed. The requirement of NAD for ligation was also studied.

Experimental procedure

Bacterial and phage strains. The *E. coli* strain 1200, endonuclease I^- and RNase I^- [7] were obtained from Dr. D. Court, and the DNA ligase-overproducing strain 594 su^- λgt 4 lop 11 lig^+ S7[8] from Dr. I. Lehman. The bacteriophage $\lambda c_1 857$ was from Dr. H. Echols' collection.

Chemicals and enzymes. Nucleoside triphosphates, deoxyadenosine monophosphate and NAD were purchased from Boehringer (Mannheim, FRG), thymidine and polyethylene glycol 6000 from Fluka (Buchs, Switzerland) and [Me-^{3}H]thymidine from the Radiochemical Centre (Amersham, England).

DNA polymerase I was purchased from Boehringer and *E. coli* DNA ligase was purified from ligase-overproducing strain 594su^- λgt 4 lop 11 lig^+ S7 according to Panasenko *et al.*,[9] except that the concentration step on a DEAE-Sephadex column was ommitted. A single protein peak exhibiting ligase activity was obtained in phosphocellulase ion exchange and the enzyme was precipitated with ammonium sulfate at 70 % saturation, dissolved in 20 mM potassium phosphate buffer (pH 6.5), 2 mM EDTA, 1 mM dithiothreitol, 10 mM ammonium sulfate, 15 % (*v/v*) glycerol and dialyzed against the same buffer. The dialyzed preparation was made up to a glycerol concentration of 50 % and stored at −20 °C.

Preparation of bacteriophage λ hydrogen-bonded [^{3}H]DNA circles, and ligation assays. Hydrogen-bonded λDNA circles were prepared essentially as described previously,[5] except that H-1 medium was replaced with LB medium supplemented with 5 mM $MgSO_4$ and 5 mM $CaCl_2$ when growing bacteria, and the phage suspension, made after precipitation with polyethylene glycol 6000, was extracted six times with chloroform.

The activities of DNA ligase preparations were assayed by the method of Gellert,[10] using hydrogen-bonded λDNA circles as the substrate. The reaction mixtures (100 μl) for studying the effects of nucleotides and DNA polymerase I on ligation contained 10 mM Tris/HCl(pH 8.0), 5 mM $MgSO_4$, 0.15 M KCl, 50 μg ml^{-1} of bovine plasma albumin and 2 μg ml^{-1} of tritium-labelled hydrogen-bonded λDNA circles. The concentrations of nucleotides, when present, were: ATP 1 mM, dNTP 10 μM of each nucleotide, dAMP 0.7 mM and NAD 26 μM. The enzymes were dissolved in 20 mM Tris/HCl(pH 8.0), 2 mM $MgSO_4$, 0.2 mM dithiothreitol and 50 μg ml^{-1} bovine plasma albumin before they were added to the reaction mixture. The samples were incubated at 30 °C for 30 min. The reaction was terminated by chilling in ice and adding 10 μl of 0.2 M EDTA and 10 μl of 0.5 M NaOH. The amount of covalently-closed circles formed was measured as described previously.[4]

A unit of DNA polymerase was taken as that defined by Richardson *et al.*[11] One unit of DNA ligase catalyzed 10 % conversion of hydrogen-bonded λDNA circles to covalent circles in the assay described by Gellert.[10]

Results

Partially purified DNA ligase preparations from *E. coli* cells are known to have some activity even in the absence of NAD,[2,12] which is the energy source of the enzyme. Table 1 shows that the enzyme purified to homogeneity from 500-fold overproducing λlysogen was also fairly active in the absence of NAD. The remaining activity in the absence of NAD was about 15 % when the optimal assay conditions for purified DNA ligase, with ammonium ions at a concentration of 10 mM, were employed. When the ammonium ions were replaced with 0.15 M KCl in the assay mixture that contained NAD, the ligase activity decreased to about 1/10 of its previous value. However, if NAD was now omitted, the activity decreased by only about 20–30 %.

Table 1. NAD requirement of purified *Escherichia coli* DNA ligase. The standard assay mixture described by Gellert[10] was employed. The activity in the standard mixture involving ammonium ions and NAD was taken to be 100 %.

Monovalent ion present in assay mixture	Ligase activity/%	
	NAD present	NAD omitted
10 mM ammonium ion	100	15
150 mM potassium ion	12	9

In extract of *E. coli,* the ligation of λDNA circles was previously found to require DNA polymerase I.[6] It was therefore of interest to see whether the pre-incubation of DNA polymerase I with the hydrogen-bonded λDNA circles at a KCl concentration of 0.15 M had any effect on the covalent closing of the circles in the subsequent incubation with DNA ligase. The experiments summarized in Table 2 showed that DNA polymerase I destroyed the ability of the circles to be ligated both in the presence and absence of dNTP. The cause of this was apparently the 5' exonuclease activity of DNA polymerase I, since the large fragment of the enzyme (Klenow enzyme) that lacks the 5' exonuclease domain[13] did not affect the ability of the circles to be ligated unless dNTP was present. The presence of dNTP presumably caused stand displacement by the Klenow enzyme, which prevented the ligation of the circles. The 3' exonuclease activity of DNA polymerase I was not involved in the inactivation, since dAMP, a specific inhibitor for 3' exonuclease activity,[14] was unable to prevent the function of the polymerase.

Simultaneous addition of DNA ligase and DNA polymerase I to the assay mixture resulted in the inhibition of the ligation if the relative concentrations of these enzymes favoured the polymerase (Table 3). This suggested that the two enzymes were probably simply competing for the same substrate. The ligation of circles in the assay mixture containing both DNA polymerase I and DNA ligase was not found to be dependent on dNTP or ATP, as is the case for *E. coli* extract.[3,5] However, ATP was observed to inhibit the ligation in the presence of dNTP in a limited range of concentrations of the two enzymes.

Table 2. The effect of DNA polymerase I and its large fragment (Klenow enzyme) on the ligation of hydrogen-bonded λDNA circles. The circles were first incubated with DNA polymerase I or Klenow enzyme at 30 °C for 30 min, and then 200 units of DNA ligase and NAD to a final concentration of 26 μM were added and the incubation was continued for another 30 min.

Enzyme used for preincubation	Amount of enzyme/units	Covalently closed circles/%				
		Nucleotide absent	ATP	dNTP	dAMP	dNTP+ATP
DNA polymerase 1	0.02	43	40	41	44	38
"	0.05	11	8	7	6	10
"	0.15	<3	<3	<3	<3	<3
Klenow enzyme	0.02	40	36	42	41	46
"	0.05	35	39	12	33	10
"	0.15	38	36	<3	38	<3

Table 3. The ligation of hydrogen-bonded λDNA circles in the mixture of DNA polymerase I and DNA ligase. The reaction conditions were those described in the Experimental section.

Enzyme/units		Covalently closed circles/%				
DNA polymerase I	DNA ligase	Nucleotide absent	ATP	dNTP	dNTP+ATP	NAD
0	200	26	38	35	39	34
0.3	200	39	36	42	38	[a]
1	200	32	36	40	6	[a]
3	200	5	6	9	<3	7
3	500	19	17	23	22	[a]

[a]Not determined.

Discussion

The *E. coli* cell has been estimated to contain about 300 DNA ligase molecules,[15] and possibly only one per cent of these is enough to satisfy the requirements of the cell.[16] The high proportion of ligase that is active without NAD suggests that NAD does not usually limit the efficiency of ligation in cells. An adequate amount of "charged ligase", i.e. ligase-AMP, is presumably always present in the cell. This is in agreement with our previous results indicating that ligation in extracts of *E. coli* cells is independent of NAD.[4,5] Instead, the ligation was found to be dependent on ATP. This NAD-independent activity was also present in extracts that were prepared in the presence of EDTA,[12] which should inhibit the formation of ligase-AMP,[17] indicating that ligase-AMP was not formed during preparation of the extract but rather was present in the cells.

The ligation with purified enzymes at a K^+ concentration of 0.15 M differs in two respects from the ligation with *E. coli* extract that has been studied before.[3-5] First, ligation was neither dependent on ATP nor even stimulated by it under any of the conditions employed and secondly, it did not require DNA polymerase I. It was also found that even when large amounts of purified DNA polymerase were added to crude extracts, the ATP-dependent ligation activity was not inhibited (data not shown). Evidently, other factors in addition to DNA polymerase I and DNA ligase are involved in the support of ligation in an ATP-dependent system.

Inhibition of ligation by ATP in a certain range of concentrations of the two enzymes in the presence of dNTP cannot be readily explained. It is

possible that ATP can somehow extend its influence to the polymerase or exonuclease activity of DNA polymerase I. ATP has previously been reported to increase the activity of DNA polymerase I in the presence of only one of the four common deoxyribonucleoside triphosphates.[18]

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