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Nick Sensing by Vaccinia Virus DNA Ligase Requires a 5′ Phosphate at the Nick and Occupancy of the Adenylate Binding Site on the Enzyme

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Vaccinia virus DNA ligase has an intrinsic nick-sensing function. The enzyme discriminates at the substrate binding step between a DNA containing a 5′ phosphate and a DNA containing a 5′ hydroxyl at the nick. Further insights into nick recognition and catalysis emerge from studies of the active-site mutant K231A, which is unable to form the covalent ligase-adenylate intermediate and hence cannot activate a nicked DNA substrate via formation of the DNA-adenylate intermediate. Nonetheless, K231A does catalyze phosphodiester bond formation at a preadenylated nick. Hence, the active-site lysine of DNA ligase is not required for the strand closure step of the ligation reaction. The K231A mutant binds tightly to nicked DNA-adenylate but has low affinity for a standard DNA nick. The wild-type vaccinia virus ligase, which is predominantly ligase-adenylate, binds tightly to a DNA nick. This result suggests that occupancy of the AMP binding pocket of DNA ligase is essential for stable binding to DNA. Sequestration of an extrahelical nucleotide by DNA-bound ligase is reminiscent of the base-flipping mechanism of target-site recognition and catalysis used by other DNA modification and repair enzymes.

The connections between DNA repair and human cancers have fueled interest in the proteins that recognize specific sites of DNA damage. Most repair pathways converge at a common final step in which the continuity of the repaired DNA strand is restored by DNA ligase, an enzyme that converts nicks into phosphodiester bonds (3, 13, 14). Nicks are potentially deleterious DNA lesions that, if not corrected, may give rise to lethal double-strand breaks.

The eukaryotic DNA ligases catalyze the ATP-dependent joining of a 5′ phosphate-terminated strand to a 3′ hydroxyl-terminated strand via three sequential nucleotidyl transfer reactions (14). In the first step, attack on the α-phosphate of ATP by ligase results in release of pyrophosphate and formation of a covalent intermediate in which AMP is linked to the ε-amino group of a lysine. The active-site lysine is located within a conserved sequence motif, KXDG (14, 25, 26, 32). The nucleotide is then transferred to the 5′ end of the 5′ phosphate-terminated strand to form DNA-adenylate, an inverted 5′-5′ pyrophosphate bridge structure, AppN. Attack by the 3′ OH of the nick on the DNA-adenylate joins the two polynucleotides and liberates AMP.

A common catalytic domain is present in all ATP-dependent DNA ligases. The domain includes six sequence motifs that are conserved among a superfamily of covalent nucleotidyl transferases, which encompasses the ATP-dependent DNA ligases, the ATP-dependent RNA ligases, and the GTP-dependent mRNA capping enzymes (26). In the crystal structure of the bacteriophage T7 DNA ligase with bound ATP, the phylogenetically conserved motifs make up the nucleotide binding site (29). What remains unclear is how DNA ligases interact with nucleic acid.

Mammalian cells contain five distinct ATP-dependent ligases (8, 20, 31). Ligase II and ligase III are strikingly similar at the amino acid sequence level to vaccinia virus DNA ligase (2, 5, 28, 34, 35). These three proteins appear to constitute a distinct subgroup within the ligase family. The vaccinia virus enzyme, like its cellular homologs, is implicated in DNA repair (9). We have shown that formation of a stable complex between vaccinia virus ligase and DNA depends on a DNA nick (24). The vaccinia virus enzyme discriminates at the DNA binding step between nicked DNA molecules that can be sealed (e.g., the molecule in Fig. 1A) versus gapped DNA molecules that cannot be ligated. Even a 1-nucleotide gap significantly reduces the affinity of vaccinia virus ligase for DNA (24). Similar specificities for binding to DNA nicks have since been documented for *Chlorella* virus PBCV-1 DNA ligase and for mammalian DNA ligase III (1, 4). Thus, these enzymes apparently have an intrinsic ability to recognize the DNA sites where their action is required.

In this study, we address the basis for nick recognition. We show that the binding of vaccinia virus ligase to DNA requires the presence of a 5′ phosphate moiety at the nick and provide evidence that DNA binding requires simultaneous occupancy of the adenylate-binding site on the enzyme. In addition, we demonstrate that the active-site lysine is dispensable for the strand closure step (step 3) of the ligase reaction.

MATERIALS AND METHODS

Enzyme purification. Wild-type vaccinia virus DNA ligase and the K231A mutant enzyme were expressed in *Escherichia coli* as fusion proteins containing a leader sequence of 10 tandem histidines as described previously (23). The proteins were purified from the soluble lysates of 200-ml bacterial cultures by sequential nickel-agarose and phosphocellulose chromatography (23). The protein concentrations of the enzyme fractions were determined by the Bio-Rad dye-binding assay, with bovine serum albumin being used as the standard.

Ligation substrates. The nicked duplex DNA substrate (Fig. 1A) was formed by mixing the 18-mer strand labeled with ^{32}P at its 5′ end with the complementary 36-mer strand and a 3′-OH 18-mer strand at a molar ratio of 1:4:4 in 0.2 M NaCl. The mixture was heated at 70°C for 10 min and then cooled slowly to 22°C. The 5′-adenylated 18-mer strand used to form the nicked DNA-adenylate substrate (Fig. 1B) was synthesized with vaccinia virus DNA ligase (24). Ten reaction mixtures (50 μl each) containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 1 mM ATP, 5 pmol of a radiolabeled 1-nucleotide gap substrate (24), and 25 pmol of vaccinia virus ligase were incubated at 22°C for 60 min. The reactions were halted by adding EDTA to 25 mM. The samples were pooled and then ethanol precipitated. The pelleted material was resuspended in formamide, heated at 95°C for 5 min, and then electrophoresed through a 12%

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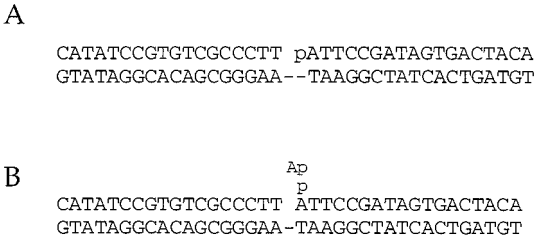


FIG. 1. Ligation substrates. The structures of the nicked duplex DNA (A) and nicked DNA-adenylate (B) substrates used in the ligation assays are shown. The labeled monophosphate end is indicated by p in panel A.

polyacrylamide gel containing 7 M urea in TBE (90 mM Tris-borate, 2.5 mM EDTA). The ^{32}P-labeled DNA-adenylate, which was separated clearly from the 18-mer input strand, was located by autoradiography of the wet gel and then eluted from an excised gel slice. The adenylated 18-mer was ethanol precipitated and resuspended in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA. The nicked DNA-adenylate substrate was formed by annealing the gel-purified adenylated strand to the complementary 36-mer and a 3′ OH-terminated acceptor strand at a molar ratio of 1:4:4.

RESULTS

Binding of ligase to nicked DNA requires a 5′ phosphate moiety at the nick. We compared the binding of wild-type vaccinia virus ligase to a nicked duplex DNA ligand containing either a 5′ phosphate or a 5′ hydroxyl at the nick. The DNAs consisted of a 5′ ^{32}P-labeled 3′ OH-terminated hairpin oligonucleotide and an 18-mer strand (either 5′ phosphate or 5′ hydroxyl terminated) annealed to the 5′ tail of the hairpin strand (Fig. 2). Vaccinia virus ligase efficiently sealed the substrate containing the 5′ phosphate-terminated 18-mer. In the linear range of enzyme dependence, 20 fmol of 5′ phosphate-containing nicks was ligated per fmol of protein (data not shown). Vaccinia virus ligase was incapable of sealing the molecule containing a 5′ hydroxyl at the nick, even in enzyme excess (data not shown).

A native gel mobility shift assay (23, 24) was used to directly examine the binding of vaccinia virus ligase to the nicked hairpin ligands. Binding reactions were performed in the absence of ATP and a divalent cation so as to preclude conversion of substrate to product during the incubation. Mixing the ligase with 0.5 pmol of ^{32}P-labeled DNA containing a 5′ phosphate at the nick resulted in the formation of a discrete protein-DNA complex that migrated more slowly than the free DNA during electrophoresis through a 6% native polyacrylamide gel (Fig. 2). The yield of this complex was proportional to input ligase. This specific complex was not detected when vaccinia virus ligase was incubated with the DNA ligand containing a 5′ hydroxyl at the nick (Fig. 2). Only trace amounts of a different band (migrating more slowly than the specific complex) were detected at the highest levels of input protein (Fig. 2). The same minor species was formed on the 5′ phosphate-containing nicked ligand; we presume that this species arises via nonspecific (i.e., nick-independent), low-affinity interaction of ligase with DNA.

This experiment shows for the first time that DNA ligase is capable of discriminating at the substrate binding step between nicked ligands containing 5′ phosphate versus 5′ hydroxyl moieties at the nick. By scanning the gel in Fig. 2 and comparing the extents of specific binding at 1 to 2 pmol of input enzyme, we calculated that the vaccinia virus enzyme's affinity for the 5′ hydroxyl-containing nicked ligand was ~1% of that for the 5′ phosphate-containing nicked DNA. This result, together with prior findings that ligase binds poorly to a DNA containing a 5′ phosphate and a 1-nucleotide gap, implies that DNA binding depends on contact with both the 3′ hydroxyl and the 5′ phosphate moieties on either side of the nick.

Catalytic properties of the active-site mutant K231A. The nick-sensing functions of vaccinia virus ligase and PBCV-1 ligase were first detected with purified recombinant enzymes that were predominantly in the adenylated form (4, 24). To address whether adenylation of the enzyme plays a role in DNA recognition, we exploited a mutated version of vaccinia virus ligase in which the active-site K231 was replaced by alanine (25). The K231A protein was purified to apparent homogeneity in parallel with the wild-type ligase (Fig. 3). Incubation of wild-type ligase with [α-^{32}P]ATP and a divalent cation resulted in the formation of a 65-kDa covalent ligase-[^{32}P]AMP complex. K231A was inert in forming the enzyme-AMP complex (Fig. 3). The K231A mutant protein was completely incapable of ligating a nicked duplex DNA containing 3′ OH and 5′ phosphate termini at the nick, even in vast enzyme excess (data not shown).

Phosphodiester bond formation at the nick requires activation of the 5′ phosphate terminus for attack by the 3′ OH terminus. Wild-type ligase accomplishes this activation by transferring the AMP to the nick to form a nicked DNA-adenylate intermediate (shown in Fig. 1B). Although K231A could not catalyze DNA-adenylate formation, it did catalyze the strand joining step when the mutant enzyme was provided with a preadenylated nicked duplex (Fig. 4). The adenylated strand used to form this substrate was synthesized by ligase-mediated AMP transfer to the strand of a DNA molecule whose 5′ end was labeled with ^{32}P and which contained a 1-nucleotide gap. Although normally undetectable, DNA-adenylate accumulates to very high levels when the 3′ OH is recessed by 1 nucleotide (24). The radiolabeled AppDNA strand was gel purified and annealed to a 36-mer template strand and a 3′ OH-terminated 18-mer to form the structure

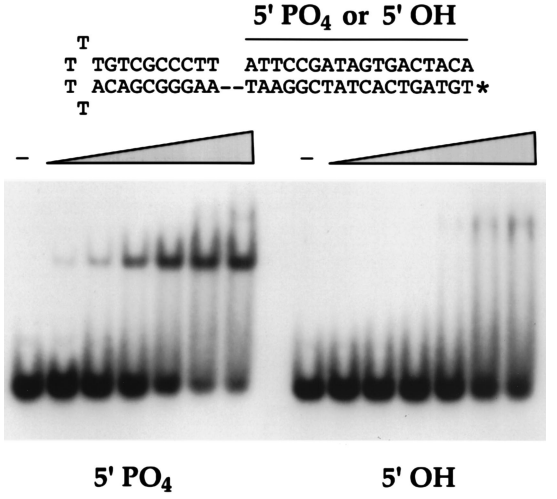


FIG. 2. Native-gel assay of the binding of vaccinia virus ligase to nicked substrates with a 5′ phosphate versus a 5′ hydroxyl group at the nick. The nicked duplex ligand used in the binding assays is shown. The 3′ hydroxyl-terminated 42-mer hairpin strand was ^{32}P labeled at the 5′ end (denoted by the asterisk). The unlabeled 18-mer strand contained either a 5′ phosphate (5′ PO_4) or a 5′ hydroxyl (5′ OH) terminus. Binding reaction mixtures (20 μl) containing 50 mM Tris-HCl (pH 8.0), 5 mM DTT, 0.5 pmol of nicked ligand, and 0.1, 0.25, 0.5, 1, 2, and 3 pmol of vaccinia virus ligase (proceeding from left to right within each titration series) were incubated for 10 min at 22°C. Control reactions (lane −) contained no ligase. Glycerol was added to 5%, and the samples were electrophoresed through a 6% polyacrylamide gel in TBE buffer at 60 V for 2.5 h. An autoradiograph of the dried gel is shown.

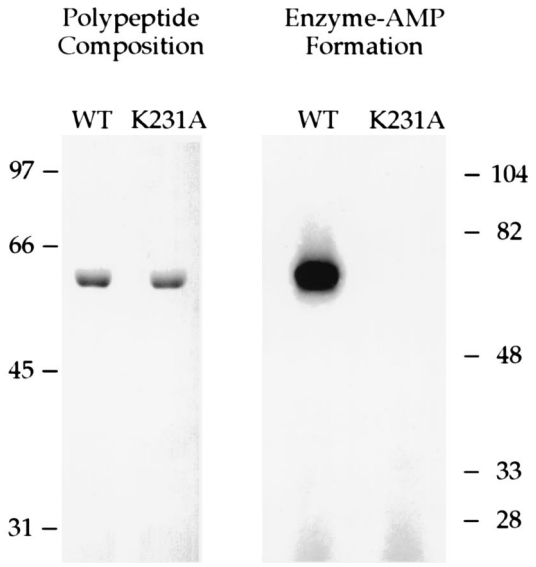
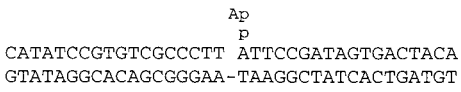


FIG. 3. Purification and enzyme-adenylate formation by wild-type and K231A ligases. Aliquots (5 μg) of the phosphocellulose preparations of wild-type vaccinia virus DNA ligase and the K231A mutant enzyme were electrophoresed through a 10% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS). The Coomassie blue-stained gel is shown on the left. The positions and sizes (in kilodaltons) of marker proteins are indicated. Enzyme-adenylate formation was assayed by label transfer from [α-^{32}P]ATP to the ligase polypeptide. Reaction mixtures (20 μl) containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, 10 μM [α-^{32}P]ATP, and 100 ng of wild-type (WT) or K231A ligase were incubated for 10 min at 22°C. The reactions were halted by adding SDS to 1%, and the samples were analyzed by SDS-polyacrylamide gel electrophoresis. An autoradiogram of the dried gel is shown on the right. The positions and sizes (in kilodaltons) of prestained marker proteins are indicated.

shown in Fig. 1B. Successful ligation was manifest by formation of an internally labeled 36-mer product. Seventy-two percent of the input substrate was ligated by K231A at a saturating concentration of enzyme during a 30-min reaction (Fig. 4). The upper limit of the extent of ligation probably reflected incomplete annealing of all three component strands to form the nicked DNA-adenylate substrate (24). The K231A mutant acted catalytically, insofar as 2.5 fmol of enzyme ligated 108 fmol of AppDNA strands (Fig. 4). The kinetics of ligation were examined in reactions containing 30 nM K231A ligase and 12.5 nM DNA-adenylate. The reaction was virtually complete in 60 s and attained 53% of the endpoint value in 10 s (Fig. 4). We conclude from these experiments that the active-site lysine of DNA ligase, which is essential for the first two nucleotidyl transfer steps, is dispensable during the strand closure step of the ligation reaction.

Nick recognition requires occupancy of the adenylate binding site on the enzyme. A native-gel mobility shift assay was employed to examine the binding of the wild-type ligase (which consists of 58% ligase–adenylate [23]) and the K231A protein (which consists of 0% ligase–adenylate) to ^{32}P-labeled nicked duplex DNA and nicked DNA-adenylate molecules. Binding reactions were performed in the absence of magnesium so as to preclude conversion of substrate to product during the incubation. Control experiments verified that ligation of nicked DNA by stoichiometric amounts of ligase-adenylate required a divalent cation, as did ligation of DNA-adenylate by K231A (data not shown). Mixing the wild-type ligase with the nicked substrate resulted in the formation of a discrete protein-DNA complex that migrated more slowly than the free DNA during electrophoresis through a 6% native polyacrylamide gel (Fig. 5). The yield of this complex was proportional to input ligase (Fig. 6). The apparent dissociation constant was ~10 nM. The K231A mutant ligase formed smaller amounts of ligase-DNA complex than did the wild-type enzyme at comparable levels of input protein (Fig. 5). By comparing the extents of binding at equivalent concentrations of enzyme, we estimated that the K231A mutation elicited a 20-fold decrement in binding affinity for nicked DNA (Fig. 6). We attribute this result to the absence of a bound adenylate moiety on the enzyme.

If occupancy of the adenylate-binding site is important for



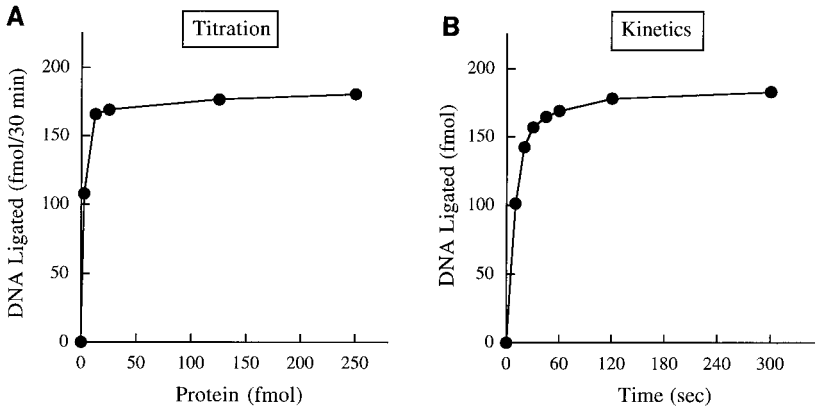


FIG. 4. Ligation of nicked DNA-adenylate by the K231A mutant ligase. (A) Ligase titration. Reaction mixtures (20 μl) containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, 250 fmol of nicked DNA-adenylate (shown at the top of the figure), and the indicated amounts of K231A ligase were incubated for 30 min at 22°C. The reactions were halted by the addition of 1 μl of 0.5 M EDTA and 5 μl of formamide. The samples were heated at 95°C for 5 min and then electrophoresed through a 17% polyacrylamide gel containing 7 M urea in TBE. The extent of ligation was quantitated by scanning the wet gel with a FUJIX BAS1000 Bio-imaging Analyzer. (B) Kinetics of strand joining. Reaction mixtures containing (per 20 μl) 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, 250 fmol of nicked DNA-adenylate, and 600 fmol of K231A ligase were incubated at 22°C. Aliquots (20 μl) were withdrawn at the times indicated and quenched immediately. Product analysis was carried out as described for panel A.

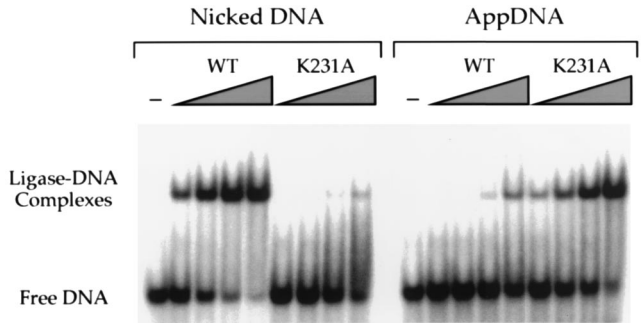


FIG. 5. Native-gel assay of the binding of wild-type and K231A ligases to nicked DNA and nicked DNA-adenylate. Reaction mixtures (20 μl) containing 50 mM Tris-HCl (pH 8.0), 5 mM DTT, 250 fmol of ^{32}P-labeled nicked DNA (left gel) or nicked DNA-adenylate (right gel), and 125, 250, 500, or 1250 fmol of the wild type (WT) or K231A ligase (proceeding left to right within each titration series) were incubated for 10 min at 22°C. Glycerol was added to 5%, and the samples were electrophoresed through a native 6% polyacrylamide gel in 0.25× TBE (22.5 mM Tris-borate, 0.6 mM EDTA) at 90 V for 2 h. An autoradiogram of the dried gel is shown. The positions of the free DNA and the ligase-DNA complexes are indicated.

substrate recognition, then we would predict that K231A would bind with high affinity to a nicked ligand containing an adenylated DNA strand at the nick. This was indeed the case (Fig. 5 and 6). The affinity of K231A for nicked-DNA–adenylate was 10-fold higher than for a plain nicked ligand. Indeed, K231A bound to the adenylated nick with nearly the same affinity as wild-type ligase bound to nicked DNA (Fig. 6). The wild-type ligase bound poorly to DNA-adenylate; this was to be expected, given that most of the ligase molecules contain a covalently bound AMP molecule that sterically hinders binding to the adenylated DNA. We presume that the low levels of binding by the wild-type enzyme to AppDNA are mediated by the nonadenylated fraction of the enzyme preparation.

Topoisomerase-like activity of vaccinia virus DNA ligase. Prokaryotic and bacteriophage-encoded DNA ligases are capable of relaxing supercoiled DNA in the presence of AMP (13, 17). We found that the wild-type vaccinia virus ligase displayed topoisomerase-like activity on supercoiled pUC19 DNA that was completely dependent on AMP (Fig. 7) and magnesium (not shown). The rapidly migrating supercoiled plasmid was converted into a mixture of more slowly migrating topoisomers and a species that comigrated with nicked circular DNA (Fig. 7). Parallel analysis by agarose gel electrophoresis in ethidium bromide confirmed that the reaction products were a mixture of relaxed covalently closed and nicked circular DNAs (not shown). AMP-dependent relaxation required a substantial molar excess of ligase relative to input DNA. This was consistent with the observation that vaccinia virus ligase bound specifically to nicks, which are obviously not present in a covalently closed circular DNA molecule. The K231A mutant protein also displayed AMP-dependent relaxation activity (Fig. 7). We conclude that the ligase-mediated supercoil relaxation reaction did not require covalent nucleotidyl transfer to the enzyme. Thus, ligase-mediated relaxation occurs solely via reversal of step 3, whereby AMP attacks the phosphodiester backbone of supercoiled plasmid DNA to yield nicked DNA-adenylate, the resulting strand break provides a swivel for relaxation of superhelicity, and the adenylated nick is resealed to restore the covalently closed circle and release AMP.

DISCUSSION

We surmise that DNA recognition by vaccinia virus ligase occurs when the adenylate-binding site is filled. This mechanism confers advantageous properties for a repair enzyme: (i) it permits ligase-adenylate to bind with high affinity to nicked DNA, while minimizing sequestration of ligase-adenylate on duplex-DNA segments where its action is not needed, and (ii) it ensures that ligase-adenylate, which is already poised to catalyze phosphodiester formation, is not competing with free enzyme for binding to sites in need of repair. The adenylate need not be bound covalently to the enzyme, as evinced by the fact that the K231A enzyme, which cannot form ligase-adeny-

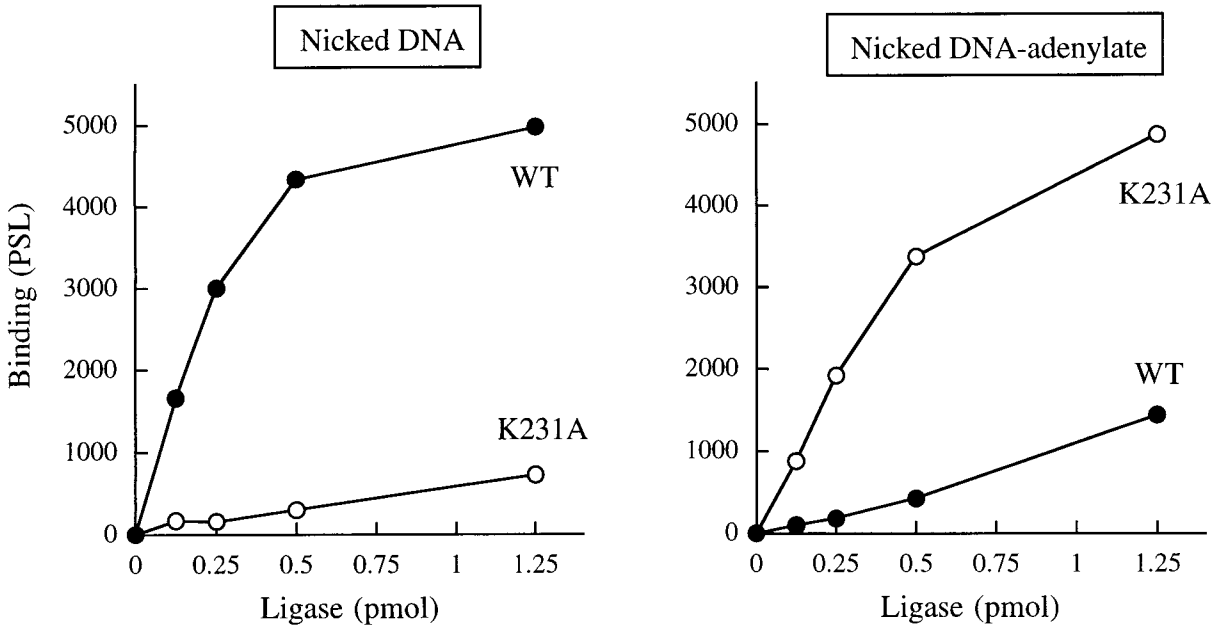


FIG. 6. Protein dependence of DNA binding. The native polyacrylamide gel shown in Fig. 5 was scanned, and the phosphorimage signal intensity of the ligase-DNA complex (photo-stimulatable luminescence [PSL]) was plotted as a function of the amount of input ligase. WT, wild type.

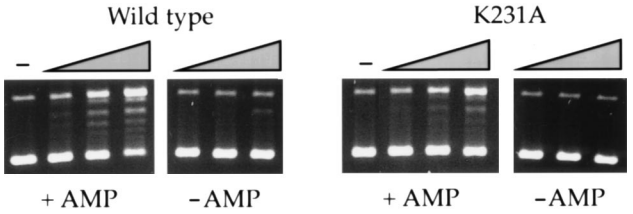


FIG. 7. Relaxation of plasmid DNA catalyzed by the wild-type and K231A ligases. Reaction mixtures (20 μl) containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, 0.3 μg (170 fmol) of supercoiled pUC19 DNA, either 5 mM AMP (+AMP) or no added AMP (−AMP), and 20, 100, and 500 ng (0.3, 1.5, and 7.7 pmol) of the indicated ligase (proceeding left to right within each titration series) were incubated for 60 min at 22°C. The reactions were stopped by the addition of SDS and EDTA to 0.2% and 20 mM, respectively. Glycerol was added to 5%, and the samples were analyzed by electrophoresis through a horizontal 1% agarose gel in Tris-glycine buffer (50 mM Tris, 160 mM glycine). The gel was stained for 15 min in 0.5 μg of ethidium bromide per ml, soaked for 30 min in water, and then photographed under short-wave UV illumination.

late, is nonetheless capable of discriminating DNA-adenylate from nicked DNA. The DNA-adenylate intermediate is normally undetectable during ligation of nicked DNA by wild-type ligase, even under conditions of enzyme excess (24). DNA-adenylate accumulates only under circumstances in which the strand-joining step of the reaction is inhibited, e.g., when there is a base mismatch on the 3′ OH side of the nick (24). Exonuclease removal of the mismatch followed by incorporation of the correct nucleotide by DNA polymerase would generate a nicked DNA-adenylate substrate (similar to that shown in Fig. 1B), closure of which would entail binding and catalysis by the unadenylated form of DNA ligase.

The nucleotide binding pocket of DNA ligase is situated such that the adenylate moieties of the ligase-adenylate—(nicked DNA) complex and of the ligase—(DNA-adenylate) complex are held by ligase outside of the DNA double helix at the 5′ side of the nick (29). This result can be viewed as a variation on the theme of base flipping (29), first documented for DNA methyltransferases (10, 19), whereby the target for site-specific DNA modification is displaced from the double helix and held within a binding pocket of the protein. The base-flipping and nucleotide-flipping paradigm has since been extended to DNA repair enzymes, in which case the misincorporated or damaged base is flipped out of the duplex into the enzyme (11, 16, 18, 21, 27, 30, 36). Alternatively, the complementary base may be flipped out to permit access of enzyme to the lesion (33). Base flipping has also been invoked to explain unpairing of a single thymine upon covalent binding of topoisomerase I to DNA (22).

Provocative functional connections between topoisomerases, DNA ligases, restriction modification enzymes, and repair proteins have emerged recently. Manifestations include the detection of topoisomerase activity intrinsic to a DNA methyltransferase (15), the transformation of a restriction endonuclease into a topoisomerase by a single amino acid change that creates a DNA ligase-like active site (6, 7), and preferential DNA cleavage by type I topoisomerase at base mismatches and sites of UV light-induced DNA damage (12, 37). Base and nucleotide flipping may be unifying themes around which specialized active sites have evolved for specific reaction chemistries.

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