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AMP-dependent DNA relaxation catalyzed by DNA ligase occurs by a nicking-closing mechanism

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ABSTRACT

In the presence of AMP and Mg^{2+}, a covalently closed duplex DNA containing negative superhelical turns was treated with DNA ligase isolated from bacteriophage T4-infected E. coli. This resulted in the gradual and not sudden loss of superhelical turns as for example in the case of type I DNA topoisomerase. All DNA products remain covalently closed. Since T4 enzyme-mediated DNA relaxation is inhibited by both pyrophosphate and by ATP this suggests that DNA relaxing and DNA joining activities probably coincide. EDTA addition in the presence of a large excess of enzyme, induces the formation of nicked DNA products while protein denaturing treatments are not very effective. Our observations might suggest an involvement of the relaxing activity of DNA ligase during the ligation process.

INTRODUCTION

DNA joining activities have been found in a variety of prokaryotic (1-5) and eukaryotic cells and tissues (6-12) and have been shown to be involved in DNA replication, recombination and repair (13-15). According to the available descriptions of the two known classes of enzymic activities so far studied, the formation of a new phosphodiester bond between adjacent termini in duplex DNA is always coupled to the cleavage of a pyrophosphate bond in diphosphopyridine nucleotide (DPN) or in adenosine triphosphate (ATP). While yeast is known to possess only one form of DNA ligase activity (15), other eukaryotes are thought to utilize two distinct forms of DNA ligase (16-17). Proofs for the mechanism of DNA ligation (18) stemmed from three types of observations: i) isolation of reaction intermediates; ii) reversal of the reaction and iii) steady state kinetic analysis with points i) and ii) strongly in favor of the proposed action mechanism for both classes of enzymes. Our observation that a number of drugs are good inhibitors of both DNA topoisomerases (19) and DNA ligases (20) brought us to reanalyse the so called "reverse reaction" of ligation. In fact, the E. coli enzyme has been shown to relax a supercoiled DNA substrate in the presence of AMP in the so called "reverse reaction" of ligation (21). The relaxation of supercoiled substrate has been interpreted as consequence of two distinct and separate events: a) adenylation of the substrate in an AMP-dependent reaction, resulting in the production of a nicked and therefore

fully relaxed substrate; b) subsequent action of DNA ligase on the adenylated substrate to produce nicked DNA, adenylated enzyme and, according to the well studied mechanism of circle-ligation (22), a population of fully relaxed circular, covalently closed DNA molecules (cccDNA). Evidence for step a) was the isolation of nicked DNA at least in part adenylated (nDNA) together with relaxed cccDNA and adenylated enzyme (21) after treatment of supercoiled substrate with E. coli DNA ligase in the presence of AMP and Mg^{2+}. Similar conclusions were drawn from more recent controls done with the Drosophyla enzyme (23). So when the question of whether DNA ligases and topoisomerases share a similar mechanism of action was originally posed (21), three main differences were found: i) cofactor requirement for ligases; ii) indipendence from the original topological state of the substrate for ligase, and iii) the nicking-closing mode of action for DNA topoisomerases when relaxing supercoiled DNA in contrast to the one-hit mechanism for DNA ligase. But after the characterization of other DNA topoisomerases (24,25) points i) and ii) were no longer properties capable of differentiating between DNA ligases and topoisomerases, while point iii) remained the major one which discriminated between the two classes of enzymes. This paper reports our findings on this aspect of the issue. Original observations about the nicking-closing mode of DNA relaxation by T4 and human enzymes were presented in poster form at the Cold Spring Harbor Laboratory meeting on 'Eukaryotic DNA Replication', on September 2nd-6th, 1987.

MATERIALS AND METHODS

DNA, nucleotides and intercalating agents.

Closed-circular double stranded DNA (cccDNA) of plasmid pAT153 (26) and of monomeric and dimeric forms of pA1 (27) DNA were prepared as described (28). A single topoisomer was prepared as previously described (29). AMP, ATP, chloroquine phosphate and ethidium bromide were purchased from Sigma; the anthracycline derivative 3'-deamino-4-demetoxy-3'-hydroxy epirubicin (DDH-Epi) was kindly supplied by Dr. F. Arcamone, Farmitalia-Carlo Erba.

Enzymes.

DNA ligase purified from T4 infected E. coli was indifferently purchased from Toyobo or Bethesda Research Laboratories. DNA ligases were routinely diluited in a buffer containing 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM DTT and 50% (v/v) glycerol. DNA ligase units (U) or milliunits (mU) were calculated as described (30). Enzymes preparations were tested for contaminating endonucleolytic or topoisomerase activities by overnight incubation in the presence of supercoiled DNA with and without ATP, in a reaction mixture identical to that described below but in the absence of AMP. No modifications of the substrate were observed.

Assay for DNA relaxation.

Except otherwise stated, reaction mixture (20 ul) contained 20 mM Tris-HCl (pH 8.0), 3 mM $MgCl_2$, 100 ug/ml BSA, 1 mM EDTA, 100 ng cccDNA, 1 mM AMP and 1 ul of an appropriate dilution of enzymes. Mixtures were incubated for 30 min at 30°C. Reactions were stopped by adding 2 ul of a solution containing 1 mg/ml bromophenol blue, 50% (v/v) glycerol and 5 mg/ml SDS.

Aliquots (7 ul) were removed and analysed on a 1.4% agarose gel with 40 mM tris-acetate, 2 mM EDTA as running buffer. Electrophoresis was at 40 volts, usually until the bromophenol blue had migrated 7 cm. The gel was then stained with ethidium bromide (1 ug/ml) for 30 min and destained with H_2O for 15 min. DNA was visualized and the gel photographed on Polaroid 55 films, with the aid of an ultraviolet illuminator.

Assay for DNA nicking.

Except otherwise stated, formation of nicked DNA (nDNA) was assayed as for DNA relaxation except that reaction mixtures were incubated for 1 min at 0°C and then, according to what has been already described (21), reactions were stopped by addition of EDTA to 20 mM. After adding 2 ul of the solution containing bromophenol blue, analysis of the nDNA was performed as just described, except that electrophoresis time was reduced.

RESULTS

The kinetic of relaxation of negatively supercoiled DNA by T4 DNA ligase has been analysed in detail by means of the agarose gel technique. A progressive reduction in the number of superhelical turns (Figure 1A) indicated that DNA relaxation occurred by a gradual course rather than by a one-hit mechanism. This particular mode of action was deduced from the appearance of intermediate, partially relaxed products of reaction. The kinetic of relaxation shown in Figure 1A was obtained by stopping the reactions by adding SDS, but identical results were obtained when the reactions were stopped by addition of EDTA (not shown). Neither nicked nor

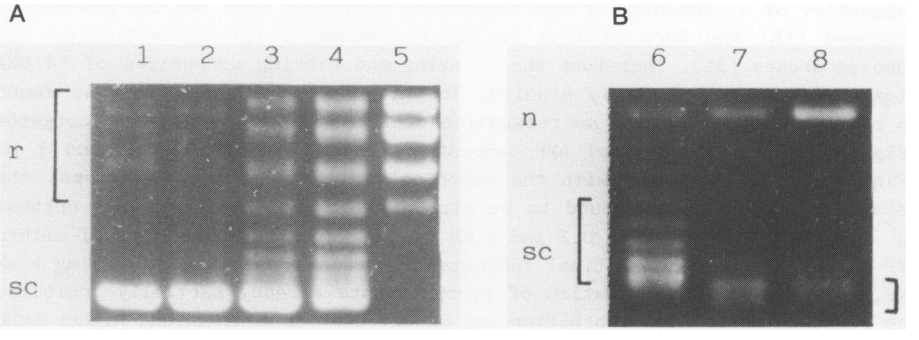

Figure 1: Kinetic of relaxation of negatively supercoiled DNA by T4 DNA ligase. Reaction mixture containing supercoiled pA1 DNA was as described in M.M.. Panel A: bacteriophage T4 DNA ligase (20 mU) was added to the reaction mixture of lanes 2 to 5. Lane 1, no enzyme. Reactions were stopped by addition of SDS containing solution at the following time: 0 min, lane 2; 2 min, lane 3; 10 min, lane 4; 30 min, lane 5. DNA analysis was performed as described in M.M.. Panel B: T4 DNA ligase (2 U) was added to lanes 7 and 8. Lane 6, no enzyme. Reactions were stopped by either SDS containing solution (lane 7) or 20 mM EDTA (lane 8) and analysed on 1.4% agarose gel in the presence of 5 uM chloroquine diphosphate to distinguish between nicked and cccDNA molecules. n=nicked, r=relaxed, sc=supercoiled.

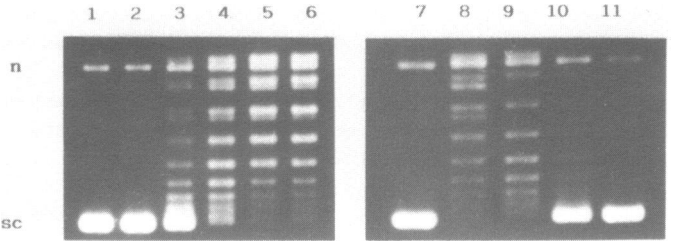

Figure 2: AMP dependence of DNA relaxation by T4 DNA ligase and effect of pyrophosphate. Reaction conditions were as described in M.M. except that a limiting amount of ligase (10 mU) was used and AMP concentrations were 0, 0.2, 0.5, 1 and 1.5 mM for lanes 2 to 6, respectively. AMP concentration was 1 mM and pyrophosphate was present at the following concentration: 0, 3, 9 and 27 mM in lanes 8 to 11, respectively. Lane 1 and 7 no enzyme. Substrate supercoiled pA1 DNA also contained small amount of nicked circles as internal marker. n=nicked, sc=supercoiled.

linear forms appeared throughout the reactions. However, when an approximately hundred times excess of enzymes was used (Figure 1A) and reaction was stopped by EDTA addition, nDNA appeared (Figure 1B, lane 8). When SDS was added nDNA also appeared, but only at a level barely detectable by our assay (Figure 1B, lane 7). Both observations: nicking-closing mode of relaxation and limited nicking in the case of SDS treatment, were strongly suggestive of a mechanism of DNA relaxation different from the one previously proposed (18) and more similar to the mechanism of DNA relaxation by DNA topoisomerases (31). Therefore the relaxing and nicking properties of T4 DNA ligase were more accurately studied. The nicking-closing activity was found to be AMP-dependent since no relaxation occurred in the absence of cofactor (Figure 2, lane 2). Optimal AMP concentration fell to between 0.5 and 1 mM (Figure 2). In agreement with the canonical activity of the T4 enzyme, the relaxation reaction was found to be strongly Mg^{2+} dependent with an optimal Mg^{2+} concentration between 0.3 and 1 mM (not shown). Concentrations of either AMP or Mg^{2+} higher than optimal inhibited the relaxation reaction. Using 1 mM AMP, an equimolar concentration of pyrophosphate already partially inhibited the reaction while 100% inhibition was observed at concentrations higher than 9 mM (Figure 2, lanes 8 to 11). When AMP (1 mM) was challenged by ATP, the relaxation reaction was strongly inhibited because a concentration of ATP a thousand fold lower than that of AMP was already partially inhibitory (Figure 3A, lane 5) while 100% inhibition was observed with 10 uM ATP. However the inhibitory action of ATP took place with a noticeable delay compared to the immediate stop produced by EDTA or SDS. In cases where ongoing relaxation reactions were stopped, greater amounts of more relaxed products appeared when 10 uM ATP was added than when EDTA was used, thus indicating that relaxation could proceed, at least temporarily, even in the presence of ATP (Figure 3B).

When the enzyme was tested on a single DNA topoisomer and the

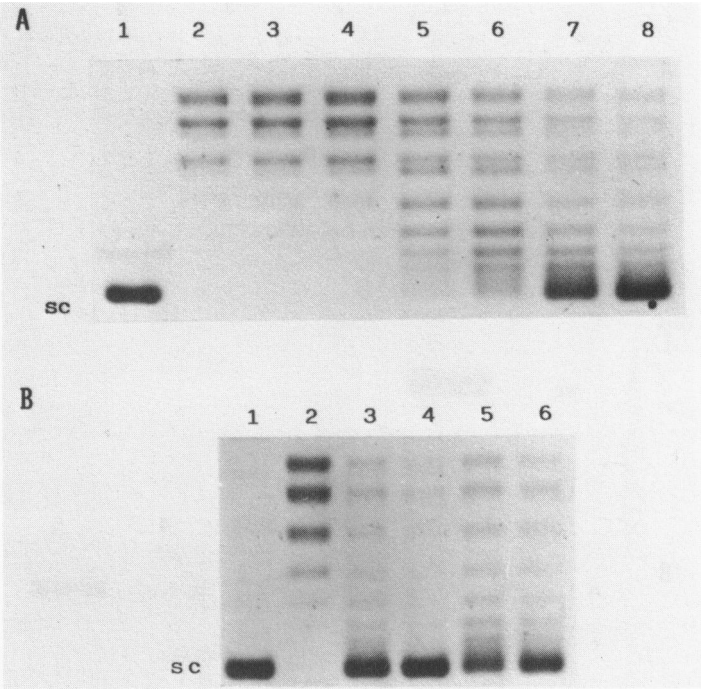

Figure 3: Panel A: effect of ATP on DNA relaxation by T4 DNA ligase. In this experiment ATP and AMP were already present at the time of DNA ligase addition (20 mU). Reaction conditions were as described in M.M. but ATP was present at 0, 0.025, 0.055, 0.5, 1, 2 and 4 uM in lanes 2 to 8, respectively. Supercoiled pA1 DNA substrate was used. Panel B: effect of EDTA and ATP on ongoing DNA relaxation by T4 DNA ligase. Reaction mixture was as in panel A. Lane 1, no enzyme; lane 2, reaction was stopped as usual after 30 min of incubation at 30°C. After 2 and 5 min either 10 uM ATP was added to reactions on lanes 3 and 5 or 20 mM EDTA to reactions on lanes 4 and 6 and incubation was terminated at time 30 min as usual. sc=supercoiled.

population of reaction products was compared to that produced by relaxing a naturally supercoiled substrate and differing by $\pm$ 1 supertwist, the patterns obtained in the two reactions were identical (Figure 4A).

The E. coli DNA ligase has been shown to relax both positively and negatively supercoiled substrates (21). We also found that T4 DNA ligase relaxes positively supercoiled substrates (Figure 4B). To generate positively supercoiled substrate, naturally supercoiled DNA was exposed to an anthracycline derivative, DDH-Epi, which shows good unwinding properties but which does not inhibit the T4 enzyme (20) while ethidium bromide does (32). The experiment was performed as follows: an amount of naturally negatively supercoiled pAT153 DNA substrate was first relaxed in the presence of a small

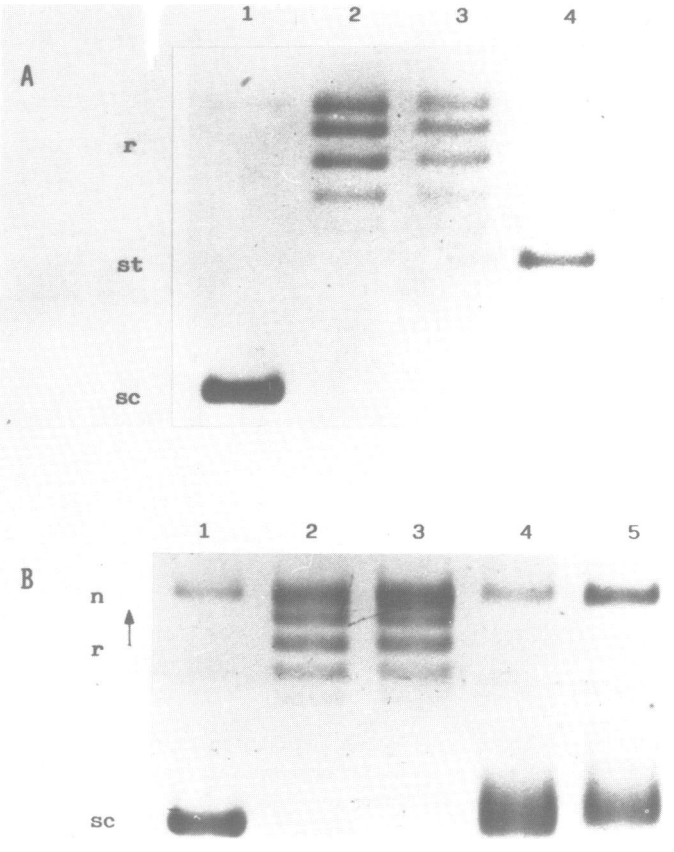

Figure 4: Change in linking number of a single topoisomer and relaxation of positively supercoiled DNA ligase. Panel A: reaction mixture containing either naturally supercoiled pAT153 DNA (lanes 1 and 2) or a single topoisomer of pAT153 DNA with -6 supertwists (lane 3 and 4) was incubated at 30°C for 30 min. In the reaction mixture of lanes 2 and 3, 20 mU of T4 DNA ligase were added. Panel B: the nicking-closing activity of T4 DNA ligase was tested on pAT153 DNA in the presence of an intercalating agent. Lane 1, pAT153 DNA supercoiled; lane 2, reaction mixture incubate for 30 min at 30°C with 40 mU of T4 DNA ligases; lane 3, same as lane 2 but after 30 min another 40 mU of enzyme were added and incubation continued for another 30 min; lane 4, reaction mixture containing 8.5 uM DDH-Epi from time 0 min, was incubated as for lane 2; lane 5, DDH-Epi 8.5 uM was added after 30 min of incubation to a mix as in lane 2, together with additional 40 mU of enzyme. Reactions were stopped with EDTA and then extracted with 10 vol. of butanol equilibrated with electrophoresis buffer. r=relaxed, sc=supercoiled, st=single topoisomer.

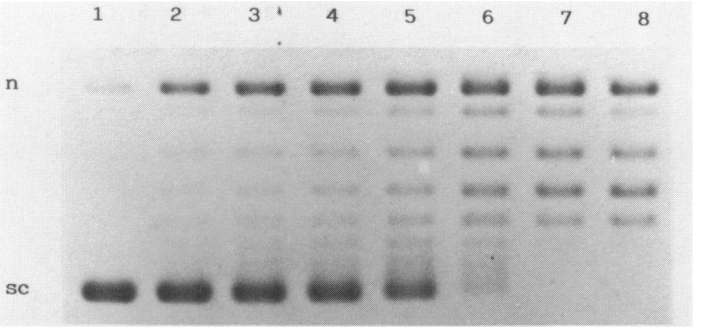

Figure 5: Kinetic of pA1 DNA relaxation by T4 DNA ligase at 4°C. Reaction mixture was as for Figure 1 except that the enzyme was present at 1 U per assay. Reactions were stopped by addition of 20 mM EDTA at times 0, 1, 2, 5, 15, 30 and 60 min of incubation, in lanes 2 to 8, respectively. Lane 1, no enzyme. n=nicked, sc=supercoiled.

excess amount of T4 DNA ligase under usual conditions (Figure 4B, lane 2). DDH-Epi was then added at a concentration sufficient to introduce 34 positive supertwists/molecule, 8.5 uM (20). After DDH-Epi addition we added an extra amount of fresh T4 DNA ligase and incubation was continued for another 30 min at 30°C. After this treatment, if the enzyme was unable to relieve positive supertwists, the substrate would appear relaxed upon drug removal. Since the reaction products appeared supercoiled (Figure 4B, lane 5), we argued that the T4 DNA ligase is capable of relieving positive supercoils. The amount of nicked DNA increased in lane 5 because of the extra amount of ligase and of the EDTA addition.

The ligase-dependent, EDTA-induced formation of nDNA was also studied. We found that while the relaxation reaction proceeds, the amount of nDNA remains costant (Figure 5). The amount of nDNA did not even change 30 min after the relaxation reaction was completed (Figure 5, lane 8) and it was still identical to the amount observed at time 0 at 0°C, before the beginning of the reaction. This observation, besides indicating that EDTA-induced DNA nicking is independent both from the topological state of the substrate and from the ongoing reaction of relaxation, also suggested that there could be a strict dependence of EDTA-induced DNA nicking on enzyme concentration. This hypothesis was confirmed by the linear dependence of EDTA-induced DNA nicking on T4 enzyme concentration at 0°C. Under these conditions we observed that DNA nicking occurs in the total absence of relaxation (Figure 6A), while no modification of the substrate was observed in the absence of added EDTA (not shown). As expected DNA nicking was confirmed as AMP-dependent, with an optimal AMP concentration of 1 mM (Figure 6B). To induce nDNA formation, Mg^{2+} had to be present at an optimal concentration between 1 and 5 mM before EDTA addition (Figure 6C). ATP was found to be a strong inhibitor of EDTA-induced

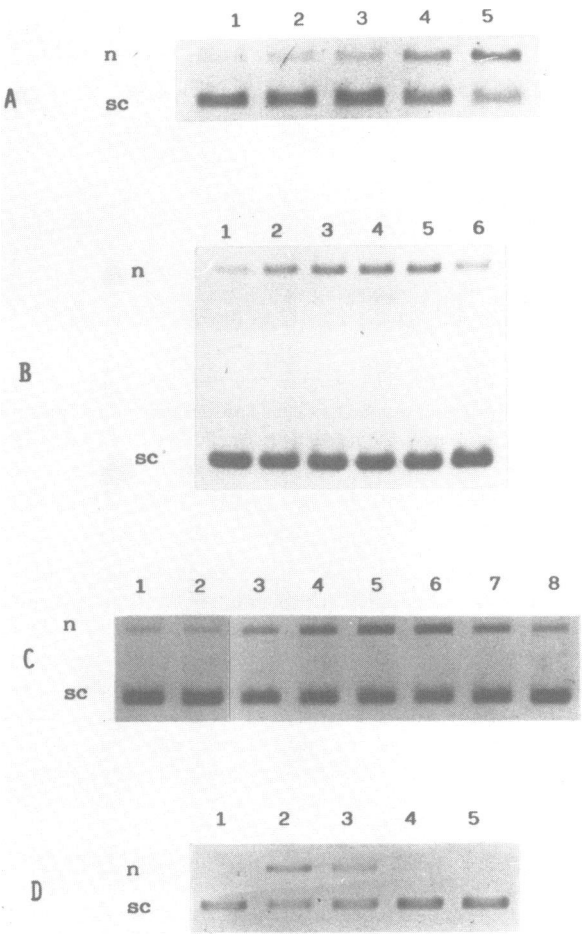

Figure 6: Properties of the EDTA-induced DNA nicking activity of T4 DNA ligase. In these experiments pA1 DNA was utilized and, except as otherwise stated, reaction conditions were as described in M. and M. A: dependence of DNA nicking on enzyme concentration. Reaction mixture contained 20 ng DNA. Enzyme U per assay were: 0, 0.21, 0.42, 0.70 and 1.4 in lanes 1 to 5, respectively. B: dependence of DNA nicking on AMP concentration. AMP was present at the following concentrations: 0, 0.2, 0.4, 1, 2, 10 mM in lanes 1 to 6, respectively. 1 U of enzyme was present in all reaction mixtures. C: magnesium dependence of EDTA-induced DNA nicking. Lane 1, untreated supercoiled pA1 DNA; lane 2, 2.5 mM EDTA; lane 3, neither EDTA or $MgCl_2$ were added; to reaction mixture of lanes 4 to 8, 0.05, 0.3, 1, 5 and 10 mM $MgCl_2$ were added, respectively. D: ATP inhibition of DNA nicking. DNA was exposed to 2 U of T4 DNA ligase (lanes 2 to 5) in usual reaction mixture containing 0, 7, 26, 130 uM ATP. Lane 1, no enzyme. n=nicked, sc=supercoiled.

DNA nicking by the T4 enzyme and 100% inhibition was observed (Figure 6D) in the same range of concentrations as those which completely inhibited DNA relaxation. All these aspects of nicking were strongly suggestive of the existence of a property connected with the enzyme itself but under the form of a DNA complex including AMP and involving a Mg^{2+} bridge. Subtraction of Mg^{2+} by EDTA most likely breaks down this complex allowing the stabilization of the already well characterized adenylated DNA and enzyme (21,33). And in fact when these hypothetical complexes were allowed to form on monomeric DNA substrate and then challenged by adding an amount of the same sequence of DNA substrate under a form (dimer) easily separable on agarose gel, broken molecules appeared in both DNA substrates after EDTA addition. In the experiments in Figure 7 the relative amounts of nDNAs in monomeric and dimeric substrates were identical whether both substrates were mixed before enzyme addition (Figure 7, lane 6) or whether one was added after enzyme addition (Figure 7, lane 5). The amount of each nicked substrate was lower when both substrates were present in the reaction mixture together than when only one substrate at a time was present, indicating the existence of an equilibrium between free enzyme and enzyme-DNA complexes at 0°C.

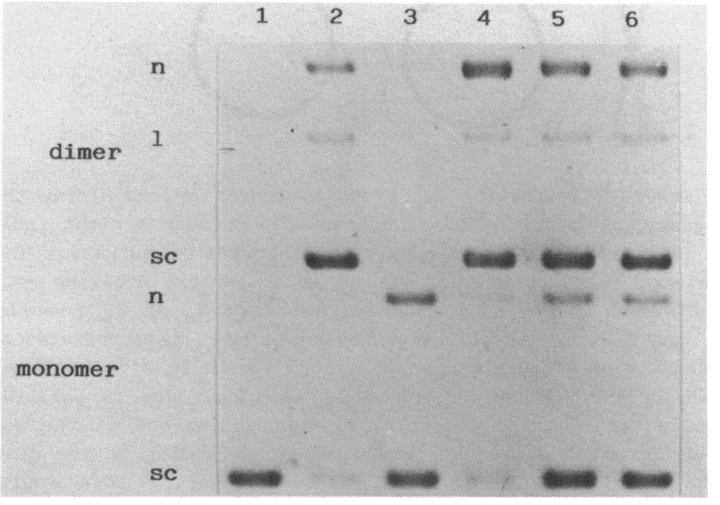

Figure 7: EDTA-induced DNA nicking of monomeric and dimeric forms of pA1 DNA, by T4 DNA ligase. Either monomeric (lane 3) and dimeric (lane 4) forms of pA1 DNA (100 ng each) were incubated at 0°C under usual conditions and in the presence of 1 U of T4 enzyme. Reactions were stopped by EDTA addition. In lane 5 monomeric form (100 ng) was incubated at 0°C in the presence of T4 DNA ligase as in lane 3, then dimeric pA1 DNA (100 ng) was added and reaction was stopped by EDTA addition. In lane 6 both forms of pA1 DNA were present at the time of DNA ligase addition. Lanes 1 and 2 controls with untreated monomeric or dimeric forms, respectively. l=linear, n=nicked, sc=supercoiled.

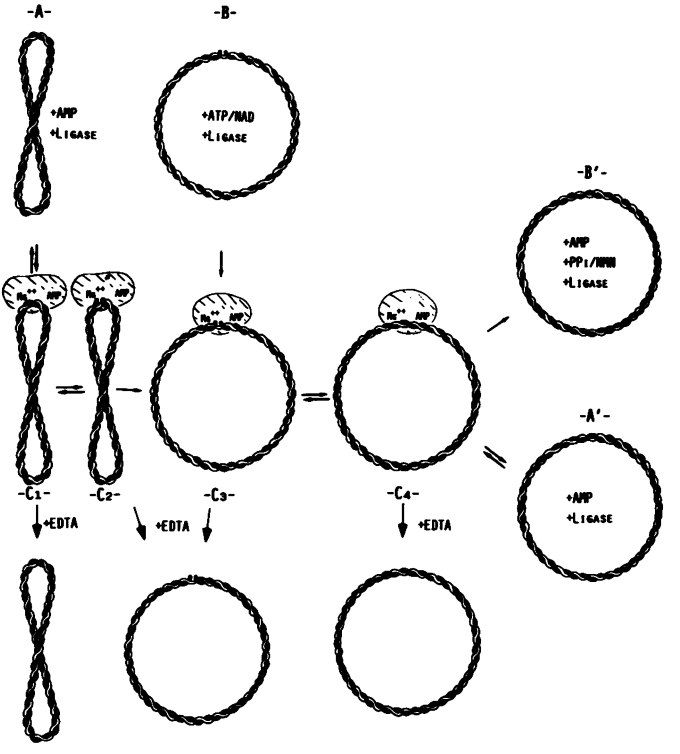

Figure 8: Scheme illustrating our proposed model for explaining the actions of DNA ligases on different DNA substrates. Substrate A might indifferently represent negatively or positively supercoiled substrate. The common intermediate of reaction should consist in a complex involving enzyme, AMP, Mg^{2+} and DNA and could exist under either form C_3 or C_4, while similar complex C_1 and C_2 are energetically unbalanced. $C_{3/4}$ intermediate complex can be indifferently obtained along route A to A' or B to B'. In the absence of PPi (or NMN) or ATP (or NAD), complex $C_{3/4}$ would also be in equilibrium with '. EDTA would destroy such a complex by subtracting one of the components. Therefore different DNA products would appear depending on the state of the DNA at the time of EDTA addition. In other words, EDTA would titrate complexes in which a phosphodiester bond is broken (C_2 and C_3).

DISCUSSION

Proof that T4 DNA ligase behaves just as AMP-dependent DNA topoisomerases would, has been presented in this paper. Bacteriophage T4 infected E. coli DNA ligase was found capable of relaxing supercoiled substrates in a stepwise manner, as the presence of partially relaxed products testifies. The T4 enzyme was also found to be capable of relaxing positively supercoiled substrate just as the E. coli enzyme was already known

to do (21). Also the E. coli and the human (8) enzymes have been analysed under the same conditions (not shown). So far the major difference between these AMP-dependent DNA-relaxing activities consists in a more processive mode of action for the E. coli enzyme and in a more distributive mode of action for the human one with respect to T4 DNA ligase. Under standard conditions (1 mM AMP) the processive mode of DNA relaxation for the T4 enzyme is deduced from the appearance at intermediate reaction times of fully and partially relaxed molecules while part of the initial substrate is still present (28). However the mode of action becomes more distributive when some parameter is altered. In lanes 3 and 9 of Fig. 2, corresponding to a lower concentration of AMP and presence of a small excess of pyrophosphate, respectively, the reaction is clearly taking place in a more distributive way exactly as it is known to occur in the case of classical DNA topoisomerases. Therefore, exactly as it happened for DNA topoisomerases, this observation is strongly suggestive of a relaxation under strict control of the enzyme. The observation that reacting enzyme is slowly inactivated by ATP is in perfect agreement with a processive mode of relaxation (Fig. 3B). More specifically, in the case of the T4 enzyme the reaction occurs by removing one superhelical twist at a time, as for type I DNA topoisomerases.

Properties such as dependence on AMP and Mg^{2+} and inhibition by ATP or pyrophosphate, strongly suggest that the catalytic activity responsible for DNA relaxation and the DNA-joining activity coincide. Our observation that during the relaxation reaction the substrate does not undergo an uncontrolled rotation allowing the complete and sudden relaxation of the DNA molecule implicate that the substrate is constantly under the control of the enzyme. Therefore our observation would not support the hypothesis that nDNA might be an intermediate of reaction catalyzed by DNA ligase (33,34). Evidently to change the topological state of the substrate DNA ligase, exactly as DNA topoisomerases, must interrupt the sugar-phosphate continuity of at least one helix. However the possibility of detecting DNA with decreasing levels of superhelical density suggests a concerted breakage and rejoining of DNA backbone bonds. In the presence of a 100 times excess of enzyme when using EDTA we observed significant increase of nicked form of substrate DNA. With SDS, however, only a slight increase was noticed. Linear DNA form was never observed in either case. According to the mechanisms of DNA ligation and of DNA relaxation previously proposed (18), if the nDNA were a real intermediate of reaction, it should also be found after SDS addition or after any enzyme-degrading treatment. But, as for SDS, neither proteinase K or pronase treatments (data not shown) were conditions sufficient to evidentiate nDNA after incubation of DNA with DNA ligase in the presence of AMP and Mg^{2+}. On the other hand nDNA, induced by EDTA addition, seems independent from the relaxation reaction (33) since its amount is remarkably constant even 30 min after all of the substrate is relaxed (Figure 5). Even considering that adenylated DNA can be utilized as substrate by ligases (33,35), the EDTA-dependence of nicking and the stepwise relaxation of supercoiled substrates allowed us to hypothesize the existence of a protein-DNA complex. This complex would be the real intermediate of reaction. According to this view Mg^{2+} might be involved in bridging DNA termini throughout the enzyme or might induce modifications in the enzyme structure

that allow proteic control of both the 5' and 3' termini of the DNA helix. Contrary to what found with DNA topoisomerases however, neither the literature nor our results, clearly prove the existence of a covalent bond between ligase and DNA. The same complex could also originate when adenylated ligase reacts with a proper, non-covalently closed substrate (36-38). This could be the real intermediate of reaction. The observation that the inhibition by ATP of DNA relaxation is delayed (Figure 3B) agrees very well with this hypothesis and the fact that enzyme molecules already involved in a complex can nick a different substrate (Figure 7), suggests the existence of a dynamic equilibrium between free and complexed enzyme. Our hypotesis of a DNA-AMP-Mg^{2+}-protein complex as the true intermediate during DNA ligase action is schematized in Figure 8. The nature of DNA termini inside the complex has not yet been investigated, however we suppose that adenylated DNA could well play a role as part of the intermediate complex.

The hypothesis of the intermediate complex would better explain fenomena such as blunt-end ligation (36,37) and sealing of gaps in douplex DNA (37). It also explains why attempts to isolate DNA-adenylate using $(pT)_7$-acetate and poly d(A) (33) or using dT_{200} chains with 2',3'-dideoxy ends substituted for 3'-hydroxyl (39) as substrates were unsuccessful. However direct binding of DNA ligase to the substrate is still to be proved.

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