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Functional characterization of the T4 DNA ligase: a new insight into the mechanism of action

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ABSTRACT

ATP-dependent DNA ligases are essential enzymes in both DNA replication and DNA repair processes. Here we report a functional characterization of the T4 DNA ligase. One N-terminal and two C-terminal deletion mutants were expressed in *Escherichia coli* as histidine-tagged proteins. An additional mutant bore a substitution of Lys159 in the active site that abolished ATP binding. All the proteins were tested in biochemical assays for ATP-dependent self-adenylation, DNA binding, nick joining, blunt-end ligation and AMP-dependent DNA relaxation. From this analysis we conclude that binding to DNA is mediated by sequences at both protein ends and plays a key role in the reaction. The enzyme establishes two different complexes with DNA: (i) a transient complex (T·complex) involving the adenylated enzyme; (ii) a stable complex (S·complex) requiring the deadenylated T4 DNA ligase. The formation of an S·complex seems to be relevant during both blunt-end ligation and DNA relaxation. Moreover the inactive His-K_{159}L substitution mutant, although unable to self-adenylate, still possesses AMP-dependent DNA nicking activity.

INTRODUCTION

DNA ligases play essential roles in DNA replication, DNA repair and DNA recombination by catalysing the formation of phosphodiester bonds at single-stranded or double-stranded breaks between adjacent 3′-hydroxyl and 5′-phosphate termini. Two major groups of DNA ligases can be distinguished on the basis of the required cofactor. Enzymes from eukaryotic cells as well as from bacteriophages of the T series and from archaebacteria all require ATP, while eubacteria DNA ligases are NAD^+ dependent (1,2).

The ligation reaction involves three successive nucleotidyl transfer reactions: (i) activation of the enzyme through the formation of a covalent protein–AMP intermediate accompanied by the release of PPi or NMN, depending on the cofactor; (ii) transfer of the nucleotide to a phosphorylated 5′-end of the nick to produce an inverted (5′)–(5′) pyrophosphate bridge structure; (iii) catalysis of the transesterification reaction resulting in joining of the nick and release of free AMP (3).

An interesting and thus far not completely analysed aspect of the mechanism of action concerns the so-called 'reverse reaction' (3), namely the AMP-dependent relaxation of supercoiled DNA. DNA relaxation occurs through a nicking/closing mechanism and results in a progressive reduction in superhelical turns according to a rather processive mode of action. This property suggests the existence of an intermediate of reaction in which the enzyme is engaged in a very stable complex with the DNA substrate (4,5).

As stated above, ATP-dependent DNA ligases are widespread in evolution from bacteriophages to mammals. While lower organisms encode a single essential DNA ligase, mammalian cells contain four different activities (designated by roman numerals I–IV) encoded by separate genes (6). DNA ligases show considerable differences in molecular mass, ranging from 41 kDa in T7 to >100 kDa for the mammalian enzymes. This difference in size is mainly accounted for by protein regions with regulatory function. DNA ligase I, the best characterized mammalian enzyme, is organized into two well-defined domains: a C-terminal catalytic domain and an N-terminal region with regulatory functions but dispensable for enzyme activity (7,8). The N-terminal domain is required both for nuclear localization and for recruitment of the enzyme at replication factories during S phase (9). In spite of their differences in size, all ATP-dependent DNA ligases share a significant level of homology. Multiple sequence alignment revealed the existence of highly conserved motifs in the catalytic domain (10). Besides the AMP binding site, no other homologies were found with the NAD-dependent DNA ligases of eubacteria. In contrast, several regions of homology have been identified between ATP-dependent DNA ligases and mRNA capping enzymes that catalyse the transfer of a GMP moiety to the 5′-diphosphate terminus of RNA via an enzyme–GMP intermediate (11). In particular five co-linear sequence elements, designated motifs I and III–VI are conserved between these two classes of enzymes. Motif I (KXDG) corresponds to the nucleotide binding site, while motif VI is the previously identified 'conserved peptide' whose function is still unknown (2). The small enzymes encoded by the bacteriophages of the T series represent a sort of 'core' structure and hence have been selected for characterization of the mechanism of action, with the idea that they will eventually shed new light on the activity of the larger mammalian enzymes. The crystal structure of the T7 DNA ligase has recently been resolved, demonstrating that the protein comprises two domains

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with a deep cleft between them. From the structure of an enzyme–AMP complex it was deduced that residues in all but motif VI contact the nucleotide molecule (12).

The recent characterization of the mammalian enzymes along with studies on the structure of T7 DNA ligase indicate a renewed interest in this class of enzymes. Along this path we undertook a functional characterization of the T4 enzyme, the prototype of the ATP-dependent DNA ligases (13). The data reported here demonstrate that the enzyme contacts DNA in two different complexes and provide a new insight into the mechanism of action of DNA ligase.

MATERIALS AND METHODS

Generation of recombinant His-tagged proteins

The T4 DNA ligase gene (GenBank accession no. X00039; 14) was PCR amplified (Amplitaq; Perkin Elmer) from T4 DNA with sense primer BL1 (5′-GGATCCA$_{524}$TGATTCTTAAAATTCTGAA-CG$_{545}$-3′) and antisense primer BLS (5′-GGATCCT$_{1987}$CATA-GACCAGTTACCTCATG$_{1967}$-3′). *Bam*HI restriction sites are underlined. The PCR product (1464 bp) containing the entire coding sequence was gel extracted (Qia-quick Kit; Qiagen) and cloned into the pCR™II TA vector (Invitrogen). After digestion with *Bam*HI the fragment was ligated into the dephosphorylated *Bam*HI site of the pTrcHis-A expression vector (Invitrogen). Recombinant clones were screened for expression of the protein after induction with 2 mM IPTG. The level of T4 DNA ligase expression was monitored by Western blotting using anti-T4 DNA ligase antibodies. A clone (pHis-T4) was selected and completely sequenced (Sequenase Version II; Amersham). To produce the three deletion mutants we used pHis-T4 as template in a series of PCR reactions. The scheme used to obtain the different clones was essentially as for the wild-type protein. PCR primers were as follows: sense primer BL80 (5′-GGATCCT$_{764}$TGACTG-GAAATGCAGCAATTGAGG$_{788}$-3′) and antisense primer BLS for clone pN-Δ80; sense primer BL1 and antisense primer BL350 (5′-GGATCC**TCA**A$_{1573}$TTTTTGAGAATAATACCTTCAAG-ACC$_{1547}$-3′) for clone pC-Δ137; sense primer BL1 and antisense primer BL430 (5′-GGATCC**TCA**A$_{1813}$TTTTGGTTTTCCA-TAATGCGAGTACGG$_{1786}$-3′) for clone pC-Δ57. *Bam*HI restriction sites are underlined; the stop codon is in bold.

To produce pK$_{159}$L bearing a single amino acid substitution at position 159, the ligase gene was amplified with two different pairs of oligonucleotides according to a procedure previously described (15). In the first amplification sense primer BL1 and antisense primer aL159 (5′-C$_{1010}$TCCATCAGCTAGTAACT-GAGCAAAGGCTGG$_{980}$-3′) were used to produce the L159-1 fragment. Mutated nucleotides are underlined. In the second amplification, sense primer sL159 (5′-G$_{989}$CTCAGTTAC-TAGCTGATGGAGCTCGGTG$_{1017}$-3′) and antisense primer BLS were used to produce the L159-2 fragment. L159-1 and L159-2, which share only a 22 nt sequence (989–1010) of the aL159 and sL159 oligonucleotides, were purified from agarose gel, mixed and further amplified with the external BL1 and BLS primers. The PCR product was cloned into the pCR™II TA cloning vector and the *Bam*HI fragment was then cloned into the pTrcHis-A expression vector.

Over-expression and purification of the recombinant proteins

Recombinant proteins were produced in *E.coli* (DH5α). An overnight culture was inoculated into 500 ml LB medium containing 100 μg/ml ampicillin and grown at 37°C with vigorous shaking to an OD_{600} of 0.7–0.9. Expression of recombinant protein was induced with 2 mM IPTG for 5 h at 37°C. Then cells were harvested by centrifugation. All subsequent procedures were performed at 4°C. Cells were resuspended in 10 ml lysis buffer (50 mM phosphate, pH 8.0, 300 mM NaCl) containing 1 mM PMSF, 1 μg/ml pepstatin, 1 mg/ml lysozyme, 0.2% NP40 and 50 mM imidazole. Resuspended cells were lysed by sonication and immediately centrifuged for 20 min at 10 000 *g* at 4°C. The supernatant was mixed with 2 ml Probond Resin (Invitrogen) equilibrated in lysis buffer containing 50 mM imidazole and stirred at 4°C for 60 min. Four washes were performed with 5 vol washing buffer (50 mM phosphate, pH 8.0, 1 M NaCl, 1 mM PMSF) containing 50 mM imidazole. The His-tagged protein was eluted with 2 × 2 ml lysis buffer containing 250 mM imidazole. The eluate was dialysed against 25 mM Tris–HCl, pH 7.5, concentrated to a volume of 200 μl, made 0.15 M with respect to NaCl and loaded on a Superdex G75 column (Pharmacia). The polypeptide composition of the fractions was monitored by silver staining of SDS–PAGE gels and Western blot analysis with chicken antibodies to T4 DNA ligase.

In the case of the N-Δ80 mutant, cells were lysed and incubated with the Probond Resin in the presence of 20 mM imidazole instead of 50 mM. The fusion protein was eluted at 250 mM imidazole, dialysed against 50 mM phosphate, pH 7.0, and applied to a mono-S column. The column was developed with a salt gradient of 50–600 mM phosphate, pH 7.0. Fractions containing the His-tagged protein were pooled and dialysed against 25 mM Tris–HCl, pH 7.5. After concentration to a volume of 200 μl, the sample was rendered 0.15 M with respect to NaCl and loaded on a Superdex G75 column (Pharmacia) equilibrated with the same buffer.

Protein concentration was determined using the BioRad Bradford reagent with bovine serum albumin as the standard. Purified proteins were diluted in 50% glycerol and stored at –20°C.

ATP binding assay

Reaction mixture (10 μl) containing 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 1 μCi [α-^{32}P]ATP (400 Ci/mmol; Amersham) and 100 ng His-tagged protein was incubated at room temperature for 20 min. The reaction was stopped by boiling for 3 min in 1× SDS gel loading buffer (16) and analysed by 10% SDS–PAGE. The extent of protein adenylation was determined by autoradiography. The autoradiographic signals were quantitated by means of an imaging densitometer (BioRad GS-670). For the deadenylation reaction PPi was added to a final concentration of 5 mM and incubation was carried on for an additional 10 min before SDS–PAGE analysis.

DNA ligation and AMP-dependent DNA relaxation assay

Nick joining activity was measured in a poly(dA)·oligo(dT)$_{16}$ assay as previously described (17). The substrate for blunt-end ligation was obtained by annealing complementary strands, both of which were 5′-end-labelled by T4 polynucleotide kinase (New England BioLabs): (+) 5′-CAAGCTTGCATGCCTGCA-3′;

(–) 5′-TGCAGGCATGCAAGCTTG-3′. A reaction mixture (50 µl) containing 66 mM Tris–HCl, pH 7.5, 1 mM DTT, 5 mM $MgCl_2$, 1 mM ATP, 15% PEG 8000, 2 pmol DNA substrate (8×10^4 c.p.m.) and purified protein (see figure legends) was incubated at 25°C for 2 h. The reaction was stopped by adding 30 µl stop solution (Sequenase). An aliquot (10 µl) was run on an 8% sequencing gel. The formation of multimers was visualized by autoradiography of dried gels. DNA relaxation of supercoiled pUC19 plasmid was performed as previously described (4).

Electrophoretic mobility shift assay (EMSA)

The substrate for DNA binding experiments is a double-stranded oligonucleotide containing a single ligatable nick. It was obtained by the annealing of three complementary strands in equimolar amounts. The sequence is shown in Figure 3. A typical reaction mixture (20 µl) containing 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 2 pmol radiolabelled substrate (1.5×10^4 c.p.m.) and different amounts of purified protein (see figure legends) was incubated at room temperature for 20 min. The reaction was stopped by adding 5 µl loading buffer (30% sucrose, 12 mM Tris–HCl, pH 8.0, 0.25% BPB, 0.25% xylene cyanol) and samples were run in native 5% PAGE in 0.5× TBE buffer at 20 mA for 2 h at 4°C. The extent of band shift was visualized by autoradiography of dried gels.

Polyclonal antibodies to T4 DNA ligase

Chicken egg yolk antibodies (IgY) against T4 DNA ligase were produced according to Gassman *et al.* (18). Two 23-week-old white laying hens were used. To induce the antibodies 15 µg T4 DNA ligase (Boehringer) were diluted in 0.01 M potassium phosphate buffer, pH 7.2, containing 0.1 M NaCl to a final volume of 500 µl and emulsified with an equal volume of complete Freund's adjuvant (Sigma). The suspension (1 ml) was injected into the pectoral muscle. Further injections of the protein emulsified with complete adjuvant were given to the hens 11 and 18 days later. Eggs were collected daily and stored at 4°C until use. IgY were extracted from individual eggs as described (18). The yolk antibodies showed a titre of 1.2×10^6, as measured in a direct ELISA assay against the purified antigen (4 µg/well). A 1:10 000 dilution in Western blot analysis could recognize a single polypeptide of the expected apparent molecular mass in an extract from *E.coli* DH5α transformed with plasmid pHis-T4. No bands were detectable in non-transformed DH5α cell extracts.

RESULTS

Previous studies have identified a class of ATP-dependent DNA ligases with identical catalytic properties: (i) ability to catalyse the ligation of nicked, blunt-ended or sticky-ended double-stranded DNA fragments; (ii) ATP-dependent self-adenylation activity; (iii) AMP-dependent DNA relaxation activity (4,5,17). All these enzymes share a set of conserved sequence motifs whose function has not yet been elucidated. The most representative of this class of enzymes, widespread in evolution from bacteriophages to humans, is the relatively small T4 DNA ligase, which was therefore selected for a functional dissection.

The coding region of the T4 DNA ligase gene was PCR amplified and cloned in the pTrcHis vector, which directs expression of His-tagged proteins in *E.coli*. Purification to homogeneity of the His-tagged T4 DNA ligase (His-T4) was

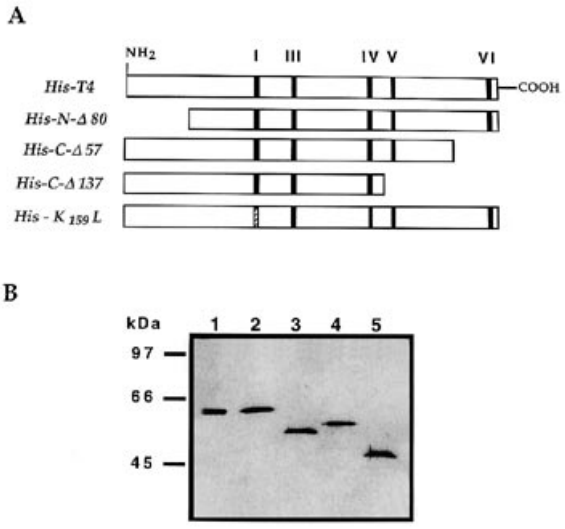

Figure 1. Schematic representation of the different T4 DNA ligase mutants expressed in *E.coli* as His-tagged proteins (**A**). The position of the conserved boxes (see text) is indicated. The His-K_{159}L mutant bears a substitution of Lys159 in the active site (site I). 0.1 µg of each purified recombinant protein was loaded onto a 10% SDS–polyacrylamide gel and revealed by silver staining (**B**). Lane 1, His-T4; lane 2, His-K_{159}L; lane 3, His-N-Δ80; lane 4, His-C-Δ57; lane 5, His-C-Δ137.

achieved through two successive chromatographic steps, as detailed in Materials and Methods, and the chromatographic behaviour of the protein was monitored by means of chicken antibodies specifically directed to the wild-type enzyme. In brief, the first step of purification consisted of a Ni^{2+} affinity column. Most of the His-T4 was retained by the column in the presence of 50 mM imidazole and eluted, along with a limited number of other proteins, after a 250 mM imidazole wash. These contaminants were removed by gel filtration on a Superdex G75 column, as estimated by silver staining of the purified enzyme analysed by SDS–PAGE (Fig. 1B, lane 1). The purified protein was then stored at –20°C in 50% glycerol and proved to be functionally stable for at least 6 months.

The His-T4 enzyme was indistinguishable from the untagged enzyme in a number of biochemical reactions, such as (i) ATP binding, (ii) nick joining, (iii) blunt-end ligation, (iv) AMP-dependent DNA relaxation and (v) DNA binding. On this basis we undertook the production of a set of His-tagged mutants bearing deletions at either end, as schematically shown in Figure 1A. The mutant His-N-Δ80 bore a deletion of the first 80 amino acids that did not affect any of the conserved motifs. In contrast, deletion of the last 57 C-terminal residues in His-C-Δ57 removed the 'conserved peptide' (motif VI) and a further 80 amino acid deletion in His-C-Δ137 resulted also in the loss of motif V. The last mutant analysed in this study (His-K_{159}L) carried a substitution within motif I (KXDG) in which the positively charged lysine was replaced by the hydrophobic leucine. A similar substitution has already been reported to inactivate both human DNA ligase I and vaccinia virus DNA ligase by abolishing their ATP-dependent self-adenylation (19,20). All these His-tagged polypeptides were purified to homogeneity as described in Materials and Methods. The mutants showed an apparent molecular mass higher than the theoretical one, in agreement with the electrophoretic behaviour of T4 DNA ligase (Fig. 1B). Proteins were then subjected to a series of biochemical assays in

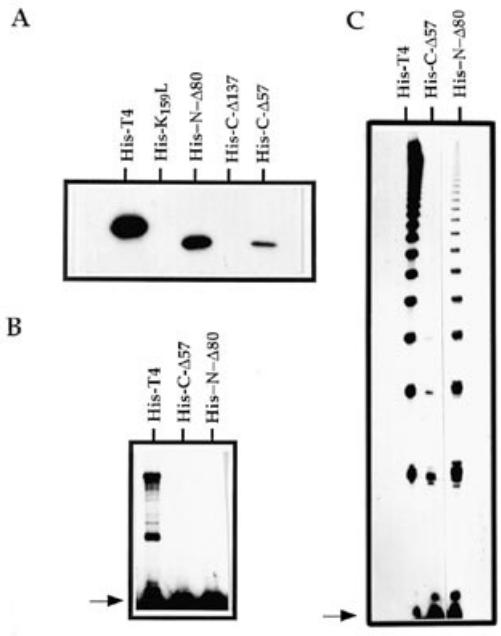

Figure 2. Self-adenylation and ligation properties of different T4 DNA ligase mutants. (**A**) 0.1 μg of purified recombinant proteins were incubated in an adenylation assay in the presence of 1 μCi [α-^{32}P]ATP and then loaded onto a 10% SDS–polyacrylamide gel. The extent of self-adenylation was revealed by autoradiography of the dried gel. The His-T4 protein and the mutants able to carry out self adenylation were challenged in blunt-end (B) and nick joining (C) assays (see Materials and Methods for details). (**B**) His-T4 (0.1 μg), His-C-Δ57 (2 μg) and His-N-Δ80 (2 μg) proteins were incubated in a standard blunt-end ligation assay. (**C**) His-T4 (0.1 μg), His-C-Δ57 (2 μg) and His-N-Δ80 (1 μg) proteins were incubated in a standard nick joining assay. Ligation products were denatured, resolved on an 8% sequencing gel and the extent of the reaction was revealed by autoradiography. The position of [^{32}P]oligo(dT)$_{16}$ is indicated by an arrow.

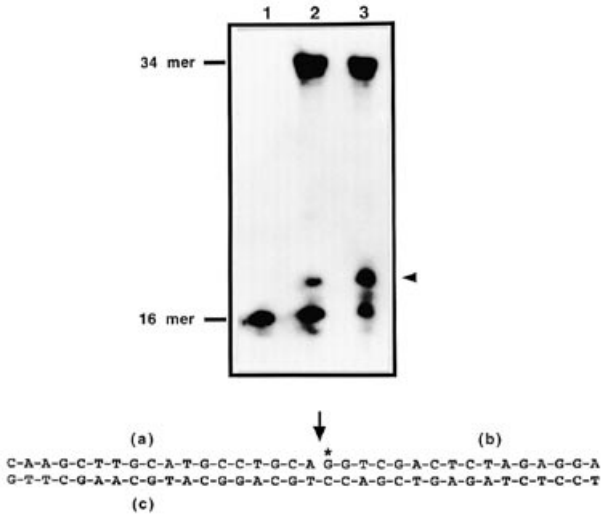

Figure 3. A 34 nt double-stranded oligonucleotide was produced by the annealing of three complementary oligonucleotides as schematically reported at the bottom of the figure. Oligonucleotide b was 5′-end-labelled with [γ-^{32}P]ATP (indicated by an asterisk). After incubation in the presence of 1 μg His-T4 in a standard ligation assay, reaction products were denatured and loaded onto an 8% sequencing gel. Ligation is revealed, after autoradiography of the dried gel, by the appearance of a 34mer resulting from the joining of oligos a and b. Lane 1, denatured substrate; lane 2, ligation in the presence of 1 mM ATP; lane 3, ligation in the absence of ATP. The position of the adenylated substrate is indicated by an arrowhead.

order to assess the effect of the introduced modifications on the catalytic properties of the enzyme.

ATP binding

All the His-tagged proteins were tested for their ability to covalently bind ATP, the first step of the ligation reaction. Equal amounts of proteins (100 ng) were incubated at room temperature for 20 min in the presence of 1 μCi [α-^{32}P]ATP and, after SDS–PAGE fractionation, the extent of protein adenylation was determined by autoradiography (Fig. 2A). The His-N-Δ80 mutant showed an adenylation level comparable with that of His-T4 (50%), ruling out a major involvement of the first 80 amino acids in the reaction. This was not unexpected, since such a region is absent in the otherwise similar T7 DNA ligase. In contrast, both C-terminal deletions affected the adenylation reaction, although to different extents. While removal of the 'conserved peptide' (His-C-Δ57) still allowed some residual activity (~10%), we failed to observe adenylation of the His-C-Δ137 mutant (Fig. 2A). As expected, the His-K$_{159}$L substitution mutant was completely inactive. In those cases in which adenylation was detected, it could be reversed by 5 mM PPi (not shown).

DNA binding

In order to characterize the DNA binding properties of T4 DNA ligase, all the tagged proteins were challenged in EMSAs. We first investigated under which conditions the His-T4 enzyme shifted a double-stranded 34mer containing a ligatable nick on one strand (see Fig. 3). It is worth underlining that the substrate, formed from three annealed oligonucleotides, contains a single phosphorylated 5′-end and therefore only nick joining, and not blunt-end ligation, can occur. Since a significant fraction of His-T4 was still adenylated at the end of the purification protocol (not shown), nick joining took place even in the absence of ATP and hence a ^{32}P-labelled 34 nt single-stranded oligonucleotide was detectable on an 8% sequencing gel (Fig. 3). As a result a deadenylated enzyme was produced after a nick joining reaction carried out in the absence of cofactor. Under these conditions a major retarded band, which increased with protein concentration, was detected by EMSA (Fig. 4A). The retarded band was detected at a protein:DNA molar ratio of 1:1 and no further bands with slower electrophoretic mobility were produced at higher ratios, suggesting that a single molecule of enzyme was bound to each double-stranded oligonucleotide. A few minor bands were also detected that altogether accounted for a few percent of the major retarded band. Because of their low relative abundance and since they were not reproducibly observed, these bands were not the subject of further investigation. It is likely that these protein–DNA complexes result from non-specific contacts between the enzyme and the substrate. The same major retarded band was also detectable in the presence of AMP or when an excess of PPi, which reverses the adenylation reaction, was added to the assay (Fig. 4B). On the other hand, band shift was affected by increasing concentrations of ATP and completely abolished at ATP concentrations higher than the K_m (Fig. 4C). The same effect was observed in the presence of αS-ATP (Fig. 4B). An informative result was obtained when dATP was used. We have previously reported (21) that T4 DNA ligase binds dATP and that the resulting complex, more stable than that

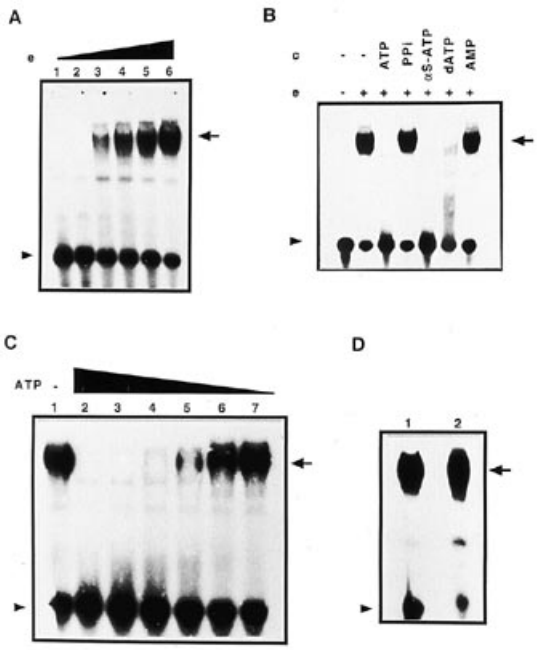

Figure 4. Characterization of the DNA binding properties of the His-T4 enzyme. The same double-stranded oligonucleotide shown in Figure 3 was used in band shift assays as described in Materials and Methods. (**A**) Band shift in the absence of ATP and in the presence of increasing concentrations of His-T4 (e). Lanes 1–6, 0.01, 0.05, 0.1, 0.5, 1 and 3 µg of protein respectively. The position of the retarded band is indicated by an arrow, while free oligo is indicated by an arrowhead. (**B**) 1 µg of protein was tested in EMSA in the presence of different cofactors (as indicated at the top of the figure) at a concentration of 1 mM. (**C**) EMSA carried out with 1 µg of protein in the absence (lane 1) or presence of decreasing amounts of cofactor (lanes 2–7, 50, 25, 12.5, 5, 2.5 and 1.25 µM respectively). (**D**) EMSA with 1 µg His-T4 (lane 1) or His-K_{159}L (lane 2).

formed with ATP, is inactive in the ligation reaction. Also, the deoxyadenylated enzyme was unable to shift the oligonucleotide (Fig. 4B). Overall, these results indicate that the stability of the enzyme–DNA complex depends on adenylation state. When either no cofactor was added or the concentration of ATP was lower than the K_m (1.4×10^{-5}) (13), thus slowing the adenylation reaction, the deadenylated enzyme seemed to stall on DNA as a stable complex (S·complex). In contrast, only a very transient complex (T·complex) should form in the presence of adenylated enzyme. Consistent with this conclusion, the His-K_{159}L substitution mutant, which was unable to carry out self-adenylation, responded efficiently to EMSA on either a nicked (Fig. 4D, lane 2) or a double-stranded (not shown) DNA, indicating that the mutated lysine residue is not involved in DNA recognition. For the sake of completeness, all the deletion mutants were challenged in EMSA in the absence of ATP. None of them was able to shift the substrate (not shown).

Ligation properties

All the mutants were challenged in both nick joining and blunt-end ligation assays. As a substrate in the nick joining reaction we used ^{32}P-labelled oligo(dT)$_{16}$ annealed to poly(dA). The ligation products were loaded onto an 8% sequencing gel and the efficiency of the reaction was qualitatively estimated by the extent of oligo(dT)$_{16}$ multimerization. A double-stranded 18mer, labelled at both 5′-ends, was the substrate in the blunt-end ligation. Again, ligation was revealed by the appearance of multimers on an 8% sequencing gel. Both His-C-Δ137 and His-K_{159}L mutants had neither nick joining nor blunt-end ligation activity, consistent with the fact that they were inactive in the self-adenylation reaction (not shown). The remaining two mutants were able to carry out nick joining, although to a lower extent than expected from their reduced ability to perform the self-adenylation step. A 10-fold molar excess of His-N-Δ80 produced a level of oligo(dT)$_{16}$ multimerization lower than that produced by 100 ng His-T4 (Fig. 2C). In the case of His-C-Δ57, only few multimers were obtained with a 20-fold molar excess (Fig. 2C), suggesting an even more dramatic effect. It is worth noting that the ligation assay appeared to amplify the differences already detected by the self-adenylation assay, just as other properties (overall protein structure or affinity for the substrate) contributed to the final joining activity.

Interestingly, both His-C-Δ57 and His-N-Δ80 were completely inactive in the blunt-end ligation assay (Fig. 2B). Their inability to carry out blunt-end ligation could correlate with their inability to form a stable complex with the substrate.

AMP-dependent DNA relaxation activity

Similarly to mammalian DNA ligase I, T4 DNA ligase can relax supercoiled DNA in an AMP-dependent reaction that could be referred to as the 'reverse reaction' (7,8). From the kinetics of the reaction and from the absence of nicked DNA in the final products it was inferred that DNA ligase acts processively through a nicking/closing mechanism similar to that proposed for DNA topoisomerases (5). In contrast to both type I and II DNA topoisomerases, DNA ligases appear to be unable to covalently bind DNA (3,5), implying that the two classes of enzymes catalyse DNA relaxation through different mechanisms. In order to better characterize this activity we analysed all the mutants in a DNA relaxation assay. As shown in Figure 5A (lanes 8–11), none of the deleted mutants had AMP-dependent DNA relaxation activity. This was unexpected where the N-terminal mutant was concerned, since it showed detectable self-adenylation and nick joining activity. Even more surprisingly, the His-K_{159}L substitution mutant, although unable to covalently bind ATP and join DNA fragments, showed AMP-dependent endonucleolytic activity. In fact, in the presence of AMP this mutant was able to fully convert a supercoiled plasmid into a circular nicked DNA (Fig. 5A). Differing from the wild-type protein (Fig. 5A, lane 2), the His-K_{159}L mutant did not show any DNA topoisomerase-like activity. Instead, after either prolonged incubation (not shown) or in the presence of high levels of protein (Fig. 5A, lane 6), linear DNA was also detected. The nicked DNA obtained as a final product was a good substrate for wild-type DNA ligase, indicating that ligatable ends were produced (Fig. 5B). In contrast, the same DNA appeared to be a poor substrate for T4 polynucleotide kinase after extensive exposure to calf intestinal phosphatase, suggesting that 5′-ends were covalently bound to an AMP molecule (not shown).

DISCUSSION

In this paper we report a functional characterization of T4 DNA ligase carried out by means of His-tagged versions of the wild-type and of four mutated enzymes (Table 1). Three of the

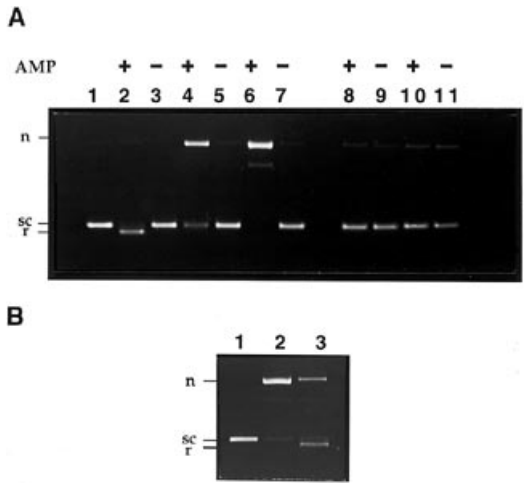

Figure 5. DNA relaxation activity of the different His-tagged proteins. (**A**) 0.2 µg pUC19 vector were used in DNA relaxation assays in the presence (+) or absence (–) of 1 mM AMP. Lane 1, substrate; lanes 2 and 3, 0.1 µg His-T4; lanes 4 and 5, 0.5 µg His-K_{159}L; lanes 6 and 7, 1 µg His-K_{159}L; lanes 8 and 9, 2 µg His-C-Δ57; lanes 10 and 11, 2 µg His-N-Δ80. (**B**) The reaction product obtained with His-K_{159}L in the presence of AMP (lane 2) was purified and an aliquot was ligated in the presence of T4 DNA ligase (lane 3). Gels were run in 1× TAE electrophoresis buffer containing 1 µg/ml ethidium bromide. Lane 1, supercoiled pUC19. n, nicked; sc, supercoiled; r, relaxed.

mutants bore either N- (His-N-Δ80) or C-terminal (His-C-Δ57 and His-C-Δ137) deletions. In the last, Lys159 in the active site, known to be covalently bound to AMP during the first step of the reaction (22), was substituted by a leucine. Several interesting conclusions emerge from this analysis. First, T4 DNA ligase seems to differ from the T7 enzyme, recently characterized at the structural level (12), with regard to the DNA binding properties. In fact, while a 26 kDa C-terminal domain of T7 DNA ligase was shown to shift a double-stranded oligonucleotide (23), both the N- and C-terminal portions of the T4 enzyme are required to detect a stable protein–DNA complex by EMSA. The failure of the T4 ligase mutants to form a stable complex with DNA appears to correlate with their inability to perform blunt-end ligation. However, since two of these deletion mutants (His-N-Δ80 and His-C-Δ57) still possess nick joining activity, we conclude that these deletions do not affect the catalytic domain. Altogether, these results suggest that the mode of binding of the enzyme to DNA plays a central role in the joining reaction.

In addition we have investigated whether enzyme adenylation could influence the stability of the ligase–DNA complex. For this analysis we used a band shift assay in which a double-stranded oligonucleotide containing a ligatable nick on one strand was used as substrate. As soon as the enzyme is added to the reaction, even in the absence of cofactor, the nick is sealed and a non-ligatable substrate with no phosphorylated 5′-ends is produced. In the presence of cofactors, such as ATP or αS-ATP, no band shift is detectable; on the other hand, DNA binding must have occurred, since the nick is efficiently closed. These data suggest that in the presence of active cofactors the protein–DNA complex is very transient (T·complex). In contrast, a stable complex (S·complex) is formed when the enzyme is deadenylated: (i) when neither proper cofactor nor analogues unable to support enzyme adenylation are present; (ii) when Lys159 in the AMP binding site is mutated; (iii) when an excess of PPi, which reverses the adenylation reaction, is added to an ATP-containing mixture; (iv) when the ATP concentration is below the K_m value and enzyme turnover is greatly reduced. On the basis of these observations we propose a three step model to explain the mode of action of DNA ligase. (i) In the presence of ATP the adenylated enzyme scans a DNA molecule through successive T·complexes in search of a phosphorylated 5′-end. (ii) Upon transfer of the adenylate group to the phosphorylated 5′-end, a deadenylated enzyme is produced that stalls on DNA until a suitable 3′-end becomes available to complete the reaction. During this phase the nucleotide binding pocket is probably still occupied by the AMP molecule, although no longer covalently bound to the K_{159} residue. (iii) The ligation reaction is completed when AMP is released and the enzyme is recycled by a new ATP molecule.

This model offers a new mechanistic viewpoint to explain blunt-end ligation. As schematically depicted in Figure 6, an S·complex between DNA and the enzyme would form upon adenylation of a 5′-end. This complex, the real intermediate of the reaction, would survive long enough to allow contact with another DNA fragment and hence completion of ligation. According to this model, blunt-end ligation would proceed through two successive second order reactions ([free enzyme] × [free DNA]→[S·complex] × [free DNA]→[free enzyme] × [ligated DNA]), rather than a single third order reaction ([free enzyme] × [free DNA] × [free DNA]→[free enzyme] × [ligated DNA]). On the basis of this we predict that low levels of ATP would increase the rate of blunt-end ligation. In agreement with our model, the efficiency of blunt-end ligation is significantly increased at an ATP concentration of 2.5 µM (R.Rossi and A.Montecucco, unpublished observation). According to this model the inability of the deletion mutants to carry out blunt-end ligation would result from their inability to enter an S·complex.

Table 1. Results of the functional characterization of the His-tagged versions of wild-type T4 DNA ligase and of four mutated enzymes

Protein	ATP binding	Nick joining	Blunt-end ligation	AMP-dependent activity	DNA binding
His-T4	+++	+++	+++	DNA relaxation	+++
His-N-Δ80	++	++	–	–	–
His-C-Δ57	+	+	–	–	–
His-C-Δ137	–	–	–	–	–
His-K_{159}L	–	–	–	DNA nicking	+++

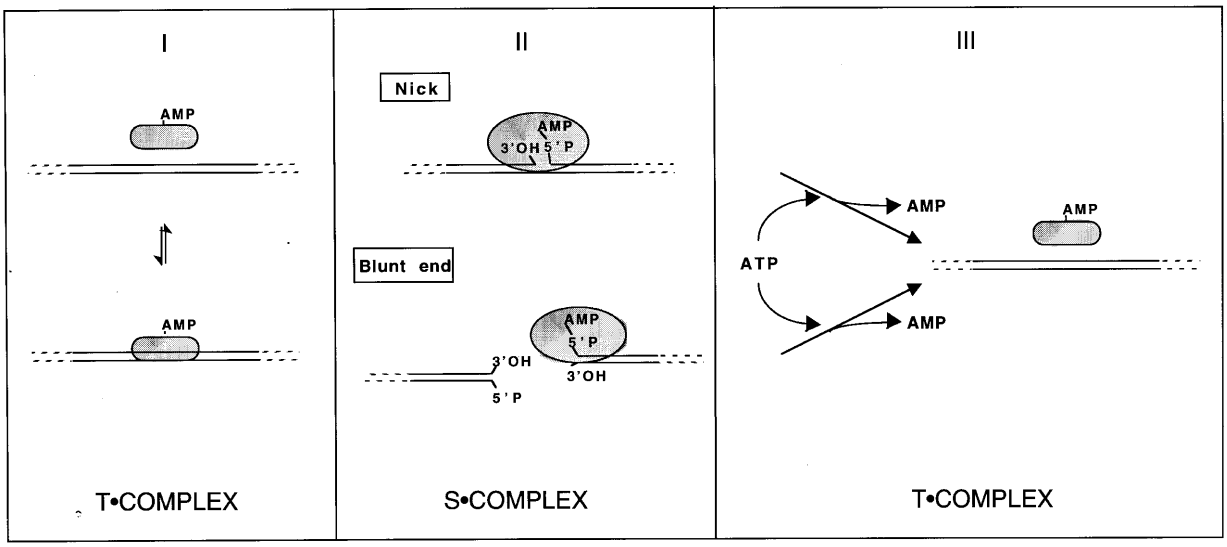

Figure 6. A three step model of the T4 DNA ligase activity. Step I: the adenylated enzyme scans the DNA molecule through successive T·complexes in search of suitable 5′-phosphorylated ends. Step II: as soon as a 5′-phosphorylated end is found, the adenylate group is transferred from the enzyme to DNA and the deadenylated enzyme stalls on it in an S·complex. Step III: when a 3-OH end is available, completion of the reaction is quick and in the presence of ATP the enzyme is readenylated to start a new cycle.

Interesting speculations can be proposed concerning the role of DNA ligase during both DNA replication and DNA repair. It has recently been reported that DNA ligase has some sequence similarity with mRNA capping enzymes and that the transfer of an adenylate group to DNA resembles addition of a GMP moiety to the 5′-end of a pre-mRNA molecule (11). Taking into account these similarities and on the basis of the data presented here, a new role for DNA ligase during both DNA replication and DNA repair can be proposed. In fact, by analogy with the RNA world where the cap at the 5′-end protects the polynucleotide from exonucleolytic activities, DNA ligase could recognize, label and protect any suitable 5′-end until a correct 3′-hydroxyl group is produced by the proper DNA polymerase. Stalling of the enzyme at the adenylated 5′-end would result in a more processive mode of action, ensuring that the same enzyme molecule that recognizes the 5′-end will carry out ligation, regardless of the time required to provide the 3′-OH group. This model could be extrapolated to the related eukaryotic DNA ligases. Since the enzyme, at least in mammals, is recruited to the replisome (9), it could bridge the replisome itself to the 5′-end of an Okazaki fragment ready to be ligated. On the other hand, during DNA repair the enzyme would label the 5′-end while the action of other enzymes is incomplete.

A last, interesting consideration concerns the 'reverse reaction' catalysed by DNA ligases in the presence of AMP. The His-$K_{159}L$ substitution mutant, although unable to covalently bind ATP, still carries out the true reverse reaction in the presence of AMP (a condition that leads to formation of the S·complex) by converting supercoiled DNA into a circular nicked DNA molecule most likely containing adenylated 5′-ends. This is a significant difference from the wild-type enzyme, which produces relaxed covalently close circular DNA molecules in a topoisomerase-like fashion (5). The topoisomerase-like activity of the wild-type enzyme would result from the high processivity of DNA ligase, which carries out consecutive nicking/closing reactions without detaching from the substrate.

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