Volume 13 Number 22 1985 Nucleic Acids Research

Stimulation of intermolecular ligation with *E. coli* DNA ligase by high concentrations of monovalent cations in polyethylene glycol solutions

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Received 12 September 1985; Revised and Accepted 31 October 1985

ABSTRACT

In the presence of high concentrations of the nonspecific polymer polyethylene glycol (PEG), intermolecular cohesive-end ligation with the DNA ligase from *Escherichia coli* was stimulated by high salt concentrations: 200 mM NaCl or 300 mM KCl in 10% (w/v) PEG 6000 solutions, and 100-200 mM NaCl or 150-300 mM KCl in 15% PEG 6000 solutions. Intermolecular blunt-end ligation with this ligase was also stimulated at 100-150 mM NaCl or 150-250 mM KCl in 15% PEG 6000 solutions. The extent of such intermolecular ligation increased and the salt concentrations at which ligation was stimulated extended to lower concentrations when we raised the temperature from 10 to 37 °C.

INTRODUCTION

Both cohesive- and blunt-end ligation with T4 DNA ligase is stimulated at high concentrations of salt: 150-200 mM NaCl or 200-250 mM KCl when 10% (w/v) PEG 6000 was present (1). This phenomenon is probably caused by increased affinity of the enzyme for the DNA substrates, because the first ligation reaction, ATP-PPi exchange (2), is not stimulated by salt in the presence of 10% PEG 6000. We think that such affinity is enhanced both by volume exclusion with PEG (3), and by changes in DNA caused by high concentrations of a monovalent cation. If this is true, monovalent cations will have such effects with other DNA ligases.

The presence of polymer makes blunt-end ligation with *E. coli* DNA ligase possible when the concentration of salt is very low (1 mM NH_4Cl); such ligation is stimulated in 19% PEG 6000 solution, but not in solutions with 15% PEG 6000 or less (4). Here, we investigate the effect of monovalent cations such as Na^+ or K^+ on ligation with *E. coli* DNA ligase in PEG solutions.

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We found that both cohesive- and blunt-end ligation was stimulated by the presence of monovalent cation in PEG solutions, and that all of the products in situations where stimulation occurred were linear oligomers from intermolecular ligation. The results with E. coli DNA ligase had something in common with those with T4 DNA ligase. We think that the stimulation phenomenon with both E. coli and T4 DNA ligase involved the same mechanism.

MATERIALS AND METHODS

Materials

E. coli DNA ligase was purified from E. coli UT481 carrying the plasmid PLG 2520 encoding the ligase (Y. Ishino et al., personal communication) as described elsewhere (5) with some modifications. The restriction endonucleases EcoRI and PvuII were purified at our laboratory. Two types of linear pBR322 DNA were prepared by digestion with EcoRI or PvuII (1); we used these DNA to assay products of cohesive- or blunt-end ligation. PEG 4000, 6000, and 20000 were purchased from Wako Chemical Industries, Ltd., Japan. Stock solutions of 40% (w/v) PEG 4000, 6000, and 20000 were made using distilled water. In this report, solutions of those polymers are expressed as (w/v) percent. [^{3}H]NAD was purchased from New England Nuclear.

Methods

The ligation activity of E. coli DNA ligase was assayed in a mixture (20 µl) containing 30 mM tris-HCl (pH 8.0), 4 mM $MgCl_2$, 1.2 mM EDTA, 100 µM NAD, 0.25 µg of linearized pBR322 DNA, and various amounts of this ligase. Samples were incubated at 10, 16, 26, and 37°C for 30 min. One unit of this ligase was defined as the amount needed to give more than 90% ligation of HindIII-digested λ DNA at 16°C for 30 min in 20 µl of the same mixture as above, except that 6 µg of the HindIII-digested λ DNA was used instead of the pBR322 DNA, and 10 mM $(NH_4)_2SO_4$ was contained. The ligation products were analysed as reported previously (1).

The enzyme-AMP complex formation was assayed in 100 µl of a mixture containing 30 mM tris-HCl (pH 8.0), 4 mM $MgCl_2$, 1.2 mM EDTA, 2 × 10^6 cpm [^{3}H]NAD (2.8 Ci/mmol), and different con-

centrations of PEG and salts. Incubation was at 37°C for 30 min. The radio activity in the acid-insoluble material was measured as described elsewhere (6) with some modifications.

RESULTS

Influence of monovalent cations on cohesive-end ligation in PEG 6000 solutions

The effect of Na^+ or K^+ on cohesive-end ligation with E. coli DNA ligase was measured in mixtures containing various concentrations of PEG 6000 (0, 5, 10, 15, and 20%). When PEG 6000 was not present, ligation was maximum at 50 mM KCl (Fig. 1, Lane 6), but it decreased as the concentration of NaCl was raised (data not shown). When either NaCl or KCl was present, ligation activity was nearly completely inhibited at 150 mM or more (Fig. 1, Lane 4). These results were similar to those reported elsewhere; that the low concentrations of K^+ (< 10 mM) stimulate the joining reaction of this ligase, but that Na^+

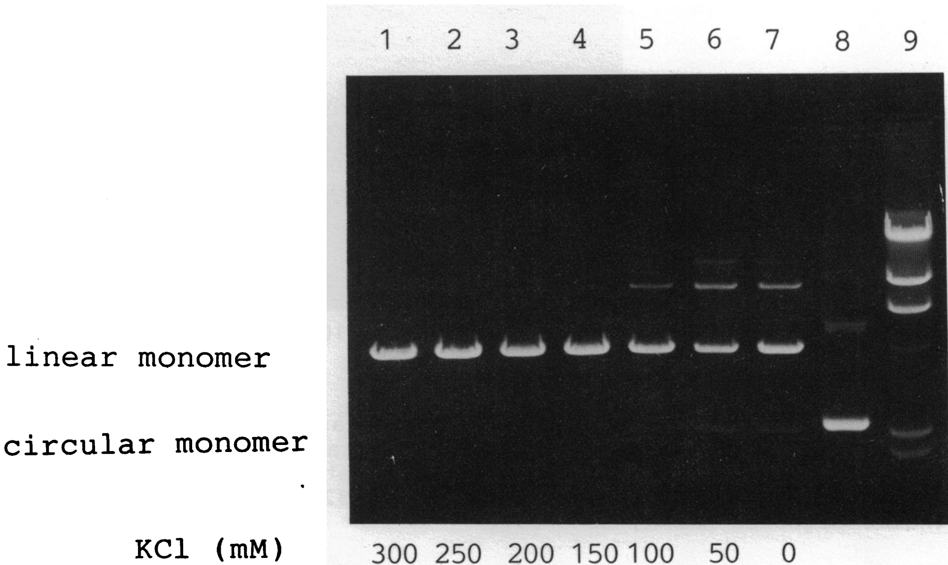

Figure 1. Effect of K^+ on cohesive-end ligation when PEG is not present. Assay mixtures were as in Methods, and contained EcoRI-digested pBR322 DNA. KCl was present at the concentrations indicated. E. coli DNA ligase (60 u) was added. Incubation was at 26°C. pBR322 DNA RFI, Lane 8; HindIII-digested λ DNA, Lane 9.

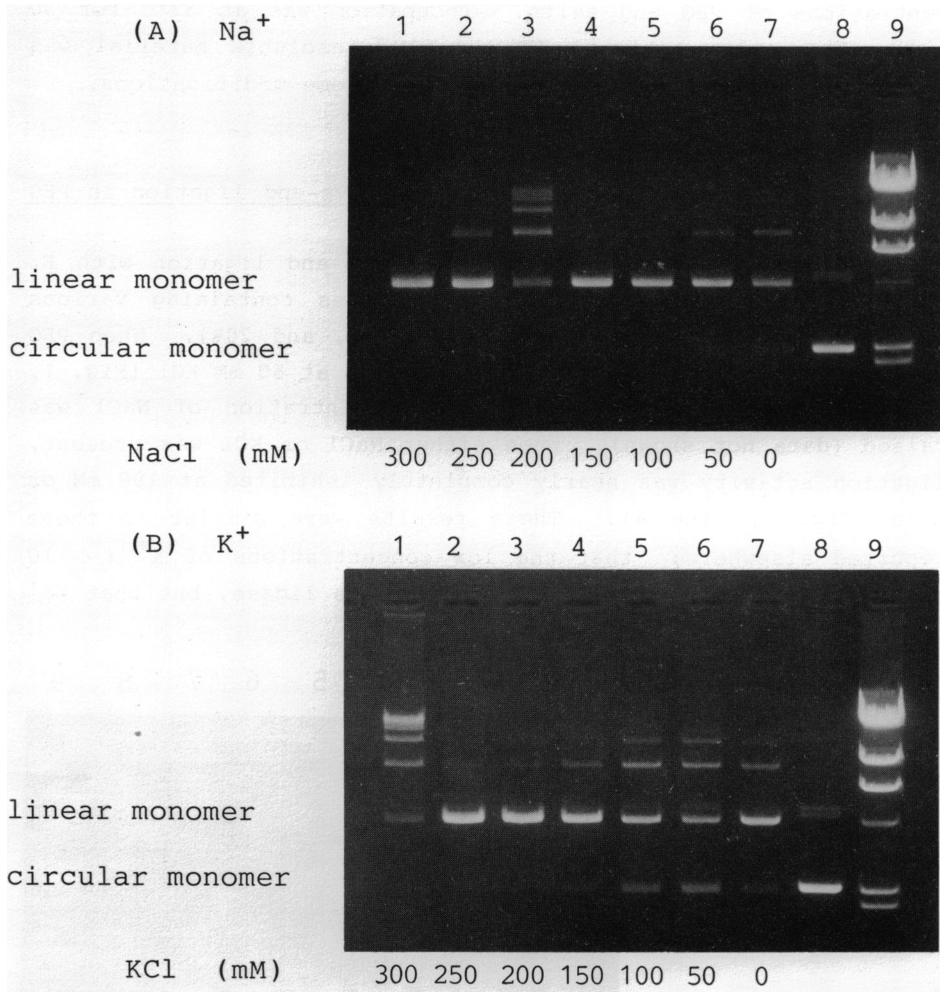

Figure 2. Effect of Na^{+} (A) and of K^{+} (B) on cohesive-end ligation in 10% PEG 6000. E. coli DNA ligase (1.2 u) was added to mixtures as in Methods, with EcoRI-digested pBR322 DNA and 10% PEG 6000. NaCl or KCl was present at the concentrations indicated. Incubation was at 26° C. pBR322 DNA RFI, Lane 8; HindIII-digested λ DNA, Lane 9.

has no effect in concentrations from 0 to 10 mM (7). The extent of ligation at 50 mM KCl was about 2 fold that when salt was absent (Fig. 1, Lanes 6 and 7). As the temperature was raised from 10 to 37°C, ligation decreased. The results with Na^{+} or K^{+} in 5% PEG 6000 were similar to those with none; both inter- and

intramolecular ligation occurred, and the extent of ligation decreased as the temperature was raised. However, the extent was several times that without PEG 6000 (data not shown).

At high salt concentrations, intermolecular ligation was stimulated in 10 or 15% PEG 6000 solutions. In 10% PEG 6000 solutions, both inter- and intramolecular ligation occurred with 0-100 mM NaCl or 0-150 mM KCl (Fig. 2A, Lanes 5-7 ; Fig. 2B, Lanes 4-7); ligation was almost completely inhibited with 150 mM NaCl or 200-250 mM KCl (Fig. 2A, Lane 4; Fig. 2B, Lanes 2 and 3). However, at 200 mM NaCl or 300 mM KCl, ligation was markedly stimulated and all ligation products were linear oligomers from intermolecular ligation (Fig. 2A, Lane 3; Fig. 2B, Lane 1). We found that the EcoRI site was actually joined by the ligase, and that these products at high salt concentrations were linear, as reported before (1). Intermolecular ligation at high concentrations of salt in 10% PEG 6000 solutions increased as the temperature was raised from 10 to 26°C, and was also stimulated at 150 mM NaCl or 200-250 mM KCl at 37°C (Fig. 3). However, both inter- and intramolecular ligation at low salt concentrations were inhibited as the temperature was raised (data not shown). Similar effects were observed in 15% PEG 6000 solutions. Figure 4 shows the results at 26°C in mixtures containing various concentrations of NaCl or KCl when 15% PEG 6000 was present. Intermolecular ligation was markedly stimulated at 100-200 mM NaCl or 150-300 mM KCl as the temperature was raised from 10 to 37°C. The extent of ligation in the mixtures with high concentrations of salt was nearly the same as that at 26°C, but ligation at low salt concentrations was inhibited as the temperature was raised (data not shown). The phenomenon in which concentrations of salt at which intermolecular ligation was stimulated extended to lower concentrations as the temperature increased was also observed in 15% PEG 6000 solutions. In the mixture containing 100 mM NaCl or 150 mM KCl, both inter- and intramolecular ligation occurred at 10°C, but intramolecular ligation was inhibited at 16, 26, and 37°C (data not shown). In 20% PEG 6000 solutions, only intermolecular ligation occurred even at low concentrations of salt; 50-100 mM NaCl or 100-150 mM KCl were the optimal

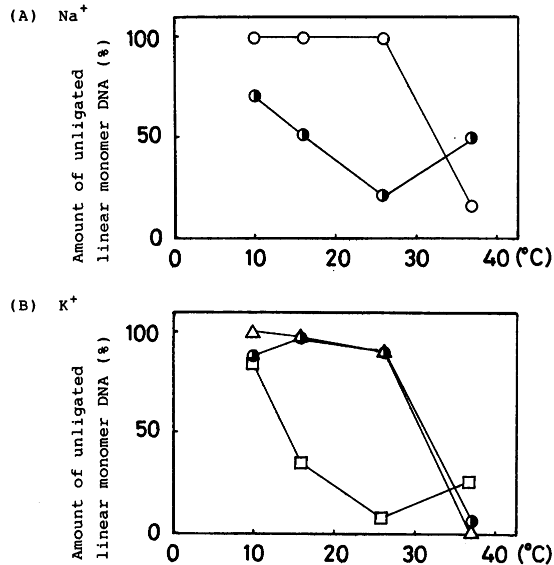

Figure 3. Effect of temperature on ligation in 10% PEG 6000 when the concentrations of Na^+ or K^+ are high. Assay mixtures were as in Methods, and contained EcoRI-digested pBR322 DNA and 10% PEG 6000; 150-200 mM NaCl (A) or 200-300 mM KCl (B) were present. E. coli DNA ligase (1.2 u) was added. Incubation was at 10, 16, 26, and 37 °C. The amount of unligated linear monomer DNA was measured using a densitometer. A: (○), 150 mM, and (◑), 200 mM NaCl; B: (◑), 200 mM, (△), 250 mM, and (□), 300 mM KCl.

concentrations, and the extent of ligation increased as the temperature was raised.

Figure 5 shows the amount of unligated linear monomer DNA at high salt concentrations (100-200 mM NaCl or 150-300 mM KCl). The results were that the extent of ligation in such situations was maximum in 15% PEG 6000 solutions, but there was no great difference in the extent of ligation at 200 mM NaCl or 300 mM KCl between 10 and 15% PEG 6000 solutions. A difference between the stimulation effect of Na^+ and K^+ on intermolecular ligation was almost undetectable at their optimal concentrations, which was higher for K^+ than for Na^+.

Influence of monovalent cations on blunt-end ligation in PEG 6000 solutions

E. coli DNA ligase cannot catalyze blunt-end ligation when

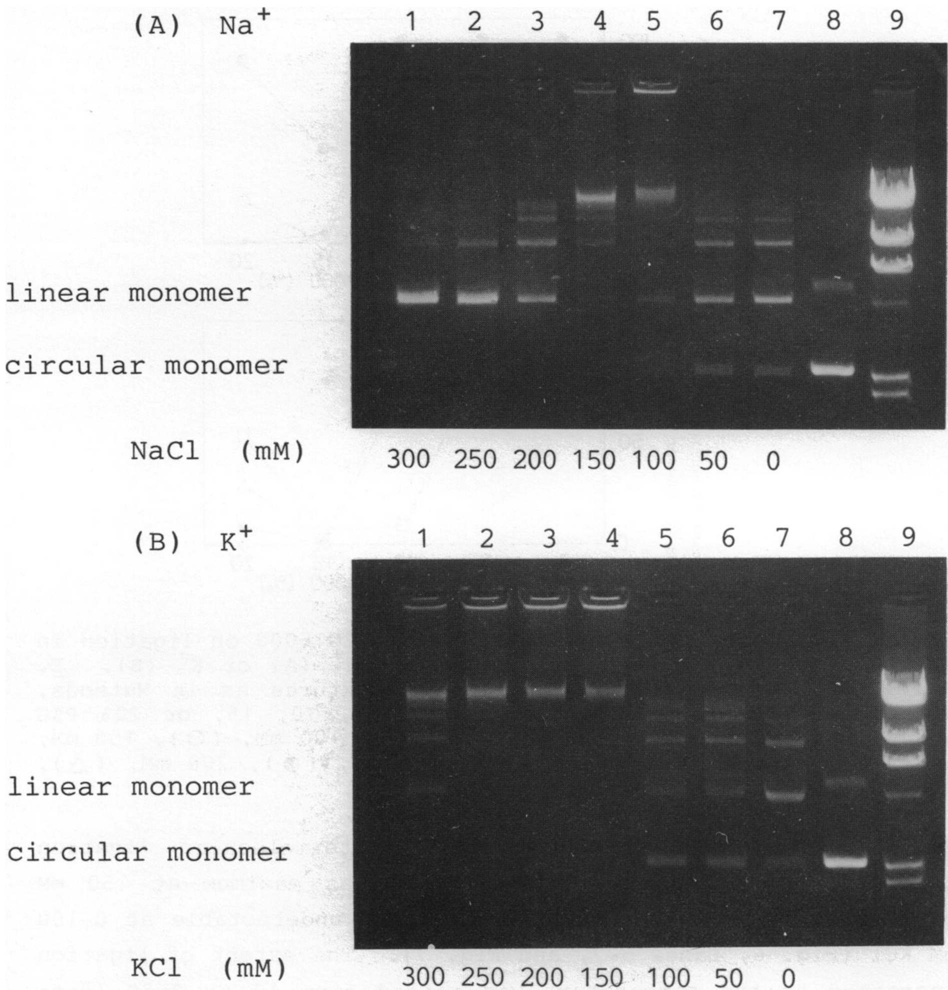

Figure 4. Effect of Na^+ (A) and of K^+ (B) on cohesive-end ligation in 15% PEG 6000. E. coli DNA ligase (1.2 u) was added to mixtures as in Methods, with EcoRI-digested pBR322 DNA and 15% PEG 6000. NaCl or KCl was present at the concentrations as indicated. Incubation was at 26°C. pBR322 DNA RFI, Lane 8; HindIII-digested λ DNA, Lane 9.

PEG 6000 is not present (8, 9), nor can it catalyze such ligation in 10% PEG 6000 solutions even at high concentrations of salt (data not shown). However, when 15% PEG 6000 was present, intermolecular blunt-end ligation was possible at only high salt concentrations. Figure 6 shows the effect of K^+ with

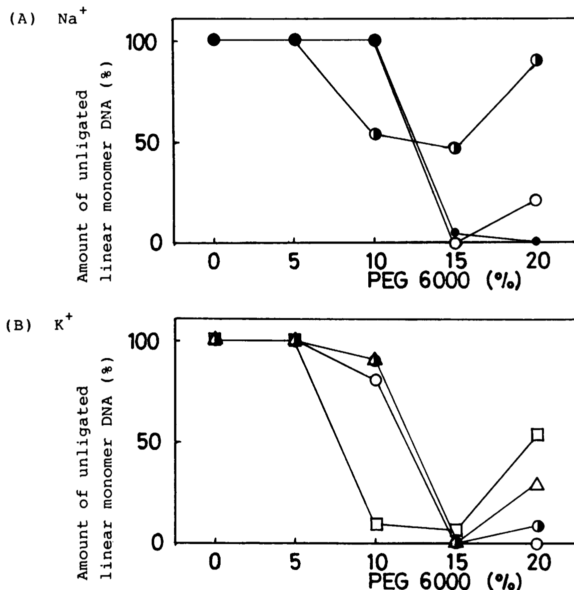

Figure 5. Effect of concentrations of PEG 6000 on ligation in the presence of high concentrations of Na^+ (A) or K^+ (B). E. coli DNA ligase (1.2 u) was added to mixtures as in Methods, with EcoRI-digested pBR322 DNA and 0, 5, 10, 15, or 20% PEG 6000. Incubation was at 26°C. A: (●), 100 mM, (○), 150 mM, and (◑), 200 mM NaCl. B: (○), 150 mM, (◑), 200 mM, (△), 250 mM, and (□), 300 mM KCl.

PvuII-digested pBR322 DNA at 26°C. Intermolecular ligation occurred at 150-250 mM KCl; ligation was maximum at 150 mM (Fig. 6, Lane 4, and Fig. 7), and almost undetectable at 0-100 mM KCl (Fig. 6, Lanes 5-7, and Fig. 7). The extent of ligation increased as the temperature was raised from 10 to 26°C (data not shown), and ligation was also possible at 100 mM KCl at 37°C (Fig. 7). In 20% PEG 6000 solutions, blunt-end ligation was possible even at low concentrations of salt; 100 mM KCl was the optimal concentration (Fig. 7), and the ligation products were linear oligomers from intermolecular ligation. In these 20% solutions, the effect of temperature was as same as for cohesive-end ligation.

Similar results were obtained with Na^+ in 15 and 20% PEG 6000 solutions; intermolecular blunt-end ligation was possible only at 100-150 mM NaCl when the temperature was 10-26°C, and

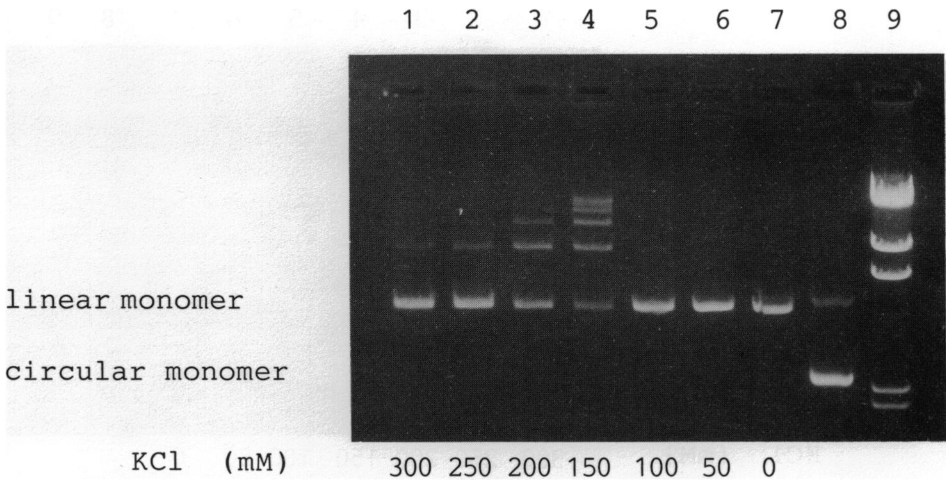

Figure 6. Effect of K^+ on blunt-end ligation in 15% PEG 6000. E. coli DNA ligase (1.2 u) was added to mixtures as in Methods, with PvuII-digested pBR322 DNA and 15% PEG 6000. KCl was present at the concentrations indicated. Incubation was at 26°C. pBR322 DNA RFI, Lane 8; HindIII-digested λ DNA, Lane 9.

at 50-150 mM NaCl at 37°C in the 15% solutions. This kind of ligation was possible even at low concentrations of NaCl in 20% PEG 6000 solutions. However, stimulation by Na^+ was only one-tenth that of K^+ (data not shown).

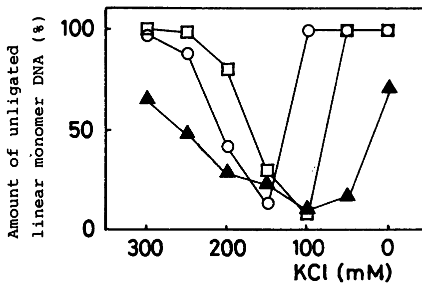

Figure 7. Extent of blunt-end ligation in 15 and 20% PEG 6000. Assay mixtures were as in Methods, and contained PvuII-digested pBR322 DNA and 15 or 20% PEG 6000. KCl was present at the concentrations indicated. E. coli DNA ligase (1.2 u) was added. Incubation was at 26 or 37°C. (○), 15% PEG 6000 at 26°C; (□), 15% PEG 6000 at 37°C; (▲), 20% PEG 6000 at 26°C.

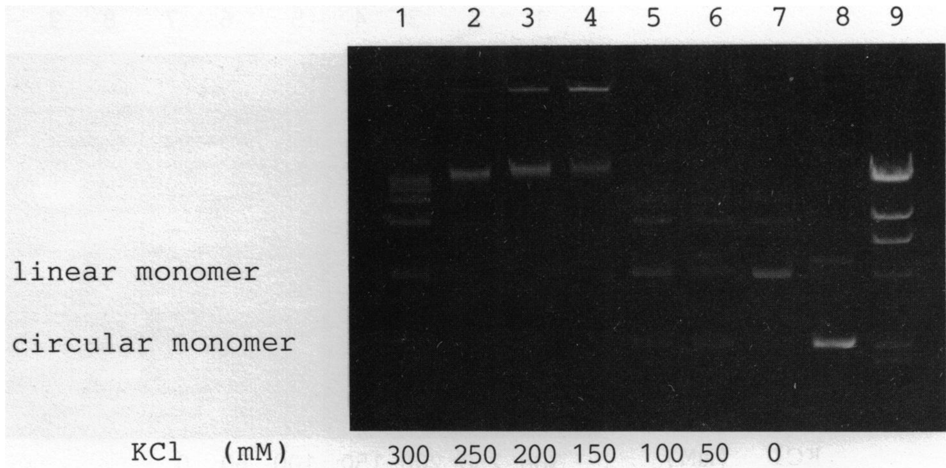

Figure 8. Effect of K^{+} on cohesive-end ligation in 10% PEG 20000. E. coli DNA ligase (1.2 u) was added to mixtures as in Methods, with EcoRI-digested pBR322 DNA and 10% PEG 20000. KCl was present at the concentrations as indicated. Incubation was at 26°C. pBR322 DNA RFI, Lane 8; HindIII-digasted λ DNA, Lane 9.

Influence of the molecular size of PEG on intermolecular ligation

The effect K^{+} on cohesive- and blunt-end ligation was also measured with 1.2 units of E. coli DNA ligase in PEG 4000 or 20000 solutions.

Stimulation of intermolecular cohesive-end ligation at high concentrations of salt was observed in 15 and 20% PEG 4000 and in 5 and 10% PEG 20000 solutions. Results in 15% PEG 4000 or 5% PEG 20000 solutions were similar to those in 10% PEG 6000 solutions; when incubation was at 26°C, intermolecular ligation was stimulated at 250 mM KCl in 15% PEG 4000 solutions, and at 300 mM KCl in 5% PEG 20000 solutions. In 20% PEG 4000 solutions, only intermolecular ligation occurred at from 0 to 300 mM KCl, and ligation was maximum at 100-200 mM KCl (data not shown). The results in 10% PEG 20000 solutions (Fig. 8) were similar to those in 15% PEG 6000 solutions; intermolecular ligation was stimulated at 150-250 mM KCl at 26°C. The extent of such ligation in PEG 4000 or 20000 solutions increased and the salt concentrations at which ligation was stimulated

Table 1. Effect of Na^+ or K^+ on formation of ligase-AMP complex in 15% PEG 6000 solutions.

Salt	Rate (%)
none	100
150 mM NaCl	91
150 mM KCl	97

extended to lower concentrations when we raised the temperature to 37°C.

Intermolecular blunt-end ligation by this ligase also occurred at high concentrations of salt in 15 and 20% PEG 4000 and in 10% PEG 20000 solutions. In 15% PEG 4000 solutions, such ligation was possible at 250 mM KCl at 26°C and at 200-250 mM KCl at 37°C, but the product amounted to only a few percents of the total DNA. When 20% PEG 4000 was present, the results were similar to those in 20% PEG 6000 solutions; ligation was maximum at 150-200 mM KCl at 26°C, and at 100-150 mM KCl at 37°C. Results in 10% PEG 20000 solutions were similar to those in 15% PEG 6000 solutions; ligation occurred at 250 mM KCl at 26°C, and at 150-200 mM KCl at 37°C (data not shown).

Influence of monovalent cations on formation of ligase-AMP complex in PEG 6000 solutions

E. coli DNA ligase first forms the enzyme-AMP complex using NAD with the elimination of NMN, and then this complex acts on the nick in DNA to form a phosphodiester linkage between the 3'-OH and 5'-phosphate termini (7). This first reaction does not require DNA. The effect of Na^+ or K^+ on this reaction was measured in 15% PEG 6000 solutions at 37°C. Table 1 shows the results obtained in mixtures containing 150 mM of NaCl or KCl, and with neither. Here, 100% activity is the activity in the mixture without salt.

The amount of the [^{3}H]NAD incorporated into the enzyme-AMP complex changed little, when high concentrations of a salt was added. These results suggest that the first ligation reaction was not stimulated by the presence of high concentrations of monovalent cations in PEG 6000 solutions.

DISCUSSION

The stimulation of intermolecular ligation with *E. coli* DNA ligase in the presence of highly concentrated PEG and monovalent cations was similar to the phenomenon we observed with T4 DNA ligase (1). Such stimulation with T4 DNA ligase occurred in 10% PEG 6000 solutions, but *E. coli* DNA ligase had this effect in both 10 and 15% PEG 6000 solutions. The results in 10% PEG 6000 solutions with T4 DNA ligase were nearly the same as those in 15% PEG 6000 solutions with *E. coli* DNA ligase. There was no difference between the stimulation effect of Na^+ and K^+ in the case of either cohesive- or blunt-end ligation with T4 DNA ligase, or cohesive-end ligation with *E. coli* DNA ligase, but K^+ gave at least 10-fold the blunt-end ligation with *E. coli* DNA ligase compared to Na^+. In spite of these differences between the two enzymes, the stimulation seems to be caused by the same mechanism, because we obtained results common to both, as follows:

(a) Formation of the enzyme-AMP complex was not stimulated by high concentrations of monovalent cations in PEG solutions.

(b) The concentrations of K^+ at which intermolecular ligation was stimulated were always higher than those of Na^+.

(c) Concentrations of PEG at which such ligation was caused by high concentrations of ,monovalent cations decreased as the molecular size of PEG increased.

(d) Concentrations of monovalent cations at which such stimulation was caused decreased as the concentrations of PEG increased.

(e) The extent of intermolecular ligation caused by the presence of highly concentrated PEG and monovalent cations increased and the concentrations of cation that caused such ligation extended to lower concentrations as the temperature was raised.

The finding in *a* indicated that such stimulation was caused by the increased affinity of the enzyme for the DNA substrates. The amount of the water around the enzyme is increased when a nonspecific polymer such as PEG is present in solution, because of its volume-exclusive effect (3). The melting temperature of DNA is raised by the presence of PEG or monovalent cations

(10-12). The effect of the cations on this property can be quantitatively interpreted as their neutralizing of the negative charges of the phosphate groups in the DNA (11, 12). Based on this evidence and our results, we think that the stimulation of intermolecular ligation by high concentrations of monovalent cations in PEG solutions is brought out by the concentration of DNA termini around the enzyme being increased by both the PEG and by neutralization of the negative charges of the phosphate groups in the DNA with cations. Finding in b supports this assumption, because the effect with Na^+ is stronger than that of K^+ (13). Results c and d indicate that the concentrations of monovalent cations around the enzyme become higher as the concentration or the molecular size of PEG increases, by its volume-exclusive effect. A concentration of DNA substrate that would stimulate intermolecular ligation could be generated at lower concentrations of cations when the concentration of PEG was high. A similar situation would be generated at lower concentrations of PEG when its molecular size was larger, because the volume-excluding effect of PEG strengthens as its molecular size increases.

We can interpret the temperature effect e as follows. The probability of the DNA terminus of one molecule approaching that of another would be increased as the temperature was raised, so that a situation in which such ligation would be stimulated could be generated at lower concentrations of cation at which its neutralizing effect was inadequate.

Therefore, we think that the concentration of the DNA substrate is increased by neutralization of the negative charges of the phosphate groups in the DNA in PEG solutions, so the stimulation of intermolecular ligation by DNA ligase is caused by any cation in PEG solution.

ACKNOWLEDGEMENTS

We thank Yoshizumi Ishino, Dr. Hideo Shinagawa, Dr. Kozo Makino, Dr. Atsuo Nakata, and other members of the Department of Experimental Chemotherapy in the Research Institute for Microbial Diseases, Osaka University for providing *E. coli* UT481 carrying the plasmid PLG 2520 encoding the ligase.

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