Bacteriophage T4 and human type I DNA ligases relax DNA under joining conditions

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ABSTRACT
Both bacteriophage T4 and human type I DNA ligases in the presence of a mixture of ATP, AMP and PPi altered the topological properties of a supercoiled substrate by a step-wise reaction eventually leading to a population of fully relaxed, covalently closed products. In the presence of only AMP and PPi DNA products containing nicks with 3'OH/5'P termini accumulated in the presence of bacteriophage T4 DNA ligase, suggesting reversal of the entire joining reaction, but not in the presence of human DNA ligase I. Both DNA ligases became deoxyadenylylated in the presence of dATP, but the joining reaction did not proceed to completion. However, with both enzymes the full relaxing reaction took place in the presence of dAMP alone and in the presence of a mixture of dATP, dAMP and PPi. In no case could the joining reaction be reversed by dAMP and PPi. Related experiments with modified derivatives of deoxyribonucleoside 5'-triphosphates and PPi gave results in accord with these observations. The AMP dependent DNA relaxation catalysed by DNA ligases was insensitive to the presence of exonuclease III. These results indicate that controlled relaxation of the substrate by both DNA ligases occurs as a separate reaction rather than by simple reversal of the joining reaction. These findings support the hypothesis that in vivo the DNA topoisomerase I ligases relax their substrate at the replication fork both during and separately from ligation of a pre-existing nick.

INTRODUCTION
Contrary to lower organisms, where a single activity is still supposed to accomplish polynucleotide joining reactions required during replication, recombination and repair, three different DNA joining activities have so far been described in mammalian cells. All three enzymes require ATP as cofactor, as does bacteriophage T4 DNA ligase, but they differ from one another in a number of biochemical properties (see ref. 1 for recent review). Several observations suggest that mammalian DNA ligase I plays a role both in DNA replication (2–5) and in DNA repair (1, 5–6) while the role played by DNA ligases II and III is still an open question (1). A number of inhibitors of DNA ligases have been described recently (7–11); nevertheless, none can discriminate between different forms of mammalian DNA ligases (12).

Upon completion of the first step of the reaction, all of the above mentioned ligases become covalently bound to an AMP residue with release of a PPi residue. The ligase-AMP intermediate may then react with duplex DNA containing a 3'-OH/5'-P nick to generate a new phosphodiester bond through a transient DNA-adenylate intermediate (1,13–14). In addition DNA ligase has been shown to react with chemically synthesised DNA containing an adenylylated 5'-terminus to generate a new phosphodiester bond (15–16). In the presence of AMP as cofactor, E.coli DNA ligase converts DNA from supercoiled to relaxed form (17). The ability of ligase to nick the substrate transiently has been taken as proof of the reversibility of the joining reaction (13,17). According to this interpretation, the relaxation of supercoiled DNA takes place initially as a simple endonucleolytic event that converts the covalently closed DNA to a nicked-adenylylated form that is free to loose all the superhelical torsion. Then the nicked-adenylylated DNA is again recognised by free ligase and resealed into a relaxed form. However more recently the AMP-dependent relaxation catalysed by DNA ligases has been shown to occur step-wise, just as in the case of the DNA topoisomerases (18–19). In mammals it is present in DNA ligase type I, it is absent in type III, while it is still to be determined in type II (20).

The analogies between the DNA relaxation reaction catalysed by some DNA ligases and the relaxation catalysed by typical DNA topoisomerases come from the observations that 1) no other changes in physical properties of the substrate can be observed; 2) a strong denaturing treatment induces breaks in the substrate, 3) the kinetics can be apparently either processive or distributive, at least judging from the distribution of the reaction products, and 4) DNA ligases can remove as little as one superhelical turn at the time (18). In the case of DNA topoisomerases a covalent DNA–protein intermediate has been isolated. On the contrary a stable DNA–DNA ligase complex has never been isolated. Nevertheless a DNA–DNA ligase-AMP-Mg intermediate complex has been postulated to explain the EDTA-induced DNA nicking catalysed by DNA ligases at 0°C, a temperature not
allowing DNA relaxation (18-19). The topoisomerase activity of certain DNA ligases for simplicity named topoligases, from topoisomerising ligases (19) suggested additional functions for this enzyme at the replicative fork during the ligation of the Okazaki fragments, i.e. to relieve the topological constraint on the lagging strand resulting from the sudden transition of the newly synthesised DNA from a nicked to an intact double stranded structure upon ligation (19,21). Nevertheless, it was still unclear whether this relaxing activity is an intrinsic property of DNA ligases that are indeed capable of maintaining the substrate relaxed immediately after a joining event. To clarify this point we have further analysed the catalytic properties of both bacteriophage T4 and human type I ATP-dependent DNA ligases in the presence of various combinations of AMP, PPI and ATP (and some of their derivatives) in order to mimic the conditions supposed to occur in vivo at the replication fork. The results of such an analysis support the hypothesis that DNA ligases are capable of topoisomerase their substrate at the completion or independently of a joining event, and provide the first evidence of the complete reversibility of the DNA joining reaction catalysed by the bacteriophage T4 DNA ligase.

**MATERIALS AND METHODS**

**Materials**

Bacteriophage T4 DNA ligase (3,000 U/mg) was purchased from Boehringer, Mannheim, F.R.G. Human DNA ligase I was purified as previously described (22). Nucleotides were purchased from Sigma Chemical Co. Nucleotide and PPI analogues were synthesised as previously described (23-26). Agarose and SDS were from BDH Chemical Ltd. All other reagents were from Merck.

**Determination of DNA-joining activity**

DNA-joining activities were determined by the method of Modrich and Lehman (27) according to which one unit of DNA ligase is the amount of enzyme activity that converts 100 nmol of poly(dA-dT) to an exonuclease III-resistant form within 30 min at 30°C.

**Determination of DNA relaxing/nicking activity**

Bacteriophage T4 DNA ligase was incubated in the following reaction buffer (20 µl): 66 mM Tris.HCl, pH 7.6, 6.6 mM MgCl₂, 1 mM dithioerythritol, 100 ng of naturally supercoiled pUC19 DNA plus cofactors as specified in the text. Human DNA ligase I was incubated in the following reaction buffer (20 µl): 20 mM Tris.HCl, pH 7.6, 3 mM MgCl₂, 1 mM dithioerythritol, 1 mM EDTA, 100 µg/ml bovine serum albumin, 100 ng of naturally supercoiled pUC19 plus cofactors as specified in the text. In both cases after incubation at 37°C for 30 min, the reaction mixtures were made 0.04% bromophenol blue, 0.01% SDS and 12% glycerol. Samples were then analysed by 1.4% agarose gel in 40 mM Tris-acetate, 2 mM EDTA, 18 mM NaCl, final pH 8. Running, staining, destaining and photographic conditions were as described elsewhere (7).

**Quantification of the number of single strand breaks in circular DNA**

After separation on agarose gel of samples containing appropriate ratios of RFI and RFII forms, the relative amounts of nicks/molecule were determined as previously described (28).

**RESULTS**

**Behaviour of bacteriophage T4 and human type I DNA ligases in the presence of a mixture of ATP, AMP and PPI**

When ATP is added at low concentration (10 µM) to a DNA relaxing reaction mixture containing the optimal concentration of 1 mM AMP, the relaxation of supercoiled DNA catalysed by bacteriophage T4 and human type I DNA ligases is inhibited (18-19). We have now analysed the effects of the simultaneous presence of ATP and both its hydrolytic products on the behaviour of bacteriophage T4 and human type I DNA ligases. When 1 mM AMP is added to a mixture containing supercoiled DNA, 1 mM AMP and 0.1-1 mM PPI, both DNA ligases relax the substrate (see Figure 1, lanes 8-9 and 16-17) according to a mechanism that is apparently indistinguishable from that of a DNA topoisomerase, at least by judging from the DNA products. Under the reaction conditions shown in lanes 8-9 and 16-17 of Figure 1, both DNA ligases ligate either poly(dA-dT) or EcoRI linearised plasmid pAT153 with an efficiency identical to that measured in the presence of 1 mM ATP alone (not shown).

**Complete reversal of DNA ligation in the presence of AMP and PPI takes place only with bacteriophage T4 DNA ligase**

We have dissected the relaxation reaction to analyse the effects of single molecules. When ATP is omitted and the reaction mixture contains only AMP and PPI, T4 DNA ligase, but not human DNA ligase I, nicks the supercoiled substrate very efficiently and even linearises it (Figure 1, lanes 6-7 and Figure 2A, lanes 3-8). As shown in Figure 2A, the accumulation of nicked products occurs at PPI concentrations varying between 0.005 and 10 mM when 1 mM AMP is present. This is, therefore, the first direct demonstration of the full reversal of the entire DNA joining reaction catalysed by a DNA ligase, at least where the DNA substrate is concerned. In fact under these conditions we cannot determine whether ATP is also reformed. At concentrations of PPI higher than 2.5 mM the nicking effect decreases markedly, but the relaxation reaction becomes increasingly inhibited, as already shown (18). After phenol-extraction of the products, the majority of the nicks produced by T4 DNA ligase during the reversal of the reaction are religatable only upon addition of new enzyme and ATP (not shown), a direct demonstration that the termini of the nicks are unmodified [3'OH/5'P].

![Figure 1. DNA relaxation catalysed by bacteriophage T4 and human type I DNA ligases in the presence of ATP, AMP and PPI. The enzymes (T4, 0.8 mU; human, 0.44 mU) were incubated as described in Materials and Methods, except that cofactors were as indicated. Samples of lanes 1 and 13 contained markers for supercoiled (RFI), EcoRI-linearised (RFII) and nicked (RFIII) pUC19 DNA.](image-url)
To our surprise we find that nicking of the supercoiled substrate by the human DNA ligase I is not stimulated by the simultaneous presence of both of the products of ATP hydrolysis (Figure 2A, lanes 12–21 and 2B, lanes 19–22). At low PPi concentrations (0.01–3 mM) the enzyme relaxes the substrate just as in the presence of AMP alone. In other words the enzyme is insensitive to the ratio AMP/PPi, but responds only to the absolute AMP concentration (see Figure 2A, lanes 12–21). At higher PPi concentrations the reaction becomes progressively inhibited (Figure 2B, lanes 21–22) as already reported (19).

We have also measured the effects of three PPi analogues, methylenediphosphonate (MDP) and two of its derivatives active against DNA polymerases, carboxyldiphosphonate (COMDP) and (difluoromethylene) diphosphonate (CF₂MDP) (25), under the conditions of DNA relaxation (Figure 2B). Their inhibitory effects against the two DNA ligases are remarkably similar, with inhibitory potencies in the order COMDP > CF₂MDP > MDP, except that some nicking appears in the case of bacteriophage T4 DNA ligase. COMDP inhibits the relaxation reaction very efficiently without inducing nicked products. CF₂MDP more closely mimics PPi, since it is inhibitory at 3 mM in both cases and since, in the case of T4 DNA ligase only, it produces a discrete fraction of nicked product at concentrations of 0.05–1 mM.

Bacteriophage T4 and human type I DNA ligases relax supercoiled DNA using dAMP as cofactor

Both DNA ligases can utilise dATP to generate a stable enzyme-deoxyadenylate complex with good efficiency (42% for T4, and 34% for human type I DNA ligases, compared to those with ATP), but dATP does not sustain a complete joining reaction in either case (29). Surprisingly we now find that dAMP can be utilised by both DNA ligases to relax supercoiled DNA (Figure 3). No other deoxyribonucleoside 5'-monophosphate added at 1 or 5 mM, including dTMP, dCMP, dGMP, dUMP, and the analogue N²-(p-n-butylanilino)dGMP (BuPdGMP), can substitute for AMP or dAMP (not shown). We have asked whether PPi, added to a dAMP-containing DNA-relaxing mixture, allows the complete reversal of the joining reaction, as would be proved by the generation of nicked/linearised substrate. As expected by the inability of dATP to drive a complete joining reaction, PPi + dAMP do not allow the accumulation of nicked products in either case. The results obtained with bacteriophage T4 DNA ligase, shown in Figure 4, indicate that at high PPi concentration the DNA relaxation is inhibited. Similar results were obtained with human DNA ligase I (not shown).

Effects of deoxyribonucleoside 5'-triphosphates on DNA relaxation in the presence of AMP

As mentioned before, the addition of low concentrations of ATP in the absence of PPi strongly inhibits the AMP-dependent relaxation of supercoiled DNA catalysed by both enzymes. This is most likely the result of the high affinity of the enzymes for ATP, resulting in the conversion of the enzymes to the adenylated form (19). We have, therefore, analysed the effects of deoxy- and deoxyribonucleoside 5'-triphosphates (dATP, dTTP, dCTP, dGTP, ddATP, ddTTP, ddCTP and ddGTP), and of the analogue 2-(p-n-butylanilino)-dATP (BuAdATP), on the AMP-dependent DNA relaxation in the absence of PPi.
**DISCUSSION**

Bacteriophage T4, *E. coli* and human type I DNA ligases alter the topological state of supercoiled DNA in the presence of AMP, according to a controlled sequence of nicking and joining events apparently indistinguishable from the action of DNA topoisomerases. These findings led to the hypothesis that replicative DNA ligases might utilise this property while joining the Okazaki fragments to mediate the sudden passage of the new double helix from a nicked structure, free to rotate, to a covalently closed, and therefore rigid, structure within the ‘replisome’ (21). This hypothesis assumes that ‘topoligases’ might relax their closed, and therefore rigid, structure within the ‘replisome’ (21).
Figure 7. Proposed model for the action of ATP-dependent DNA ligases. The classical model of DNA ligase action is represented by part A of the scheme. This scheme is valid for all known DNA ligases. However our present data indicate that the ligation reaction is fully reversible only in the case of bacteriophage T4 DNA ligase. As shown in part B of the scheme, DNA relaxation occurs independently of the joining reaction or, as suggested by our data in the presence of a mixture of ATP, AMP and PPI, it could take place also at the end of the joining event. Our model differs from the previous interpretation only where the AMP-dependent properties of some ligases are concerned. According to our data, DNA topoligases relax supercoiled DNA not by completely reverting the entire joining reaction back to the DNA-adenylate state, but instead by a partial reversal. This requires a complex involving DNA-ligase-AMP in which DNA ligase can break a phosphodiester bond while still controlling the DNA termini. This additional step of the reaction appears to be a characteristic of DNA topoligases only.

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Enzymes remains unaffected. These observations contrast with the previous interpretation of the AMP-dependent DNA relaxation as complete reversal of the multistep reaction of ligation. In fact, under joining conditions, the reverse reaction should be unfavoured. We have already ruled out the possibility of trace contamination of topoisomerases in both bacteriophage T4 and our DNA ligase preparations since the topoisomerising activity of DNA ligases is entirely AMP dependent and is absent in the presence of ATP alone (18–19).

So far, the reversibility of the DNA ligation reaction has been demonstrated by the ability of adenylated DNA ligases to regenerate ATP or NAD in the presence of PPI or NMN, respectively, and by the ability of the enzymes in the presence of AMP to transiently nick and reseal a covalently closed DNA substrate. However, during the relaxation catalysed by topoligases, nicked DNA cannot be detected unless the so called 'reverse reaction' of ligation is stopped by EDTA or by a strong denaturing treatment, just as in the case of DNA topoisomerases (18–19). By utilising supercoiled DNA, AMP and PPI and analysing the reaction product on agarose gel, we have now shown that bacteriophage T4 DNA ligase nicks the substrate very efficiently, whether the reaction is stopped with or without EDTA. This observation is confirmed by the use of PPI analogues that are found both capable of inhibiting the relaxation reaction and to favour the nicking of the substrate. We consider these facts as strong evidences of the complete reversal of the multistep reaction of ligation catalysed by bacteriophage T4 DNA ligase. Interestingly, the human DNA ligase I is unable to reverse the entire joining reaction in the presence of AMP and PPI. This difference is surprising since both enzymes are capable of relaxing a supercoiled DNA in the presence of AMP. If the DNA relaxation is really due to the complete reversal of the multistep reaction, the addition of PPI should strongly force the reaction of ligation backwards to generate nicked DNA also in the case of the mammalian enzyme. The inability of the human enzyme to reverse the entire joining reaction could therefore be interpreted as the DNA relaxation being a step independent of the joining reaction. However it must be noted that PPI, its analogues and the deoxyxynucleotides at very high concentrations inhibit the topoligase, but they never induce nicking of the substrate in the presence of the human DNA ligase I and AMP. This behaviour, beside distinguishing the human DNA ligase I from the T4 DNA ligase, could indicate that the role played by PPI in the case of the T4 DNA ligase may be played by other regulatory factors in the case of the human DNA ligase. In this regard it is noteworthy to remember that in the case of the bacterial DNA ligases PPI is replaced by NMN, while a specific protein inhibitor of human DNA ligase I that affects the joining but not the self-adenylation reaction has been recently described (30).

We find that in the presence of a mixture of ATP, AMP and PPI both DNA joining and relaxation can occur. We also show that, beside dATP and BuAdATP at high concentration, deoxyribonucleoside 5'-triphosphates do not affect significantly the topoisomerising activity of T4 DNA ligase. In this regard we find that T4 DNA ligase discriminates between purines and pyrimidines. In fact, only the triphosphates of (d)deoxyribo- pyrimidines can induce DNA breaks just as PPI does when tested in the presence of AMP. One explanation could be that the enzyme recognises the base moiety first, and only later the PPI moiety. The results obtained with PPI analogues are quite informative in view of the search for potential inhibitors of DNA ligases and for the understanding of the interaction between PPI and DNA ligase.

By using a DNA deoxyadenylate as substrate, Harvey et al. (16) have shown that T4 DNA ligase can generate a new phosphodiester bond at a rate 0.6% of that observed with DNA adenylate. It is also known that dATP does not sustain the joining reaction catalysed by either T4 or human type I DNA ligases (29). In agreement with these notions, we now show that in the presence of dAMP and PPI the reaction does not significantly revert even if both DNA ligases can be deoxyadenylated (29). Therefore, the observation that dAMP is quite efficient in allowing the topoisomerisation of supercoiled substrate catalysed by both enzymes, without favouring the reversal of the joining reaction when PPI is present, suggests that the DNA adenylate is not an intermediate of the relaxation reaction. Moreover, while the 2'OH moiety of AMP might play a role in a central step of the DNA joining reaction, it probably does not play any role during the topoisomerisation step. This is in agreement with our inability to detect the DNA-adenylate intermediate using [32P]AMP in the relaxation assay (unpublished results). Thus the inability to produce nicks in the DNA substrate even in the presence of PPI strongly supports the hypothesis that the AMP-dependent topoisomerisation is not simply the consequence of the relaxation of nicks accumulated as a result of the reverse reaction. This possibility is remote since we also show here that the presence of an enzymatic activity capable of removing any free nicked DNA (exonuclease III) does not affect the AMP dependent DNA relaxation property of DNA topoligases. The model presented in Figure 7 takes into consideration all of our observations that cannot be explained by the old model and stresses the difference existing between DNA ligases and DNA topoligases. In addition we suggest the possibility that the covalently closed DNA produced upon ligation can be maintained.
relaxed in the presence of AMP or of ATP+AMP+PPi. Therefore, we consider the possibility that DNA topoligases indeed operate in order to both join and maintain the newly synthesised DNA in a relaxed state, allowing quick interactions with replication proteins, before more efficient and specific enzymes take care of topological and superhelical problems.

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