

Label-Free Electrochemical Monitoring of DNA Ligase Activity

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This study presents a simple, label-free electrochemical technique for the monitoring of DNA ligase activity. DNA ligases are enzymes that catalyze joining of breaks in the backbone of DNA and are of significant scientific interest due to their essential nature in DNA metabolism and their importance to a range of molecular biological methodologies. The electrochemical behavior of DNA at mercury and some amalgam electrodes is strongly influenced by its backbone structure, allowing a perfect discrimination between DNA molecules containing or lacking free ends. This variation in electrochemical behavior has been utilized previously for a sensitive detection of DNA damage involving the sugar–phosphate backbone breakage. Here we show that the same principle can be utilized for monitoring of a reverse process, i.e., the repair of strand breaks by action of the DNA ligases. We demonstrate applications of the electrochemical technique for a distinction between ligatable and unligatable breaks in plasmid DNA using T4 DNA ligase, as well as for studies of the DNA backbone-joining activity in recombinant fragments of *E. coli* DNA ligase.

In all cells, nucleic acids are continually synthesized, broken, and rejoined in a variety of fundamental processes. In addition to their primary importance in cell biology, enzymes that break and join the backbone of nucleic acids provide indispensable tools for molecular biological manipulation of nucleic acids. Consequently, much is now understood about the reaction mechanisms by which these enzymes act. One of the most well-known classes of these types of enzymes are DNA ligases, which are essential constituents of all organisms due to their crucial roles in DNA replication and repair.^{1–3} Importantly, two types of DNA ligase have been identified that are categorized by whether NAD⁺ or ATP is used as the source of adenylate that is used to activate the enzyme. The essential DNA ligases of eukaryotes, archaea, and viruses are ATP-dependent while those of bacteria are NAD⁺-dependent. This distribution of specificity has led to the suggestion that

NAD⁺-dependent DNA ligases may provide useful targets for broad-spectrum antibacterial compounds.^{1–3}

Besides electrophoretic techniques commonly used for the detection of DNA strand breaking or joining (ligation), several interesting alternative approaches have recently been developed. For example, stem–loop structure-forming oligonucleotide templates have been applied to detect DNA ligation on the basis of the molecular beacon concept.⁴ Hairpin oligonucleotide substrates, tethered at one end to a surface and bearing a label at the other, were used to detect ligation of a nick within the hairpin stem.^{5,6} Retention of the label signal upon ligation of the nick, or loss of the signal upon cleavage of the DNA sugar phosphate backbone, has been monitored using either fluorescence⁵ or electrochemical⁶ techniques.

The widespread interest in DNA ligases has focused attention on development of improved assays to monitor their enzyme activities. The potential for electrochemical methods to be used in sensing DNA strand breakage has been established (reviewed in refs 7–9). In particular, techniques working with mercury-based electrodes have been reported to exhibit a high sensitivity for the detection of single-strand breaks (ssb).^{10–16} These techniques also provide excellent discrimination between DNA molecules containing or lacking free DNA ends (such as nicked or covalently closed circular plasmid DNAs, respectively^{10,17}). Differences between the ac voltammetric responses of the two duplex DNA forms originate

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from their intrinsic properties, such as adsorbability at the mercury (or amalgam) surface and susceptibility to the potential-induced surface denaturation (reviewed in refs 8 and 18). Thus, voltammetric determination of ssb with mercury-based electrodes is inherently label-free. Previously we proposed an electrochemical sensor for the detection of DNA damaging (strand-breaking) agents¹⁰ and applied it in studies of DNA cleavage by enzymes,¹⁹ reactive oxygen species,¹¹ or intermediates of chromium(VI) electroreduction.¹² In combination with DNA repair nucleases, the voltammetric method was also applied to detect damage to DNA bases.¹³

In this work, we use an analogous approach to monitor an inverse process, i.e., the ability of DNA ligases to seal ssb present in plasmid DNA. We demonstrate the applicability of ac voltammetry with the mercury electrode to detect plasmid DNA ligation, to differentiate between ligatable and unligatable breaks, and its usefulness in studies of the DNA ligase activity of recombinant fragments of *Escherichia coli* DNA ligase (LigA).²⁰

EXPERIMENTAL SECTION

Materials. Supercoiled DNA of the pL1 plasmid (derived from the single-*BbvC* I site plasmid described in ref 21 kindly donated by Dr. Darren Gowers, University of Portsmouth, UK) was isolated as described.¹⁰ The open (nicked) circular (ocDNA) form of pL1 was prepared by treatment with the DNA nicking mutant of *BbvC* I restrictase, *Nb.BbvC* I²² (New England BioLabs), followed by ethanol precipitation. T4 DNA ligase (*T4 lig*) was purchased from Promega, T4 endonuclease V (*endoV*) was from Epicenter, and ATP and NAD⁺ were from Sigma. Overexpression and purification of the *E. coli* ligase A recombinant proteins (full length LigA and its fragments ΔBRCT, BRCT, and C-term) was performed as described previously for T4 DNA ligase²³ and *E. coli* LigA.²⁰ Other chemicals were of analytical reagent grade.

UV Irradiation and endoV Cleavage. Supercoiled pL1 DNA was irradiated with a 254-nm UV lamp (Desaga) from a distance of 4 cm in clear polypropylene Eppendorf tubes containing 50 μL of 50 μg mL⁻¹ scDNA solution in 100 mM Tris-HCl, pH 7.6. The irradiation time was 30 s, corresponding to a dose of 5.2 J cm⁻². Then the DNA (20 μg mL⁻¹) was incubated with 1 unit of the endoV/mL in 100 mM KCl, 50 mM Tris, 1 mM EDTA, pH 7.1, followed by ethanol precipitation.

DNA Ligation. The nicked-pL1 DNA was incubated with *T4 lig* in 30 mM Tris-HCl (pH 7.8) containing 10 mM MgCl₂ and 1 mM ATP at 16 °C. If not stated otherwise, 0.01 U of *T4 lig*/μg of nicked-pL1 sample was used (0.01 U of *T4 lig* is defined as the amount of enzyme required to catalyze the ligation of greater than 95% of the *Hind*III fragments of 1 μg of λ-DNA at 16 °C in 20 min); the incubation time was 30 min. Incubation of the DNA with the recombinant protein (LigA or its fragments, always 25 pmol

of the protein/50 pmol of pL1) was performed in 50 mM Tris (pH 7.8), containing 10 mM MgCl₂ and 25 μM NAD⁺ at 25 °C for 30 min. For more details see ref 20.

Gel Electrophoresis. Plasmid DNA samples were separated in 1% agarose gel containing 0.3 μg mL⁻¹ of ethidium bromide. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) of the recombinant proteins was performed as follows: 0.5–1 μg of the protein samples was mixed with loading buffer containing 2% SDS, heated at 95 °C for 5 min, and loaded onto a 5% stacking/12.5% separation gel containing 0.1% SDS in 25 mM Tris/192 mM glycine buffer, pH 8.3. After separation, the gel was stained with Coomassie Brilliant Blue.

Electrochemical Analysis. Alternating current voltammetric measurements were performed with an Autolab analyzer (Eco Chemie) in connection with VA-Stand 663 (Metrohm). The three-electrode system included hanging mercury drop electrode (HMDE) as working, Ag/AgCl/3 M KCl as reference, and platinum wire as auxiliary electrode. The measurements were done in an ex situ (adsorptive transfer stripping) mode.^{10,18,19} DNA was adsorbed at the electrode from 5-μL aliquots at open current circuit for an accumulation time $t_A = 120$ s, followed by washing with deionized water, and transfer into blank supporting electrolyte (0.3 M NaCl and 0.05 M Na₂HPO₄) in a standard voltammetric cell. Ac voltammetric (acv) measurements at the HMDE were performed with initial potential -0.6 V, frequency 230 Hz, amplitude 10 mV, scan rate 10 mV s⁻¹, and negative scan direction; in-phase component of the ac current was measured. All measurements were performed at room temperature in air.

RESULTS AND DISCUSSION

Ac voltammetry with mercury^{10,11,13,19} or some types of silver amalgam electrodes^{13–15} has been utilized as a sensitive label-free technique for detection of DNA ssb. Double-stranded (ds) DNA molecules possessing the ssb (such as ocDNA, Figure 1A) can undergo extensive potential-induced surface denaturation, resulting in formation of regions of single-stranded DNA (ssDNA) around the ssb.^{8,10,18} These ssDNA regions produce a specific tensammetric signal, peak 3 (Figure 1B), which has been attributed to segmental desorption or reorientation of polynucleotide chains adsorbed at the electrode surface via nucleobases (reviewed in refs 8, 9, and 18). Double-stranded DNA lacking free ends, such as covalently closed circular DNA (cccDNA, Figure 1A) does not yield this signal because unwinding of the cccDNA is restricted for topological reasons. The qualitative difference between ac voltammetric behavior of cccDNA and that of ocDNA renders the electrochemical technique applicable for monitoring of the breaking or joining of the DNA sugar phosphate backbone.

Figure 1 illustrates changes in the electrochemical behavior of the pL1 plasmid DNA after introduction of an ssb by the *Nb.BbvCI* endonuclease^{22,24} and upon sealing the break with the T4 DNA ligase (*T4 lig*). The pL1 plasmid possesses a single *BbvC* I site,²⁴ and thus, its treatment with the mutant enzyme results in formation of a single nick per plasmid monomer. Enzymatically untreated pL1 produced an ac voltammogram showing negligible peak 3 (the very small peak 3 was most likely due to a trace

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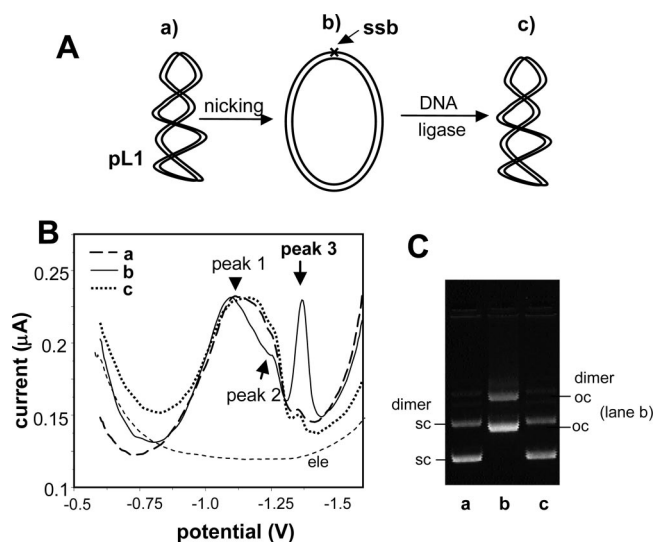


Figure 1. Ac voltammetry used to measure ligation of breaks in plasmid DNA. (A) Scheme of a single nick creation in the pL1 plasmid DNA with the *Nb.BbvC* I endonuclease and sealing the break with a DNA ligase: (a) covalently closed circular (supercoiled) pL1 DNA; (b) open (nicked) circular DNA; (c) cccDNA after ligation. The cccDNA molecules are schematically depicted as supercoiled in both (a) and (c), which is valid under conditions of the agarose gel electrophoresis run in the presence of ethidium bromide (panel C) [It should be noted that during the gel electrophoresis saturation of the cccDNA with the intercalating dye ethidium bromide causes it to migrate as positively supercoiled in both lanes a and c. In the absence of ethidium bromide, the DNA was negatively supercoiled prior to *Nb.BbvC* I treatment (lane a) but relaxed after ligation of the nick (lane c)]. (B) Ac voltammograms: a, pL1 cccDNA before *Nb.BbvC* I cleavage; b, pL1 ocDNA; and c, the same DNA after treatment with T4 DNA ligase (12.5 mU/μg of DNA, 30 min, in the presence of 10 mM Mg²⁺ and 1 mM ATP). (C) Ethidium bromide-stained agarose gel electrophoresis of the same DNA samples (marking of lanes on the gel corresponds to marking of curves in panel B). For other details, see Experimental Section.

amount of nicked DNA in the cccDNA preparation, Figure 1B, curve a). The same DNA migrated during agarose gel electrophoresis as supercoiled (sc) DNA, producing two major bands (Figure 1C, lane a), which were confirmed to be monomeric and dimeric forms of the same plasmid (data not shown). Conversion of the cccDNA into ocDNA resulted in a change of the plasmid mobility in the agarose gel (due to relaxation of the superhelix turns, Figure 1C, lane b) and in appearance of a well-developed peak 3 on the ac voltammogram (Figure 1B, curve b). After the ocDNA was treated with the T4 DNA ligase, the peak 3 was depressed (Figure 1B, curve c) and mobility of the pL1 DNA in the agarose gel was increased again (Figure 1C, lane c), indicating that the ssb was repaired.

Effects of Enzyme Concentration and Time of Ligation. We prepared reaction mixtures containing 30 μg mL⁻¹ pL1 ocDNA, both cofactors (1 mM ATP and 10 mM Mg²⁺), and various concentrations of T4 lig. Ac voltammograms of the DNA were recorded after a 30-min incubation at 16 °C. Figure 2 shows peak 3 intensity steeply decreasing with increasing amounts of the enzyme added to the reaction mixture between 0 and 3 mU of the ligase per microgram of DNA. For 12.5 mU μg⁻¹, the peak height was reduced to ~10% of the intensity observed for ligase-untreated ocDNA and did not change with addition of further

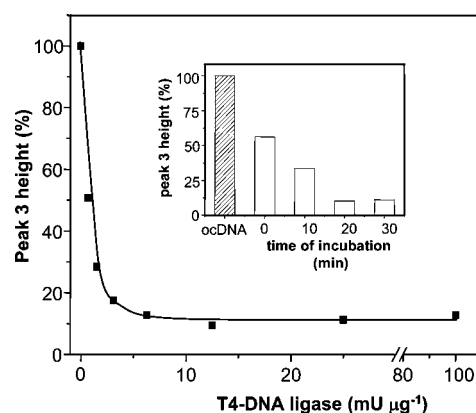


Figure 2. Analysis of peak 3 intensity. This provides a measure of the ligation activity of T4 DNA ligase. Effects of the amount of T4 lig (per microgram of DNA) on the peak 3 intensity were measured after 30-min incubation of pL1 ocDNA with the enzyme in the presence of 10 mM Mg²⁺ and 1 mM ATP. Inset: dependence of the peak 3 height on the time of pL1 ocDNA incubation with T4 lig (12.5 mU μg⁻¹). Peak 3 height corresponding to ligase-untreated ocDNA was taken as 100%.

amounts of the enzyme. Such behavior indicated completion of the ligation of repairable ssb (see below) in the pL1 DNA.

The effects of the time of DNA ligation on the peak 3 intensity was measured during reactions with 12.5 mU of T4 lig/μg of pL1 ocDNA (Figure 2 inset). The reaction mixture was prepared, and immediately after addition of the enzyme, an aliquot was withdrawn from which the DNA was adsorbed at the electrode surface. Measurement performed under such conditions revealed an ~45% decrease of the peak 3 height, compared to the signal intensity corresponding to untreated ocDNA (shaded column in Figure 2, inset). Thus, a considerable portion of the ssb was sealed during the short time interval needed for the sample mixing and during the 2-min period of DNA accumulation at the electrode. After 10 min of the enzymatic treatment, the relative peak 3 intensity decreased to ~35%, and after 20 min, the reaction was apparently completed (with ~10% of peak 3 remaining).

Ligatable and Unligatable Single-Strand Breaks. The above experiments revealed that a certain portion of the ssb (indicated by peak 3) remained unsealed even when high excesses of the enzyme and long reaction times were applied. DNA ligases can only join DNA strands with the 3'-OH, 5'-phospho configuration (Figure 3A,a) and base-pair complementarity at the nick junction is usually required.^{25,26} Hence, while DNA ends created by endonucleases such as DNase I,¹⁹ common restriction endonucleases, and the DNA nicking *Nb.BbvC* I enzyme²² are substrates for DNA ligases, interruptions of the DNA sugar-phosphate backbone resulting from nonspecific DNA damage during the sample handling may be unligatable. Furthermore, it is now clear that different types of DNA ends, such as those indicated in Figure 3A, may form in genomic DNA due to certain types of damage or due to the disruption of some types of DNA processing events.^{27,28} Thus, it is important to establish new methodologies that allow analysis of the repair of different types of ends.

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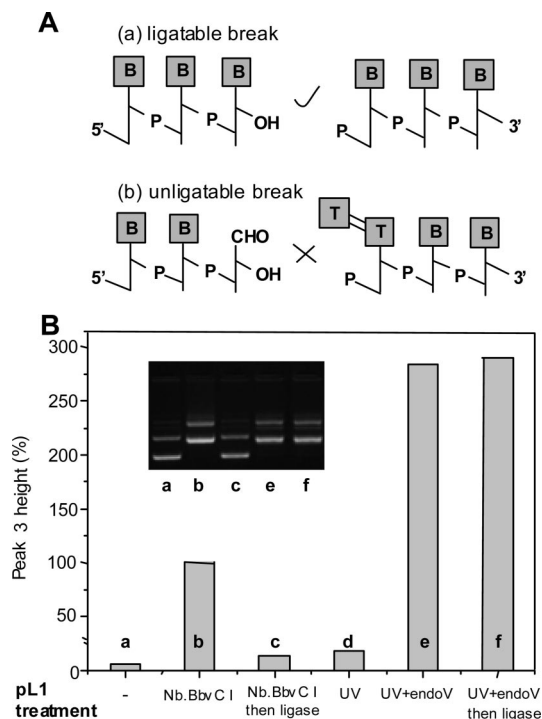


Figure 3. Use of ac voltammograms to report on the chemical groups available at DNA ends. (A) Scheme of the configuration of (a) the ligatable ssb created by (for example) the *Nb.BbvCI* enzyme (3'-OH, 5'-phospho; base-pair complementarity at the junction), and (b) an unligatable break created by the T4 endonuclease V (*endoV*) at sites of pyrimidine cyclobutane dimers in UV light-exposed DNA. (B) Schematic representation of peak 3 intensities obtained for (a) pL1 cccDNA prior to any treatment, (b) ocDNA prepared with the *Nb.BbvCI* enzyme, (c) cccDNA resulting from *T4 lig* treatment of the ocDNA, (d) the cccDNA irradiated with UV light, (e) UV-irradiated DNA treated with *endoV*, and (f) as in (e) but after incubation with the *T4 lig*. Peak 3 height corresponding to the single nick-containing ocDNA (sample b) was taken as 100%. Inset, agarose gel electrophoresis of the same DNA samples (marking of lanes corresponds to marking of samples in the column graph).

As mentioned above, the supercoiled pL1 preparation contained (before *Nb.BbvCI* digestion) a certain amount of DNA possessing free ends, which gave rise to the small peak 3 (Figures 1B and 3B). After *T4 lig* treatment of the *Nb.BbvCI*-nicked pL1, the height of peak 3 was about twice that of the input scDNA preparation (Figure 3B). It is likely that the increase in the residual signal intensity was, at least partly, due to some DNA damage having occurred during the manipulation of the samples. DNA ends arising from this accidental DNA damage (together with those present in the input pL1 cccDNA preparation) may include lesions that cannot be repaired by DNA ligase.

An example of unligatable ssb is the product of UV-irradiated DNA cleavage by T4 endonuclease V (*endoV*), an enzyme involved in repair of pyrimidine dimers (common products of DNA damage by UV light).^{13,29} The enzyme, possessing N-glycosylase and AP lyase activities, cleaves the N-glycosidic bond of the 5'-pyrimidine of the dimer, followed by interruption of the phosphodiester bond 3' to the resulting abasic site (the final product is depicted in Figure 3A,b).³⁰ We created this type of lesion in the pL1 DNA via exposure to UV light followed by treatment with the *endoV*.¹³

Figure 3B shows that UV irradiation alone caused a small increase of the peak 3, while the subsequent enzymatic digestion gave rise to a large peak 3, the intensity of which was almost three times as high as the signal yielded by the *Nb.BbvCI*-treated pL1. The higher peak 3 intensity indicated the presence of multiple breaks per pL1 molecule¹⁹ induced by the combination of (UV + *endoV*) treatment under the given conditions, in contrast to the single nick-containing ocDNA produced by the *Nb.BbvCI* enzyme. Notably, upon treatment with the combination of UV + *endoV*, agarose gel electrophoresis showed that the integrity of the circular DNA molecules in solution was similar to that produced by the *Nb.BbvCI* enzyme (see inset in Figure 3B). This observation confirms that the electrochemical approach provides a more sensitive analysis for the presence of ssb in DNA. Incubation of the (UV + *endoV*)-treated DNA with *T4 lig* did not result in any decrease of the peak 3 intensity, suggesting that no significant ssb sealing took place (Figure 3B). Results of the gel electrophoresis supported these conclusions (Figure 3B, inset).

Analysis of DNA Ligase Activity in Recombinant *E. coli* LigA Fragments. High-resolution structures that have recently been obtained for a range of enzymes that ligate nucleic acids provide significant insights into the molecular mechanisms by which these enzymes work (reviewed in^{2,31}). All NAD⁺-dependent DNA ligases are likely to consist of a modular architecture, as observed for LigA enzymes from *E. coli* and *Thermus filiformis*.^{2,32} Within the four distinct domains of LigA are several well-characterized protein folds, including a zinc finger, an oligomer-binding h-barrel, a helix-hairpin-helix motif, and a specialized version of the BRCT domain²⁰ (see Figure 4A). It is now well established that significant DNA binding activity resides within the C-terminal domain of the enzyme but that this region cannot covalently join breaks in DNA. To assess whether the changes in voltammetric signal were really due to nick-joining as opposed to simple binding and protection of the DNA breaks, we made use of a variety of recombinant fragments of *E. coli* LigA that we have previously characterized biochemically.²⁰

We prepared reaction mixtures containing *Nb.BbvCI*-treated pL1 ocDNA, the relevant cofactors (for the LigA, NAD⁺ was used instead of ATP), and the purified LigA constructs.²⁰ After 30-min incubation at 25 °C, the DNA was analyzed as above. The peak 3 was strongly depressed (reaching the same intensity as observed after ocDNA treatment with *T4 lig*, Figure 4D) for full-length LigA and for the ΔBRCT fragment. On the contrary, no change in the peak 3 intensity was detected for the C-terminal fragment and the isolated BRCT domain, indicating that no nick-joining had occurred. Control agarose gel electrophoresis experiments (Figure 4C) confirmed the electrochemical data. These observations are in agreement with the previous study, which revealed that the full-length LigA and ΔBRCT fragment have DNA ligase activity, but the C-terminal and BRCT fragments are unable to join DNA breaks.²⁰ Thus, the electrochemical approach developed here is not influenced by simple binding of the proteins to DNA ends, meaning that it is a robust technique for analysis of processes that join DNA nicks.

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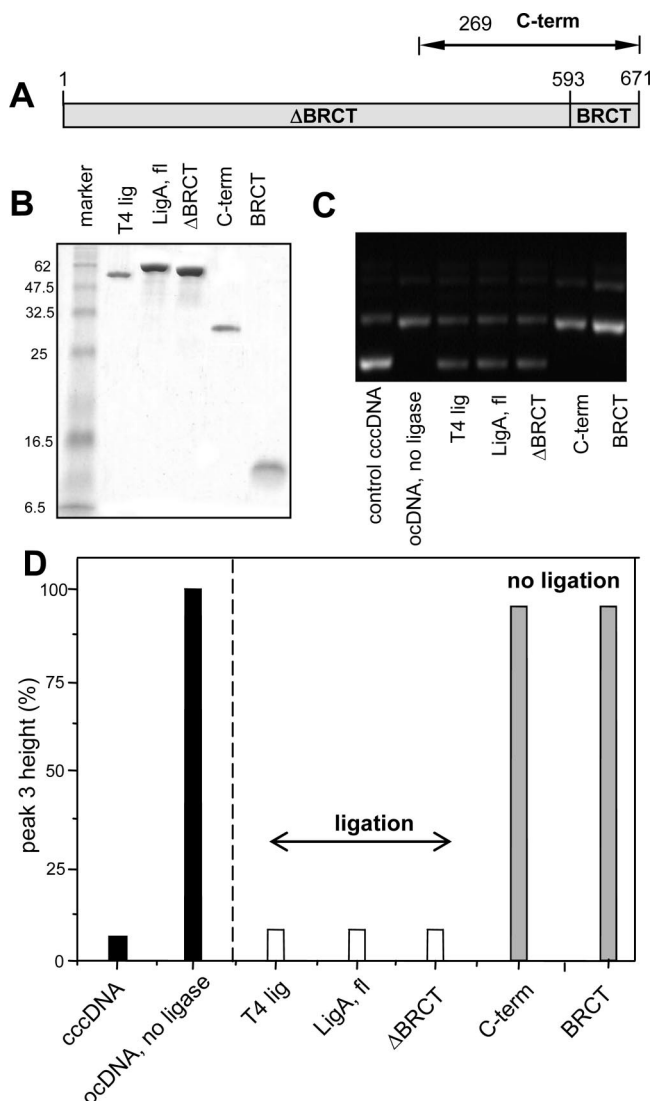


Figure 4. Application of the electrochemical technique to probing of DNA ligase activity in fragments of *E. coli* DNA ligase, LigA. (A) Simplified map of the full length (fl) LigA molecule showing the fragments Δ BRCT, C-term, and BRCT. (B) SDS-PAGE of proteins tested. The DNA backbone-joining activity of the LigA fragments was monitored by agarose gel electrophoresis (C) or ac voltammetry. (D) Single nick-containing ocDNA was incubated with individual LigA constructs (25 pmol of the given protein/50 pmol of nicked-pL1) in the presence of relevant cofactors (10 mM Mg^{2+} and 25 μ M NAD^+) at 25 $^{\circ}C$ for 30 min (for *T4 lig*, the same conditions as in Figure 1 were used). Peak 3 height corresponding to ligase-untreated ocDNA was taken as 100%.

CONCLUSIONS

In this paper, we extended applications of the label-free electrochemical sensing of DNA strand breaks through detection of DNA ligation. We show that sealing of an ssb within plasmid ocDNA can easily be detected via measurement of the DNA ac voltammetric peak 3. Results of the electrochemical analysis were in excellent agreement with agarose gel electrophoretic monitoring of topological changes within the plasmid DNA upon introduction or ligation of the ssb. Compared to the electrophoretic method, the electrochemical technique is faster, label-free and reagent-less, and easily amenable to parallelization, if the HMDE is replaced by a solid amalgam electrode.^{13–15} Moreover, the electrochemical approach detects a wider dynamic range of ssb since the peak 3 intensity can respond to more ssb per circular plasmid molecule¹⁹ (which is not possible using native agarose electrophoresis unless the DNA circular structure is disrupted). We demonstrate utilization of the electrochemical method in studies of the DNA ligation activity in truncated variants of an *E. coli* DNA ligase LigA²⁰ and in distinguishing between ligatable and unligatable strand breaks in plasmid DNA. It is now clear that the studies of processes influencing the production and resolution of DNA breaks should make use of a variety of techniques that facilitate studies of different facets of the reactions of ligases on DNA. Since the assay presented here could have a wide applicability in probing DNA backbone breaking/joining enzymatic activities as well as in analysis of the diverse products of DNA damage, it provides another useful addition for the toolbox that can be used to analyze these complex processes.

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