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Analysis of ligation and DNA binding by *Escherichia coli* DNA ligase (LigA)

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Abstract

NAD^+-dependent DNA ligases are essential enzymes in bacteria, with the most widely studied of this class of enzymes being LigA from *Escherichia coli*. NAD^+-dependent DNA ligases comprise several discrete structural domains, including a BRCT domain at the C-terminus that is highly-conserved in this group of proteins. The over-expression and purification of various fragments of *E. coli* LigA allowed the investigation of the different domains in DNA-binding and ligation by this enzyme. Compared to the full-length protein, the deletion of the BRCT domain from LigA reduced in vitro ligation activity by 3-fold and also reduced DNA binding. Using an *E. coli* strain harbouring a temperature-sensitive mutation of *ligA*, the over-expression of protein with its BRCT domain deleted enabled growth at the non-permissive temperature. In gel-mobility shift experiments, the isolated BRCT domain bound DNA in a stable manner and to a wider range of DNA molecules compared to full LigA. Thus, the BRCT domain of *E. coli* LigA can bind DNA, but it is not essential for DNA nick-joining activity in vitro or in vivo.

Keywords: BRCT domain; DNA ligase; Ligation; NAD^+; Protein–DNA binding

1. Introduction

DNA ligases join gaps in the phosphodiester backbone of DNA and are essential enzymes in all cells [1–3]. One of the first DNA ligases to be purified and analysed biochemically was that from *Escherichia coli*, which is encoded by *ligA* and consists of 671 amino acids (molecular weight of 74 kDa) [1]. During ligation by LigA, a covalent enzyme–adenylate intermediate is formed, with the adenylate group (AMP) being obtained from NAD^+. Thus, these bacterial enzymes are termed NAD^+-dependent; note that we refer to them as NAD^+-ligases for convenience. As with all DNA ligases, the AMP is linked to a conserved lysine in the catalytic motif of the enzyme [1–4]. Currently, functional NAD^+-ligases have not been detected in humans, leading to speculation that they could be useful targets for broad-spectrum antibiotics [5–12].

Open reading frames predicted to encode NAD^+-ligases are present in the genome of every bacterial species that has been sequenced so far [4,13]. These predicted proteins are of fairly uniform size and have extensive amino acid sequence homology: a basic BLAST alignment to *E. coli ligA* detects typical amino acid sequence identity of 35–50% across all sequences. High-resolution structures have been obtained for the N-terminal adenylation domain of NAD^+-ligase from *Bacillus stearothermophilus* [5] and *Enterococcus faecalis* [14] and for the full-length NAD^+-ligase from *Thermus filiformis* [6]. The good agreement

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between these structures and the high degree of homology across the full length of their sequences suggest that all NAD^+-ligases are likely to consist of a modular architecture, as observed for the *T. filiformis* enzyme [3, 6]. Within the four distinct domains of LigA are several well-characterised protein folds, including a zinc-finger (Zn), an oligomer-binding (OB) β-barrel, a helix–hairpin–helix motif (HhH) and a BRCT domain (Fig. 1A). A model for the ligation reaction of NAD^+-ligases was proposed from the X-ray crystallography structure of the *T. filiformis* enzyme that suggests two regions of the enzyme interact independently with DNA [3,6].

Very recently, crystallographic studies of human DNA ligase I — an ATP-dependent enzyme — demonstrated that the enzyme encircled its DNA substrate and redirected the path of the double helix to expose the nick termini for the strand-joining reaction [15]. Structures of several other partial or complete ATP-dependent DNA ligases have been solved at high resolution [16–18]. Conservation between the active sites of these proteins suggests that all are likely to follow similar mechanisms for joining of DNA ends. Indeed, it was proposed that the domains of NAD^+-ligases may also encircle DNA substrates in a manner analogous to human DNA ligase I [15]. Currently, however, the molecular details of DNA binding to NAD^+-ligases have not been determined experimentally.

BRCT domains take their name from their original identification at the C-terminus of the protein encoded by the breast cancer susceptibility gene *BRCA1* [19–22]. Homologous sequences have been identified in a range of proteins involved in DNA repair and cell-cycle checkpoint control. BRCT domains are usually 80–100 amino acids in length and are characterised by the hydrophobic nature of their sequence rather than a high degree of amino acid identity. High-resolution structures have been obtained for several BRCT domains [18,23–26]. These studies confirm that BRCT domains have a similar core structure and support suggestions that these motifs are autonomous folding regions of polypeptides [21,22]. BRCT domains promote interactions between proteins

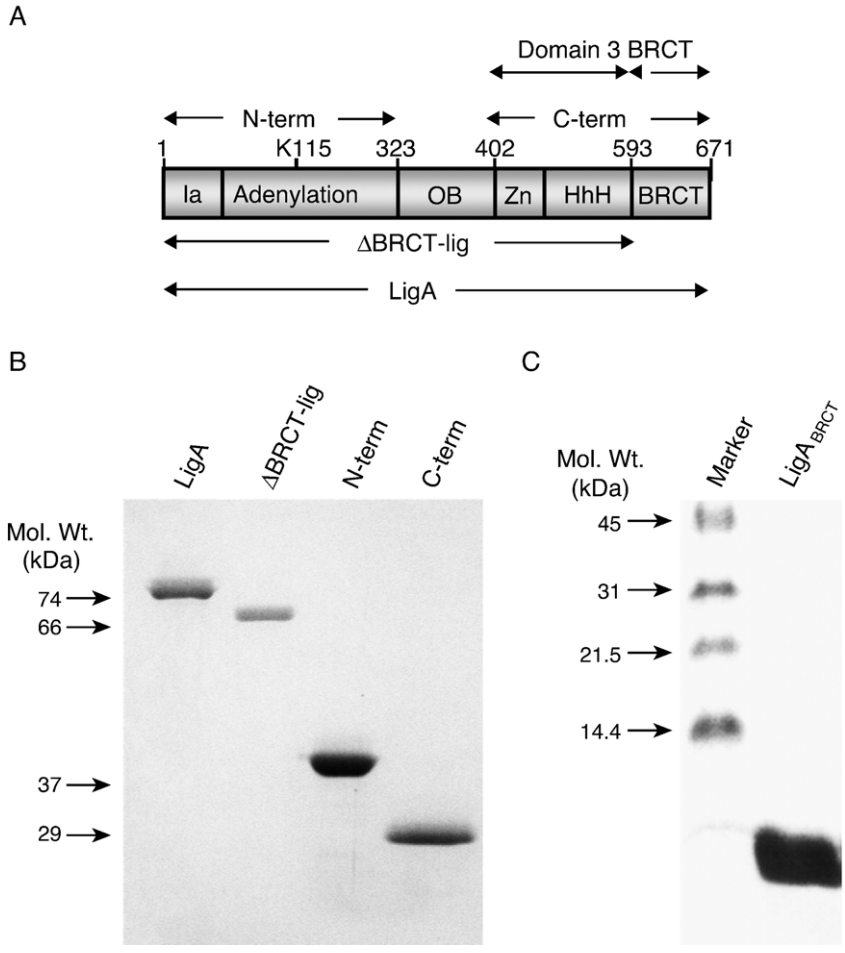


Fig. 1. Predicted domain structure of *E. coli* NAD^+-dependent DNA ligase (LigA). For (B) and (C), arrows show the sizes of marker proteins analysed on the same gel. (A) Schematic diagram illustrating the various fragments of *E. coli* LigA used during this study. This naming scheme follows that identified from the crystallographic structure of *T. filiformis* LigA and is used throughout the text. (B) SDS-PAGE analysis of various LigA fragments used during this study. Purified proteins were electrophoresed on a 10% SDS-polyacrylamide gel. (C) Analysis of purified $BRCT_{LigA}$ on an 18% SDS-polyacrylamide gel. The marker is the low range molecular weight marker from Bio-Rad.

and, in some cases, bind to phosphorylated motifs in polypeptides (reviewed in [21,22]). Other studies have shown that BRCT domains can bind to DNA, including those within the human proteins TopBP1, XRCC1 and BRCA1 [27,28].

Since BRCT domains are not present in all ATP-dependent DNA ligases, it is apparent that this motif is not required for the ligation activity of all ligases [4]. However, this region is highly conserved within the majority of NAD^+-ligases [4,29], suggesting that it performs a useful and necessary function. In the high-resolution structure of the NAD^+-ligase from *T. filiformis*, the structure of the BRCT domain was difficult to resolve, implying that it was quite mobile, although its core structure was similar to other BRCT domains [3,6].

The BRCT domains of NAD^+-ligases are one of the least well conserved members of this family of polypeptide domains [19,20,29,30], implying that it may have different functions compared to other members of this family. To elucidate further details of the role of the BRCT domain in DNA nick-joining by NAD^+-ligases, we have analysed the role of this domain in the macromolecular interactions of *E. coli* LigA. We present evidence that the BRCT domain from *E. coli* LigA promotes the formation of a stable complex between LigA and DNA, but it is not essential for DNA nick-joining activity in vitro or in vivo.

2. Materials and methods

2.1. Cloning of DNA ligase fragments and mutant

Various regions of *E. coli* NAD^+-ligase (LigA) (Fig. 1A) were cloned from sequences amplified by PCR as follows:

Full-length ligase (LigA, 671 amino acids) was amplified using 5′ primer (5′-CAT ATG GAA TCA ATC GAA CAA C-3′) and 3′ primer (5′-GGA TCC TCA GCT ACC CAG CAA ACG CA-3′).

Ligase lacking the BRCT domain (ΔBRCT-lig, amino acids 1–592 of LigA) was amplified using 5′ primer (5′-CAT ATG GAA TCA ATC GAA CAA C-3′) and 3′ primer (5′-GGA TCC TTA TTC CGC GTT GAT AAC GAT CG-3′).

The N-terminal domain (N-term, amino acids 1–323 of LigA) was amplified using 5′ primer (5′-CAT ATG GAA TCA ATC GAA CAA C-3′) and 3′ primer (5′-GGA TCC TAA AAG GTC ATC TGC TCC TGC G-3′).

The C-terminal domain (C-term, amino acids 402–671 of LigA) was amplified using 5′ primer (5′-CAT ATG GTT GTA TTC CCG ACG CAT TG-3′) and 3′ primer (5′-GGA TCC TCA GCT ACC CAG CAA ACG CA-3′).

Domain 3 (amino acids 402–592 of LigA) was amplified using 5′ primer (5′-CAT ATG GTT GTA TTC CCG ACG CAT TG-3′) and 3′ primer (5′-GGA TCC TTA TTC CGC GTT GAT AAC GAT CG-3′).

The region corresponding to the BRCT domain ($BRCT_{LigA}$, amino acids 593–671 of LigA) was amplified using 5′ primer (5′-CAT ATG GAG ATT GAC AGC CCG TTT GC-3′) and 3′ primer (5′-GGA TCC TCA GCT ACC CAG CAA ACG CA-3′).

For each PCR amplification, the 5′ primers contained an *Nde*I site, and the 3′ primers contained a *Bam*HI site. PCR products were cloned using the Zero Blunt TOPO® Cloning kit (Invitrogen). All DNAs cloned from PCR products were sequenced to confirm that no mutations were introduced during PCR. Fragments were excised from the TOPO vectors using the *Nde*I and *Bam*HI sites and cloned into pET16b (Novagen). Proteins over-expressed from this vector carry an extra 21 amino acids (2.5 kDa) at the N-terminus of the protein, due to the addition of a 10-His tag and a Factor Xa cleavage site. To allow the over-expression of proteins in *E. coli* GR501, all fragments of LigA were excised from pET-16b vectors using the *Nco*I and *Bam*HI sites and cloned into p*Trc99*A (Amersham Pharmacia).

A mutation of the active site lysine to alanine (K115A) was created using the QuikChange Mutagenesis kit (Stratagene). Plasmid pET-16b harbouring full LigA was mutated by amplification using 5′ primer (5′-GGT GCT GTG AGC TGG CGC TGG ATG GTC TTG C-3′) and 3′ primer (5′-GCA AGA CCA TCC AGC GCC AGC TCA CAG CAC C-3′). The underlined bases highlight the mutation that alters the wild-type codon for lysine (AAG) to alanine (GCG).

2.2. Protein purification

For protein expression, all *E. coli* cultures were grown at 37 °C in LB containing ampicillin (100 μg/ml) and chloramphenicol (50 μg/ml). The pET16b clones were transformed into *E. coli* BL21 (DE3) pLysS, plated on LB-agar containing antibiotics and grown overnight. Single colonies were inoculated into 5 ml liquid media, grown overnight and diluted 100-fold into 50 ml fresh media. After growth to mid log phase (OD_{600}=0.4–0.6), protein expression was induced by the addition of IPTG to 0.4 mM. Cells were harvested after 4 h further growth, and proteins were purified from the soluble fraction on 2 ml Ni^+–agarose columns (Novagen His-bind resin). The proteins were dialysed into 20 mM Tris–HCl pH 7.5, 50 mM NaCl, 20% glycerol, except for $BRCT_{LigA}$, which was dialysed into 20 mM Tris–HCl pH 7.5, 300 mM NaCl, 20% glycerol. Representative examples of some purified proteins are shown in Fig. 1B and C.

2.3. Preparation of DNA substrate

A double-stranded DNA 40-bp substrate carrying a single strand nick between bases 18 and 19 was used as the standard substrate for gel shift and ligation assays, as described previously [31–33]. This substrate was created in

TBE buffer by annealing an 18-mer (5′-GTA AAA CGA CGG CCA GTG-3′) and a 22-mer (5′-AAT TCG AGC TCG GTA CCC GGG G-3′) to a complementary 40-mer (5′-CCC CGG GTA CCG AGC TCG AAT TCA CTG GCC GTC GTT TTA C-3′). The 18-mer contained a fluorescein molecule attached at the 5′ end, and the 22-mer was phosphorylated at the 5′ end. A 40-bp DNA substrate without a nick was prepared in a similar manner — a fluorescein-labelled 40-mer (5′-GTA AAA CGA CGG CCA GTG AAT TCG AGC TCG GTA CCC GGG G-3′) was annealed to the complementary 40-mer. In some experiments, the fluorescein-labelled 40-mer was used as a single-stranded DNA substrate.

2.4. Gel-mobility shift assay

The nicked 40-bp DNA, the non-nicked 40-bp or single-stranded 40-mer were used as substrates in the gel-mobility shift assays. To ensure that there was no ligation of the nicked 40-bp DNA during incubations with LigA, the 22-mer was not phosphorylated. In a total volume of 20 μl containing ligation buffer (details below), the relevant protein was mixed with 30 pmol of oligonucleotide substrate and left at room temperature for 5 min. Protein amounts added were varied between 15 pmol and 1.5 nmol, as described for specific experiments. After the addition of an equal volume of 50% sucrose, the samples were loaded on to a 10% polyacrylamide gel (20 × 20 cm) and run at 4 °C in 1× TBE for 5 h at 5 V/cm. DNA was visualised on the gel using a Molecular Dynamics Storm phosphorimager.

2.5. Analysis of ligation activity

The nicked 40-bp substrate was also used for in vitro ligation assays. Generally, the fluorescein-labelled substrate (50 pmol) was mixed with enzyme (35 pmol) and ligation buffer (50 mM Tris (pH 7.8), 10 mM $MgCl_2$, 10 mM dithiothreitol, 26 μM NAD^+, 25 μg/ml BSA) in 10 μl reactions, and incubated at 25 °C for up to 1 h. For time-course experiments, the ligation was performed in 50 μl reactions (250 pmol substrate and 35–175 pmol enzyme), and 10 μl volumes were removed at the relevant times. To analyse the extent of ligation, the samples were mixed with an equal volume of formamide loading buffer, heated to 95 °C, and loaded on to a 15% polyacrylamide–urea gel (20 × 20 cm) and run at 15 V/cm for 4 h in 1× TBE. Reaction products on the gel were visualised using a Molecular Dynamics Storm phosphorimager.

To assay for ligation activity in vivo, we used *E. coli* GR501, which has a temperature-sensitive mutation in *ligA* [33,34]. Cells were transformed with p*Trc99*A over-expressing full-length and fragments of LigA and grown overnight at 30 °C on LB-agar containing ampicillin (100 μgl/ml). Single colonies were streaked on to fresh LB-agar plates and grown at 30 °C or 43 °C.

3. Results

3.1. The BRCT domain is not required for the nick-joining activity of E. coli LigA

The regions of *E. coli* LigA required for DNA nick-joining activity were investigated using a variety of fragments (Fig. 1A). The BRCT domains of NAD^+-ligases are particularly well conserved and we were interested to examine whether this region had a role in ligation.

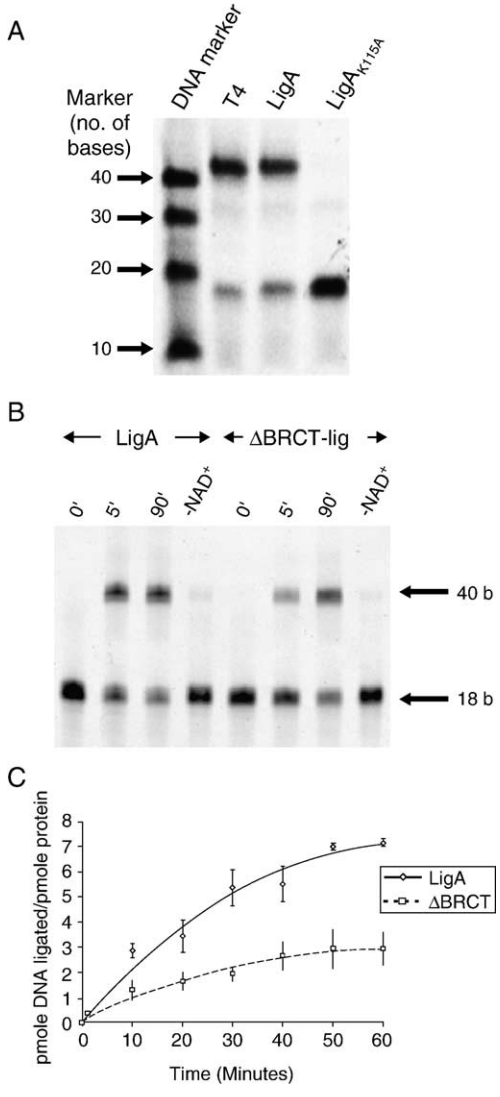


Fig. 2. Effects of mutations on the in vitro ligation activity of *E. coli* LigA. Extents of in vitro ligation at 25 °C of a 40-bp fluorescein-labelled DNA substrate with a single strand nick (see Fig. 5A for details) were analysed on 15% polyacrylamide gels containing 7 M urea. (A) Mutation of amino acid 115 from Lys to Ala abolishes the ligation activity of LigA. (B) Gel electrophoretic time-course assay of the ligation activity of full-length LigA and ΔBRCT-lig. The sample labelled "−NAD^+" contained no NAD^+ and acts as a control to identify the amount of enzyme that is pre-adenylated. (C) Quantitation of detailed time-course reactions as in panel B. Data points are as follows: diamond and full line — LigA; square and dashed line — ΔBRCT-lig. Error bars show standard error across three identical experiments.

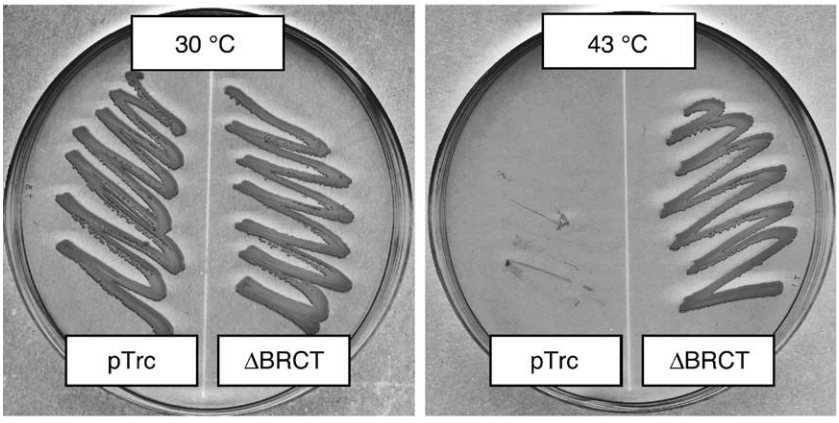
Proteins were over-expressed and purified by affinity chromatography with an in-frame N-terminal His-tag, as has been used in studies of a number of different DNA ligases [7,13,32,33,35,36]. Analysis on SDS-PAGE showed that the His-tagged proteins were >90% pure (see Fig. 1B and C for representative examples of purified proteins). Western blots with a polyclonal antibody to LigA [33] detected no contamination with chromosomally-expressed *E. coli* LigA in the over-expressed proteins (data not shown).

The ability of proteins to seal single strand nicks at 25 °C was assayed using a fluorescein-labelled 40-bp DNA substrate (see Fig. 5A). Full-length LigA had nick-joining activity of approximately 0.1 mol ligation events (mol protein)$^{-1}$ min^{-1} (Fig. 2A). No in vitro DNA ligation activity was detected for LigA$_{K115A}$ (Fig. 2A) or for fragments of LigA (N-term, C-term, Domain 3, BRCT) that did not contain the N-terminal adenylation domain and a significant part of the C-terminal domain on the same polypeptide (data not shown).

In time-course experiments, ΔBRCT-lig had significant in vitro DNA ligase activity, although this was less than full LigA (Fig. 2B). Note that there was a low level of activity of both proteins without the addition of NAD$^+$ to the buffer. This has been observed with several other DNA ligases (e.g. see [8,13,32,33]) and it occurred because the over-expressed enzymes were pre-adenylated, allowing a single turnover of each enzyme molecule. Detailed time-course experiments showed that ΔBRCT-lig was about three times less effective at joining nicks than was full LigA (Fig. 2C). This is in good agreement with recent observations for similar fragments from other NAD$^+$-ligases [29,37]. Additional preliminary experiments confirmed that LigA lacking the BRCT domain (ΔBRCT-lig) could join double-strand breaks in plasmids, though at a much reduced rate compared to full LigA (data not shown).

The activity of ΔBRCT-lig in the nick-joining assay was increased upon the addition of purified BRCT domain to the ligation reaction mixture (data not shown). However, the addition of an equivalent concentration of BSA had a

A



B

Fragment of EcLigA	Growth of *E. coli* GR501	
	30 °C	43 °C
LigA	+	+
ΔBRCT-lig	+	+
N-term	+	-
C-term	+	-
BRCT	+	-
LigA$_{K115A}$	+	-

Fig. 3. Complementation of growth in *E. coli* GR501 by different fragments of LigA. *E. coli* GR501 was transformed with p*Trc99*A over-expressing various fragments of LigA and grown at 30 °C and 43 °C on LB-agar containing ampicillin (100 μgl/ml). (A) The over-expression of ΔBRCT-lig complements the temperature-sensitive mutation and allows *E. coli* GR501 to grow at 43 °C as well as 30 °C. *E. coli* GR501 transformed with p*Trc99*A alone was used as a control for cells containing only temperature-sensitive *ligA*. (B) Complementation experiments were performed for different fragments of LigA. For each temperature, good growth and no growth is indicated by "+" and "–", respectively.

similar effect, suggesting that this effect was not due to trans-complementation. A more likely explanation of the increased DNA ligation in the presence of exogenous BRCT domain was that it was due to macromolecular crowding, as has been observed in other studies of DNA ligases [38].

The essential nature of LigA is illustrated by *E. coli* strain GR501, which contains a temperature-sensitive mutation to LigA that renders the strain non-viable at temperatures of 42 °C or above [33,34]. This strain has been used previously to confirm ligation activity in the *E. coli* of human DNA ligase I [39], bacteriophage T7 [40] and several bacterial DNA ligases [11,32,33]. Control experiments showed that *E. coli* GR501 was non-viable at 43 °C when transformed with p*Trc99*A alone (Fig. 3A). As expected, the over-expression of full-length *E. coli* LigA from p*Trc99*A allowed growth at this temperature [32,33]. The over-expression of ΔBRCT-lig from p*Trc99*A also permitted the growth of *E. coli* GR501 at 43 °C (Fig. 3A). Thus, LigA lacking the BRCT domain can function as a DNA ligase in *E. coli* GR501. All other proteins prepared during this study were unable to allow the growth of *E. coli* GR501 at its non-permissive temperature (Fig. 3B).

These experiments show that the BRCT domain is not required for the ligation activity of *E. coli* LigA in vitro and in vivo, but it does improve the rate of DNA nick-joining in vitro.

3.2. DNA binding properties of the BRCT domain of E. coli LigA

The crystal structure of the *T. filiformis* NAD^+-ligase confirmed that several protein folds that can bind DNA are present in the C-terminal half of the enzyme, such as a Zn^{2+}-finger domain, a HhH motif and a BRCT domain (Fig. 1A) [6]. Interestingly, the preliminary structural analysis of *E. coli* NAD^+-ligase suggested that the region equivalent to the BRCT domain may be important for DNA binding [41]. To establish more precisely the regions of *E. coli* LigA that are important for DNA binding, we used the well-established method of gel-mobility shift assays [42,43].

Gel-mobility shift assays were performed with all fragments of LigA and a fluorescein-labelled 40-bp DNA molecule with a central single strand nick (see Fig. 5A). The binding of the DNA was observed for both full-length LigA (Fig. 4A) and the C-terminal domain (data not shown), in support of previous studies of NAD^+-ligases from *B. stearothermophilus* and *Staphylococcus aureus* [8,31]. By contrast, domain 3 of LigA failed to produce gel-shifts of nicked DNA (Fig. 4A). In these experiments, the buffer used for electrophoresis was 1×TBE, which is a rather stringent buffer for DNA binding proteins, meaning that only quite strong interactions will be detected. Note that the Zn^{2+}-binding domain and HhH domain are present in domain 3, and both of these domains have been predicted or suggested to be involved in DNA binding by NAD^+-ligases [6,15,29,37]. Fig. 4A shows that the Zn^{2+}-binding and HhH domains of *E. coli* LigA do not bind DNA in a stable manner under the conditions of gel-shift mobility experiments used here. Thus, if these regions interact with DNA, they are likely to require other regions of the protein to stabilise the interaction. Since no function was detected for the isolated domain 3, it is possible that the protein used here was mis-folded and did not retain its usual biochemical activity. Additional high-resolution studies of NAD^+-ligases in complex with DNA are required to resolve whether the Zn^{2+}-binding and HhH domains interact with DNA.

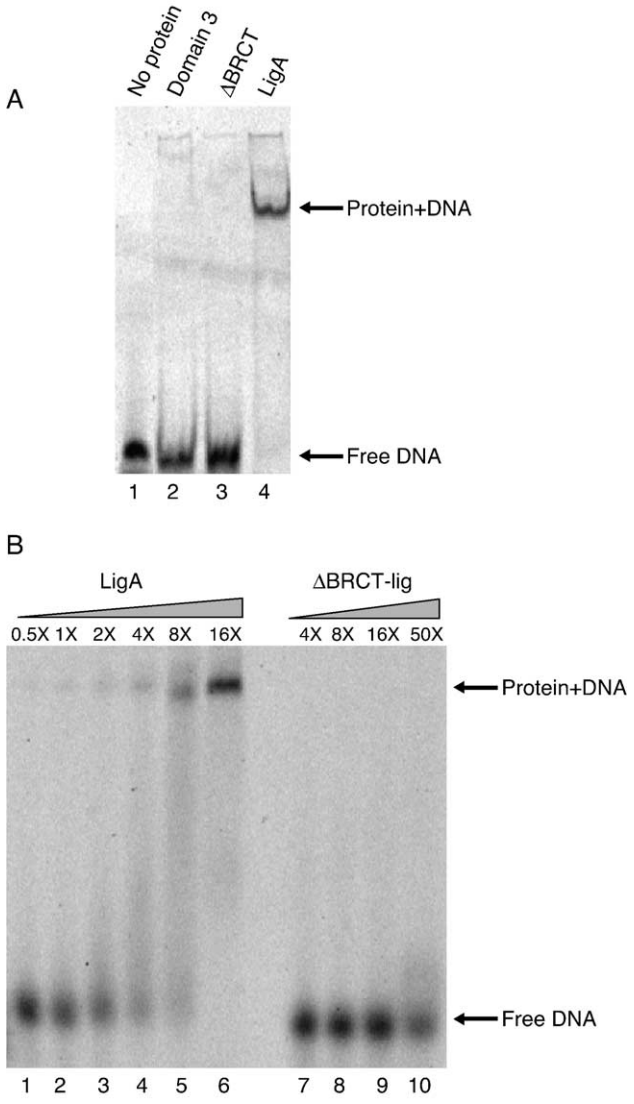


Fig. 4. DNA binding analysis of *E. coli* LigA to "nicked" dsDNA. Gel-mobility shift assays used a 40-bp DNA substrate (30 pmol) with a central single strand nick (see Fig. 5A) and named fragments of *E. coli* LigA. Binding was assayed on 10% polyacrylamide gels run at 4 °C in TBE for 5 h at 100 V. (A) Full LigA binds nicked dsDNA much better than domain 3 or ΔBRCT-lig. Approximately 300 pmol of protein was added as follows: lane 1 — DNA only; lane 2 — domain 3; lane 3 — ΔBRCT-lig; and lane 4 — full-length LigA. (B) A range of protein: DNA concentrations were tested for full LigA and ΔBRCT-lig. The relative molar excess of protein over DNA increases from left to right, as shown for each lane. Note that the deletion of the BRCT domain reduces the binding affinity of *E. coli* LigA to "nicked" dsDNA.

With only a moderate excess of protein:DNA, ΔBRCT-lig did not gel-shift nicked DNA (Fig. 4A). This is in contrast to recent observations that used much higher protein:DNA amounts (>100:1) for similar fragments from other NAD^+-ligases [29,37]. To evaluate the differences between these results, full-length LigA and ΔBRCT-lig were tested for their ability to bind DNA over a range of different protein concentrations (Fig. 4B). For full-length LigA, approximately 50% of the protein was complexed to DNA at a 4:1 ratio of protein:DNA. Using a standard method to calculate the dissociation constants (K_D) of protein–DNA complexes [43], this level of binding is consistent with a K_D for the complex of ~5 μM. By contrast, ΔBRCT-lig showed no tight binding to the DNA even at a 50:1 ratio of protein:DNA, suggesting that the K_D for the complex was greater than 100 μM. The large amounts of protein used in these experiments preclude the accurate calculation of the dissociation constants (due to dissociation of the protein–DNA complex during electrophoresis). However, since the observations with the two proteins were made under the same conditions, they infer that the BRCT domain allows *E. coli* LigA to make a more stable association with DNA.

The above observations could be explained if the BRCT domain functions as either a distinct DNA-binding domain in LigA or if it forms an essential part of the DNA-binding activity associated with the C-terminal fragment. To assess whether the BRCT domain can bind to DNA when isolated from the remainder of LigA, over-expressed $BRCT_{LigA}$ was used in the gel-mobility shift assay with several different DNA substrates (Fig. 5A). The ability of $BRCT_{LigA}$ to produce gel-shifts of DNA was compared to full-length LigA (Fig. 5B). $BRCT_{LigA}$ bound in a stable manner to the "nicked", "double-stranded" and "single-stranded" 40-bp DNA substrates (lanes 2, 4 and 6, Fig. 5B). Note that, under the conditions of these experiments, the "ssDNA" may form intra-strand base-pairing and, thus, may not be completely single-stranded. The 40-bp DNA

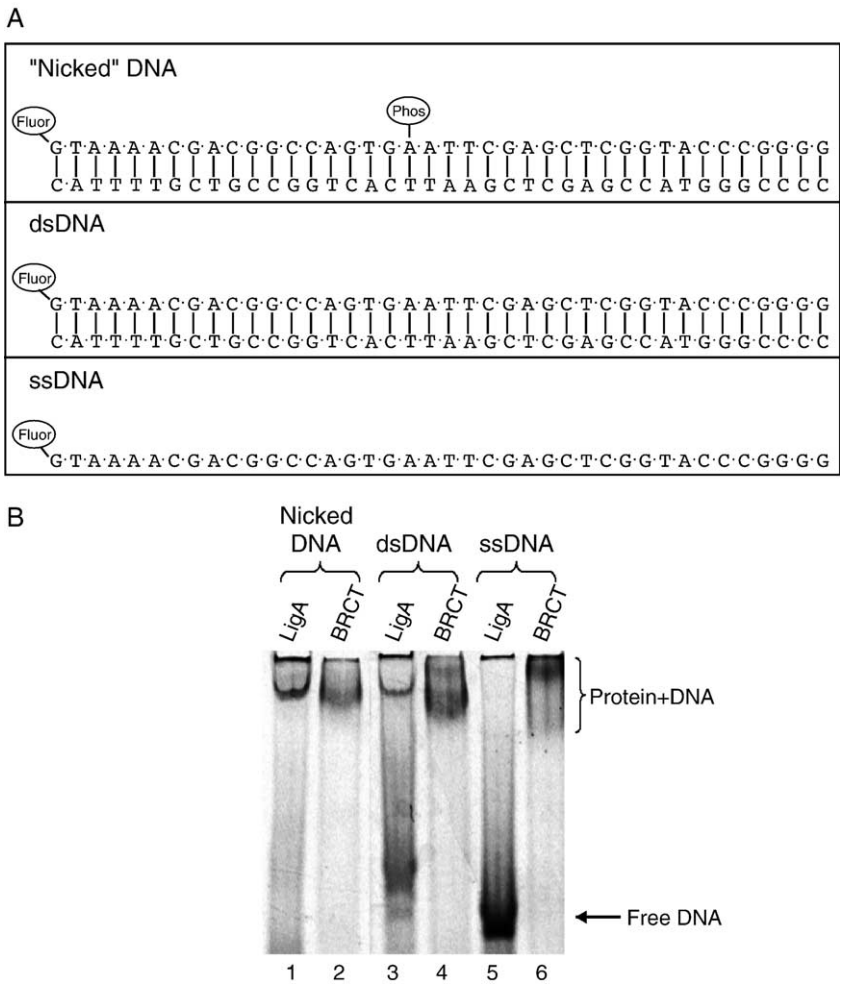


Fig. 5. Different binding affinities of full *E. coli* LigA and the isolated BRCT domain to various DNA substrates. (A) The various substrates used in the DNA binding assays. (B) Gel-mobility shift assay binding of LigA and BRCT to nicked double-stranded DNA, fully double-stranded DNA or single-stranded oligonucleotide. Lanes 1 and 2 — 40-bp DNA substrate with single strand central nick and full-length LigA (lane 1) or BRCT domain (lane 2). Lanes 3 and 4 — 40-bp dsDNA substrate and full-length LigA (lane 3) or BRCT domain (lane 4). Lanes 5 and 6 — ssDNA 40mer and full-length LigA (lane 5) or BRCT domain (lane 6). A 10-fold molar excess of protein over DNA was used in each case.

substrate with a nick was also bound strongly by LigA (lane 1, Fig. 5B). The 40 bp dsDNA was shifted by LigA, but a smear with faster mobility was also observed, suggesting that binding was not tight (lane 3, Fig. 5B). In contrast to the $BRCT_{LigA}$ domain, full-length LigA did not produce a stable gel-shift of the single-stranded 40-bp DNA (lane 5, Fig. 5B). We note that the complexes of the different DNA substrates with $BRCT_{LigA}$ have mobilities that are similar or slower than with full LigA. Such observations suggest that there may be differences in the oligomeric state of the protein–DNA complexes detected here. Thus, additional biophysical studies are required to provide a detailed description of interactions mediated between DNA and $BRCT_{LigA}$.

In summary, gel-shift assays show that isolated $BRCT_{LigA}$ binds to a wider range of substrates compared to full-length LigA. Importantly, full-length LigA binds most strongly to substrates that can be ligated.

4. Discussion

NAD^+-ligase (LigA) is an essential enzyme in *E. coli* and it is likely that homologous proteins are essential for all bacteria [4]. In general, bacterial NAD^+-ligases have sequences that are highly conserved and it is believed that such similarity carries through to their structure [2,3]. A region that is particularly well conserved among NAD^+-ligases is the BRCT domain that occurs at the C-terminus of the polypeptide, suggesting that this domain provides a useful and necessary function for the enzyme. We present in vitro and in vivo experiments showing that this region is not essential for ligation activity of the enzyme although its presence does improve the efficiency of the in vitro nick-joining reaction.

It is firmly established that some BRCT domains are required to make important protein–protein contacts [21,22,44]. Thus, we used *E. coli* LigA fragments in yeast two-hybrid assays, which have allowed the detection of protein–protein interactions mediated by other BRCT domains [45–47]. Bait constructs were made for the C-terminal domain, $BRCT_{LigA}$ and full-length LigA and were screened for interactions against a library representative of all *E. coli* proteins or between specific sequences present within LigA. These experiments did not detect interactions with proteins known to be involved in DNA metabolism (data not shown). When evaluating the significance of the yeast two-hybrid results, it is important to bear in mind that this assay has many potential flaws and it has particular problems in detecting weak interactions. Negative results (i.e. no interactions) may be dependent on the library or gene fragments used as prey or on the particular strains and vectors used in the experiments [48]. Thus, before it can be convincingly concluded that the $BRCT_{LigA}$ domain is not important for protein–protein interactions, different types of analyses should be performed.

4.1. DNA binding by NAD^+-dependent DNA ligases

A high-resolution structure of *T. filiformis* NAD^+-ligase suggested that two independent DNA binding activities reside within these enzymes [3,6]. Two aspects of this study support the predictions from the X-ray crystallographic studies. Firstly, gel-mobility shift experiments suggest that the BRCT domain represents the “non-catalytic” DNA binding activity of the enzyme (or at least a critical part of it). Secondly, since the deletion of the BRCT domain does not completely abolish ligation activity, other parts of LigA must be able to bind to DNA. This latter point supports the proposal for multiple independent DNA binding activities residing within the protein.

The crystal structure of NAD^+-ligase from *T. filiformis* identified several regions that have previously been shown to bind DNA, including an OB-fold, Zn^{2+}-finger and HhH motif [6]. Recent crystallographic studies identified that human DNA ligase I encircles its DNA substrate and suggested that NAD^+-ligases may perform the same feat by interactions between domain Ia and the HhH domain [15]. The data presented here suggests that regions within domain 3 (the Zn^{2+}-finger and HhH motif) do not bind strongly to DNA. Therefore, these regions may require the presence of other regions to stabilise their interaction with DNA. This may be due to domain Ia [15], but the BRCT domain may also be involved in forming stable protein–DNA complexes, as suggested by the current data and that obtained recently using other NAD^+-ligases [29,37]. It is clear that additional high-resolution studies are required to resolve the molecular details of DNA binding to NAD^+-ligases.

In this study, the combination of ligation assays and gel-mobility shift experiments allows the differentiation of two DNA binding sites. Since both of these DNA binding sites are not observed in the gel electrophoresis experiments reported here, the strength of the interactions from the two sites must be different. This is confirmed by estimations that the dissociation constant for the protein–DNA complex is low μM for *E. coli* LigA, but at least 20 times higher for LigA lacking the BRCT domain (Fig. 4B). Data obtained with the isolated BRCT from LigA did not permit the analysis of its Km for nicked DNA. The Km for nicked DNA of full LigA observed in these studies is similar to that obtained for some other NAD^+-ligases [49], but weaker than observed in other studies, especially when a phosphate was present at the nick [50,51]. Currently, we are testing a range of biophysical techniques that should resolve these discrepancies in binding data and allow a more complete thermodynamic analysis of the reaction between LigA-BRCT and DNA.

As discussed above, the detection of DNA binding by the BRCT domain of *E. coli* LigA confirms recent observations using LigA from thermophilic bacteria [29,37]. These studies are also consistent with previous analyses of NAD^+-ligases from *B. stearothermophilus* and *S. aureus*,

which showed that stable DNA binding was provided by a C-terminal fragment that contains the BRCT domain [8,31]. Furthermore, almost a decade ago, before the discovery of BRCA1 and BRCT motifs, the region equivalent to the BRCT domain was predicted to be involved in DNA binding [41]. The preservation of the BRCT domain in NAD^+-ligases from a diverse range of organisms suggests that it provides a useful function to the ligase inside the cell. We suggest that in vivo studies are required to determine if DNA binding is part of this "useful function".

Although we did not examine the specific nature of the BRCT–DNA complex, it is clear that DNA does not need to be completely double-stranded to form a stable complex with the $BRCT_{LigA}$ domain (Fig. 5B). Thus, $BRCT_{LigA}$ is less sensitive to the structure of the DNA molecule, in contrast to full-length LigA, which only produces stable gel-shifts with double-stranded DNA containing "nicks" i.e. a good substrate for nick-joining. The fact that $BRCT_{LigA}$ is less sensitive to DNA structure suggests that the BRCT domain may form an electrostatic interaction with the phosphate backbone of DNA. Notably, the theoretical isoelectric point (p*I*) of the isolated $BRCT_{LigA}$ domain is 4.92, indicating that this fragment will be negatively charged at the pH (7.8–8.3) used in these experiments. Thus, it is unlikely that the complete $BRCT_{LigA}$ domain would have a favourable ionic attraction with the negatively-charged backbone of DNA. To assess the distribution of charge across $BRCT_{LigA}$, we generated a molecular model of the this domain using SWISS-MODEL [52] (data not shown). The model suggests that $BRCT_{LigA}$ contains a central groove containing several positively charged amino acids, which would provide favourable interactions with the DNA backbone. To determine the exact nature of the interaction between DNA and $BRCT_{LigA}$, a high-resolution structure of the complex is required.

It is likely that all BRCT domains have a similar core structure, as confirmed by several high-resolution structures [6,18,23–26]. So, is the DNA binding by BRCT domains from NAD^+-ligases relevant to BRCT domains in general? Previous studies have shown that DNA may be bound by the BRCT domains within the human proteins TopBP1, XRCC1 and BRCA1 [27,28], but it is not clear whether BRCT–DNA complexes have any in vivo significance. Current experimental evidence does not suggest that all BRCT domains are important for DNA binding, but further studies are required to identify how prevalent this function may be within this family.

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