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DOI:

[10.1042/BSR20160003](https://doi.org/10.1042/BSR20160003)

*Document Version*

Publisher's PDF, also known as Version of record

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*Citation for published version (APA):*

Pergolizzi, G., Wagner, G. K., & Bowater, R. P. (2016). Biochemical and structural characterization of DNA ligases from bacteria and archaea. *Bioscience Reports*, 36(5), [e00391]. <https://doi.org/10.1042/BSR20160003>

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# Biochemical and structural characterization of DNA ligases from bacteria and archaea

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## Synopsis

DNA ligases are enzymes that seal breaks in the backbones of DNA, leading to them being essential for the survival of all organisms. DNA ligases have been studied from many different types of cells and organisms and shown to have diverse sizes and sequences, with well conserved specific sequences that are required for enzymatic activity. A significant number of DNA ligases have been isolated or prepared in recombinant forms and, here, we review their biochemical and structural characterization. All DNA ligases contain an essential lysine that transfers an adenylate group from a co-factor to the 5'-phosphate of the DNA end that will ultimately be joined to the 3'-hydroxyl of the neighbouring DNA strand. The essential DNA ligases in bacteria use  $\beta$ -nicotinamide adenine dinucleotide ( $\beta$ -NAD<sup>+</sup>) as their co-factor whereas those that are essential in other cells use adenosine-5'-triphosphate (ATP) as their co-factor. This observation suggests that the essential bacterial enzyme could be targeted by novel antibiotics and the complex molecular structure of  $\beta$ -NAD<sup>+</sup> affords multiple opportunities for chemical modification. Several recent studies have synthesized novel derivatives and their biological activity against a range of DNA ligases has been evaluated as inhibitors for drug discovery and/or non-natural substrates for biochemical applications. Here, we review the recent advances that herald new opportunities to alter the biochemical activities of these important enzymes. The recent development of modified derivatives of nucleotides highlights that the continued combination of structural, biochemical and biophysical techniques will be useful in targeting these essential cellular enzymes.

**Key words:** adenylate, antibacterial compounds, ATP, DNA ligase, enzyme inhibitors,  $\beta$ -NAD<sup>+</sup>.

Cite this article as: Bioscience Reports (2016) 36, e00391, doi:10.1042/BSR20160003

## DNA LIGASES

The family of enzymes classified as nucleotidyl transferases play important roles in the metabolism of nucleic acids due to their ability to transfer phosphorus-containing groups between biomolecules. An important member of this family are the DNA ligases, which are essential to all living cells due to their roles in DNA replication, repair and recombination [1–7]. DNA ligases participate in a range of cellular reactions, with their role particularly well characterized for the joining of Okazaki fragments that are synthesized on the lagging (discontinuous) strand during replication. The biochemical activity of DNA ligases results in the sealing of breaks between 5'-phosphate and 3'-hydroxyl termini within a strand of DNA. DNA ligases have been differentiated as being adenosine-5'-triphosphate (ATP)-dependent (EC 6.5.1.1) or NAD<sup>+</sup>-dependent (EC 6.5.1.2) depending on the co-factor (or

co-substrate) that is used during their reaction [2,3,8]. Typically, more than one type of DNA ligase is found in each organism [1–3,6,9,10]. Here, we review current biochemical and structural knowledge of these enzymes from bacteria and archaea. In addition, we go on to highlight modifications of co-factors that alter the biochemical activities of these important enzymes, with a particular focus on altered forms of  $\beta$ -nicotinamide adenine dinucleotide ( $\beta$ -NAD<sup>+</sup>). Recent developments in identifying such compounds as selective inhibitors of a range of NAD<sup>+</sup>-dependent enzymes is encouraging for their development as potential drug candidates [8,11,12].

DNA ligases from several sources were first isolated by several research groups during the 1960s and their reaction mechanisms were soon elucidated [13]. It was quickly realized that DNA ligases are extremely useful tools in molecular biological manipulations of DNA, which has led to their widespread usage in biotechnological applications [7,14,15]. In the case of both

**Abbreviations:**  $\beta$ -NAD<sup>+</sup>,  $\beta$ -nicotinamide adenine dinucleotide;  $\beta$ -NMN,  $\beta$ -nicotinamide mononucleotide; ADL, ATP-dependent DNA ligase; ATP, adenosine-5'-triphosphate; BRCT, breast cancer carboxy-terminal; HhH, helix-hairpin-helix motif; NDL, NAD<sup>+</sup>-dependent DNA ligase; NHEJ, non-homologous end joining; NTase, nucleotidyltransferase domain; OB site, oligonucleotide binding site.

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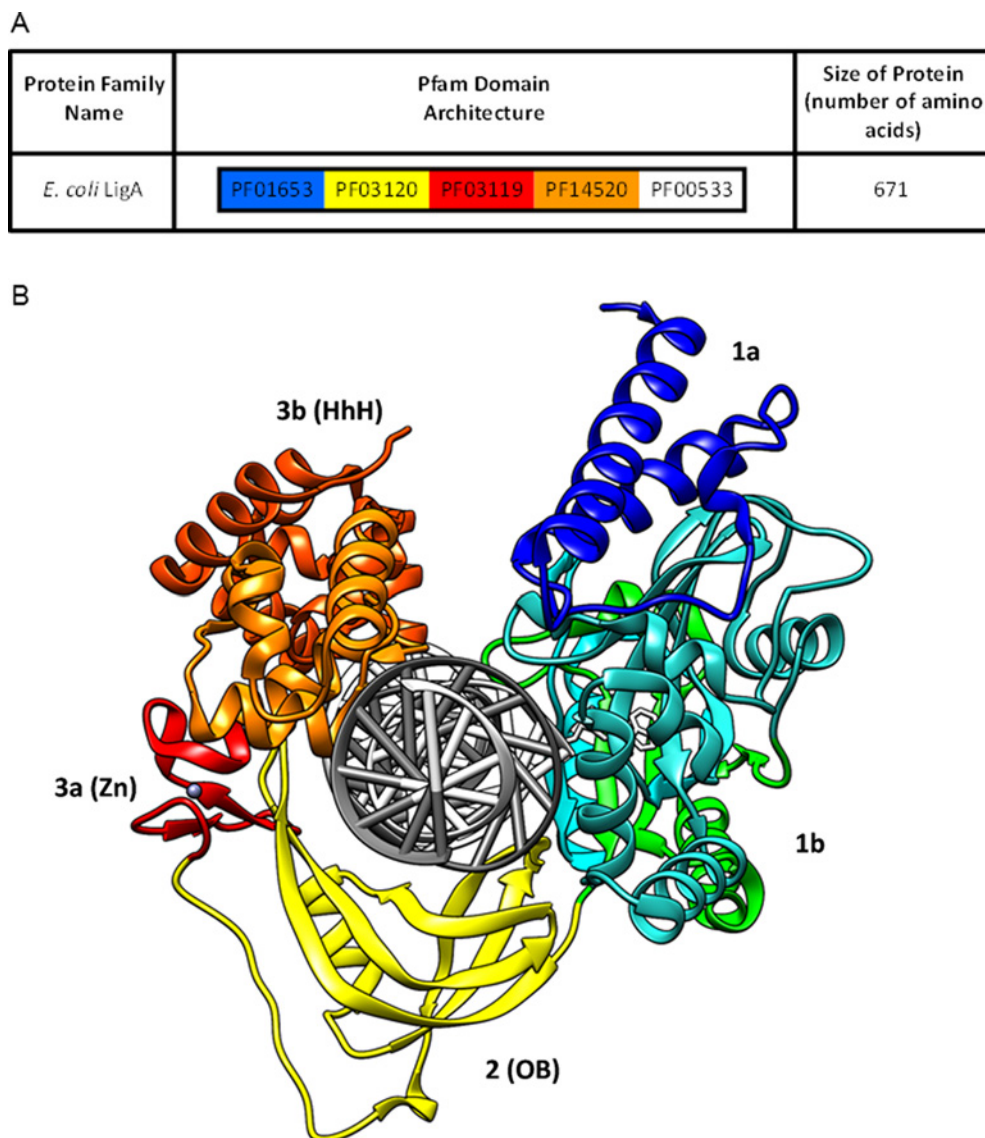
Protein Family Name	Pfam Domain Architecture	Size of Proteins (number of amino acids)
LigB		860
LigC		512
LigD1		764
LigD2		868
LigD2		764
LigD3		419
LigE		499

**Figure 1 Conserved domains within the ATP-dependent DNA ligases from bacteria and archaea**

Schematic diagram indicating the position of conserved domains within the ATP-dependent DNA ligases identified in bacterial and archaeal genomes. Only protein domains matching to Pfam families [16] are referred to and polypeptide regions with no match are not depicted. Domains are not shown to scale. Sizes refer to numbers of amino acids in the largest protein within each domain architecture according to the Pfam database. Note that two related forms of LigD2 are observed within sequence databases. Details are adapted from [10]: Williamson, A., Hjerde, E. and Kahlke, T. (2016) Analysis of the distribution and evolution of the ATP-dependent DNA ligases of bacteria delineates a distinct phylogenetic group 'Lig E'. *Mol. Microbiol.* **99**, 274–290.

ATP-dependent DNA ligases (ADLs) and  $\text{NAD}^+$ -dependent DNA ligases (NDLs), the joining of DNA breaks occurs via a ping-pong mechanism and the biochemical details are well-characterized for many DNA ligases [1–7]. (For NDLs, the reaction mechanism is discussed further below in reference to Figure 5.) Briefly, in the first interaction between the enzyme and co-factor,  $\beta\text{-NAD}^+$  (or ATP) binds to the active site of the enzyme; there, a nucleophilic lysine residue attacks the phosphate of the adenosine in the co-factor with subsequent release of the leaving group,  $\beta$ -nicotinamide mononucleotide ( $\beta\text{-NMN}$ ) for NDLs (or  $\text{PP}_i$  for ADLs). After the positioning of the DNA nick in the proximity of the adenylated-ligase intermediate, the nucleophilic phosphate in the 5' position of the DNA attacks the phosphoramidate bond to form an adenylated-DNA intermediate. Finally, the nucleophilic hydroxyl group in the 3' position of the DNA attacks the new pyrophosphate bond with formation of a phosphodiester bond between the 5' and 3' positions of the DNA and release of AMP. As will be discussed within this review, structural studies using a range of enzymes have demonstrated that large-scale conformational movements take place for ligation reactions performed by NDLs and ADLs.

Although they all perform the same biochemical reaction, DNA ligases have been identified with a range of structures that incorporate a core 'adenylation domain' linked to other well-defined domains (Figures 1 and 2) [3,5–7]. The adenylation domains of ADLs and NDLs perform a similar enzymatic reaction, but they have key differences within their structures, as indicated by their presence as distinct domains within the Pfam database of proteins [16]. To highlight the key similarities and differences across ADLs and NDLs, we will briefly review the structural knowledge that informs biochemical function for the two classes of DNA ligases from bacteria and archaea. Notably, the evolutionary relationship between archaea and eukaryotes has recently been re-evaluated [17–19]. It is now clear that many genes have been transferred 'horizontally' from and into archaeal genomes, and this is almost certainly true for genes that encode DNA ligases. Thus, an evolutionary understanding of these genes may not be straightforward and it may be more useful for certain types of organisms than others. Nonetheless, detailed phylogenetic studies of essential cellular genes such as DNA ligases may offer useful information in relation to the evolution of eukaryotic cells, but this potential will not be considered in detail within this review.



**Figure 2** Conserved domains within the  $\text{NAD}^+$ -dependent DNA ligases from bacteria

(A) Schematic diagram indicating the approximate size and position of domains conserved within the  $\text{NAD}^+$ -dependent DNA ligase (LigA) identified in *E. coli*. Only protein domains matching to Pfam families [16] are included and polypeptide regions with no match are not depicted. Domains are not shown to scale. Note that these Pfam domains are present in the majority of bacterial  $\text{NAD}^+$ -dependent DNA ligases, though some contain additional Pfam domains that are not present in *E. coli* LigA. (B) High-resolution structure of *E. coli* LigA in complex with nicked adenylated DNA from PDB 2OWO [55], visualized by UCSF Chimera. The various domains are indicated by different colours and relate to Pfam domains as follows: subdomain 1a (light blue) and subdomain 1b (green/cyan) is equivalent to PF01653; OB-fold/domain 2 (yellow) is equivalent to PF03120; subdomain 3a (red) is equivalent to PF03119; subdomain 3b (orange) is equivalent to PF14520; the adenylated-DNA molecule is shown in grey. Note that the BRCA1 C-terminus (BRCT) domain (equivalent to PF00533) is not resolved in the crystallographic structure.

## STRUCTURE AND FUNCTIONS OF ATP-DEPENDENT DNA LIGASES

ADLs occur in the genomes of all domains of life [1–3,6,9,10]. Viruses contain a diverse range of ADLs, whereas eukaryotes contain three main types of ADLs, although mitochondrial and

nuclear localization signals direct some versions of the proteins to specific organelles [20]. Biochemical and structural details of DNA ligases from different organisms have been reviewed [1,3–7,9,20]. In this review we focus attention on DNA ligases from bacteria and, to a limited extent, archaea.

ADLs encoded within the genomes of archaea are relatively homologous in size and sequence, whereas those from bacteria

have a wider diversity of total size and domain arrangements [2,10,14]. In the majority of archaea studied to date, the only functional genes for DNA ligases encode proteins that are of the ATP-dependent type, so the proteins they encode participate in all aspects of DNA metabolism. By contrast, where ADLs are found in bacteria, it is always in addition to, rather than instead of, NDLs and it is the  $\text{NAD}^+$ -dependent versions that are essential [21], which we discuss below in more detail. The physiological requirements for so many different bacterial ADLs are not fully clear, though they appear to be not essential and are sometimes only expressed under particular growth conditions [2,22–26]. Furthermore, significant numbers of bacteria do not possess any genes for ADLs [2]. High-resolution structures exist for several ADLs from archaea [27–33] and bacteria [34,35].

In addition to their core adenylation domain, many ADLs include extra domains and motifs that enhance DNA binding, bring additional enzymatic activities or link them to macromolecular complexes that are important in specific aspects of DNA metabolism. With this knowledge it is not surprising that ADLs undergo large-scale conformational movements during the DNA ligation reactions. To assist comparison of the different types of ADLs (Figure 1), we follow the approach of Williamson et al. [10] and use the terminology from the Pfam database of proteins [16]. Distinct domains have a specific Pfam identifier, and those identified in ADLs are often found in other proteins involved in DNA metabolism. Note that PF01896 corresponds to DNA primase, which synthesizes short fragments of nucleic acid, usually RNA; PF01896 is not always recognized within DNA ligase sequences in the Pfam database, but these regions are usually annotated region as the Pol domain of LigD, which corresponds to this domain [10]. From this type of analysis of protein families it becomes clear that ADLs have significant diversity in their domains and in the way these are organized within a single polypeptide (Figure 1).

Where bacteria do encode ADLs within their genomes, the number of genes varies widely – though it is relatively consistent within each bacterial genus [2,10]. Three classes of these enzymes (LigB, LigC and LigD) appear to have descended from a common ancestor within bacteria. It is important to note that the various domains (and their corresponding biochemical activities) are arranged in different organizations within the LigD class of enzymes (Figure 1), which have been given different names [10]. This same recent comparison of the domain structure of different types of ADLs and their distribution among bacteria also delineated an additional group, termed LigE (Figure 1). These enzymes possess several unique features, suggesting that the genes encoding LigE-type proteins were horizontally transferred into bacteria in a separate event from other bacterial ADLs.

Functional characterization of the ADLs has delineated that the four classes of genes contain different organization of the structural domains found within the Pfam database [10]. The arrangement of the domains varies between species, but there are clear groupings of functional domains, as illustrated in Figure 1. The different domains are likely to promote interactions between the DNA ligase and other proteins, ensuring that the DNA-sealing polypeptide is targeted to specific processes or types of DNA

ends. A number of observations suggest that ADLs assist the efficiency of DNA repair, with the best evidence being available for the non-homologous end-joining (NHEJ) system that repairs double-stranded breaks in DNA. The LigD proteins are required for efficient NHEJ that involve interactions with Ku proteins that also participate in NHEJ, but the LigC proteins also act as a back-up system [22–26,34,36–40]. Furthermore, the multiple enzymatic modules that are present within the large LigD proteins include a DNA-primase/polymerase domain and in some cases a phosphoesterase domain, and these activities are able to alter the structure and sequence of the ends before repairing them [41–45].

Bacterial ADLs have been demonstrated to have DNA end-joining activities by a variety of methods and assays, but the non-essential nature of these genes suggests that they are not functional as replicative DNA ligases. This is clearly different from the situation in the majority of archaea, where ADLs must participate in replication in each organism. The widespread presence of genes for ADLs in bacteria suggests that their protein products have important activities in DNA metabolism with their host cells. It seems likely that the various classes of ADLs have different physiological functions and, indeed, the function of a specific class may vary in different organisms. A number of possible functions have been suggested for these enzymes [10,22–26], but further research is required to clarify the usefulness of the various types of ADLs in different organisms.

## STRUCTURE AND FUNCTIONS OF $\text{NAD}^+$ -DEPENDENT DNA LIGASES

In contrast with the widespread presence of functional ADLs across cell types, the majority of functional NDLs have only been identified within the genomes of all bacteria and some viruses. In bacteria the genes are essential, presumably due to the requirement of their protein product for efficient DNA replication [21]. As discussed in detail below, the essential nature of the NDLs for bacterial viability make them a possible target for novel anti-bacterial drugs. Novel chemicals that are developed for such approaches also provide useful compounds for biochemical characterization of DNA ligases.

Some archaeal organisms encode NDLs, including *Methanomethylophilus alvus* [46] and, potentially, the recently discovered lokiarchaeota [19]. In contrast with the majority of archaea studied to date, genetic- and microbiology-based studies have confirmed the cellular requirement of an NDL homologue in the halophile *Haloferax volcanii* [47,48]. Phylogenetic analysis suggests the genes encoding these enzymes were most probably acquired as a result of horizontal gene transfer from bacteria [47,49]. Biochemical and structural characterization of NDLs encoded by archaeal genomes will illuminate the evolutionary history of these enzymes and help with the development of drugs that are specifically targeted against bacterial NDLs.

NDLs from different bacteria have been structurally characterized to better understand their activities and reaction mechanism,

**Table 1 Essential amino acids and their roles in the NTase domains of  $\beta$ -NAD<sup>+</sup>-dependent DNA ligases from *E. coli* [59,60] and *M. tuberculosis* [54,61]**

'Roles' refer to the biochemical mechanism of DNA ligases, as shown in Figure 5.

	Residue	Interaction	Role
<i>E. coli</i>	K115	Part of KXDG conserved motif; nucleophilic attack to adenosine phosphate of $\beta$ -NAD <sup>+</sup>	Formation of adenylated-ligase intermediate
<i>M. tuberculosis</i>	K123		
<i>E. coli</i>	D117	Part of KXDG conserved motif	Stabilization of active site
<i>M. tuberculosis</i>	D125		
<i>E. coli</i>	G118	Part of KXDG conserved motif	Stabilization of active site
<i>M. tuberculosis</i>	G126		
<i>E. coli</i>	E173	Coordination of O-2' of the adenosine ribose in $\beta$ -NAD <sup>+</sup> and AMP	Involved in formation of adenylated-ligase intermediate and sealing of nick
<i>M. tuberculosis</i>	E184		
<i>E. coli</i>	K290	H-bonding with N-1 on adenine ring	Stabilization of adenine conformation
<i>M. tuberculosis</i>	K300		
<i>E. coli</i>	Y225	Stacking interaction with adenine ring	Stabilization of adenine conformation
<i>M. tuberculosis</i>	H236		
<i>E. coli</i>	E113	H-bonding with N-6 on adenine ring	Stabilization of adenine conformation
<i>M. tuberculosis</i>	E121		

and to aid the development of possible inhibitors against these enzymes [3–5,7,8]. High levels of homology have been found between sequences of bacterial NDLS, showing highly conserved motifs and the same modular architecture. The sequence similarity with ADLs is quite poor, as expected because of the different co-factor; however, NDLS and ADLs share a similar biochemical reaction mechanism, as highlighted above [2,5,7,8].

The first crystallographic structure of an NDLS was a partial structure that included the adenylation domain from the enzyme from *Bacillus stearothermophilus* [50]. Shortly after, a more complete and biochemically informative structure was obtained for the NDLS from *Thermus filiformis* [51]. Other high-resolution structures have been resolved for the homologous enzymes from *Enterococcus faecalis* [52,53], *Mycobacterium tuberculosis* [54], *Escherichia coli* [55,56], *Haemophilus influenzae* [57], *Streptococcus pneumoniae* [56] and *Staphylococcus aureus* [58]. Many of the structures contain only fragments of the enzyme, but some have ligands incorporated. Comparison of the different structures reveals that the NDLS have a modular architecture consisting of four main domains (Figures 2A and 2B), which incorporate a number of domains from the Pfam database [16]. This rather complex conformation leads to several important features for the reaction mechanism of NDLS. Domain 1 (incorporating PF01653) is formed by two subdomains; subdomain 1a, at the N-terminus, binds the  $\beta$ -NMN moiety of  $\beta$ -NAD<sup>+</sup>, whereas subdomain 1b is the adenylation domain, or the nucleotidyltransferase domain (NTase), since it binds the AMP moiety of  $\beta$ -NAD<sup>+</sup>. Domain 2 is the oligonucleotide binding site (OB), analogous to PF03120. Domain 3 consists of subdomain 3a, a Cys<sub>4</sub>-type zinc finger (equivalent to PF03119), and subdomain 3b, containing a helix–hairpin–helix motif (HhH) (equivalent to PF14520). Domain 4, at the C-terminus, is a BRCT domain (breast cancer carboxy-terminal), analogous to PF00533. Note that, typically, the BRCT

is not resolved in crystallographic structures of NDLS (e.g. as shown in Figure 2B).

The catalytic core of NDLS consists of subdomain 1b and domain 2 (OB-fold), flanked by N- and C-terminal domains arranged in a C-shape that clamps the double-stranded DNA [2–5,7,8]. Subdomain 1a has been shown to interact with the  $\beta$ -NMN moiety of  $\beta$ -NAD<sup>+</sup> and, indeed, it is not present in ADLs [59]. Its presence is fundamental to initiate the ligation reaction of NDLS, but is not essential in the case of the interaction of preadenylated-enzyme with DNA; this means that subdomain 1a is required for the  $\beta$ -NAD<sup>+</sup> binding and the adenylation step. From the crystallographic studies, it was possible to identify an 'open' enzyme conformation, in which  $\beta$ -NAD<sup>+</sup> was bound to the solvent exposed NTase domain. Therefore, the closure of subdomain 1a to bury  $\beta$ -NAD<sup>+</sup> puts  $\beta$ -NMN in an apical position as a leaving group during the nucleophilic attack of the lysine residue on the adenosine phosphate; the subdomain 1a has to swivel by almost 180° to switch from the 'open' to the 'closed' conformation [52].

Subdomain 1b contains the adenylation domain of NDLS, or the core of the DNA ligase. Several residues in the NTase have been found to be fundamental for the enzyme activity, with modifications bringing a considerable rate decrease or inactivation of the enzyme. The essential amino acids in the NTase, their interactions and their biochemical roles are summarized in Table 1 for the NDLS from *E. coli* [59,60] and *M. tuberculosis* [54,61]. The high conservation of NDLS is highlighted by the presence of similar residues in NDLS from the two organisms, with the only difference being that the stacking interaction to adenine occurs with Tyr-225 in EclgA, while it is with His-236 in MtLigA.

Domain 2 contacts the double-stranded DNA, covers the nick and helps in positioning it into the NTase domain. Its action is supported by subdomain 3b, also referred to as HhH, which binds both DNA strands in a non-specific sequence manner across the



minor groove. The OB-fold, NTase and HhH have been shown to clamp the double-strand DNA across 19 bp [55].

Subdomain 3a, the Cys<sub>4</sub>-type zinc finger, holds the OB-fold and HhH at the appropriate structural positions during DNA binding [51,55,62]. Finally, the C-terminal domain 4, BRCT, has a role for the DNA ligases that is still not fully explained. Indeed, from the crystal structures of different enzymes, the BRCT domain appears as a flexible unit, with a structure that cannot to be properly resolved, and it is apparently not essential for the adenylation step [51,55,63]. Indeed, NDL proteins not including BRCT in their modular structures are still able to perform their ligation reaction, albeit at a lower rate [62–64]. However, DNA binding was shown to be tighter for an enzyme with a BRCT domain compared with one not containing it [64]. Therefore, the BRCT domain could stabilize the DNA binding on NDLs, even though it does not interact directly with DNA. Another possible function for the BRCT domain could be as a signal transducer of the DNA-damage response; indeed, other well-characterized proteins use this domain for protein-protein interactions to transmit signalling responses [51].

Overall, the modular structure of NDL allows it to catalyse the ligation reaction by coordinating considerable movements of the different domains. It is now clear that similar large-scale conformation movements of the enzymes take place for ligation reactions performed by the majority of NDLs and ADLs.

## ASSAYS OF DNA JOINING BY DNA LIGASES

The reaction catalysed by DNA ligases leads to changes in the ends or backbone structure of DNA and such changes can be detected using a range of biochemical and biophysical assays [65]. Here we briefly review assays that are particularly useful in analysing the influence of small molecules on the activity of DNA ligases.

Gel electrophoresis is a convenient method for detecting the sizes of molecules, and it has been widely used among the primary biochemical studies of DNA ligases that are reviewed here (e.g. see [64,66,67]). However, gel electrophoresis is rather time-consuming and laborious and, thus, is not ideal for drug discovery purposes. Therefore, to facilitate biochemical analyses of DNA ligases and development of inhibitors against them, attention has focused on development of alternative assays to monitor DNA joining activities. Some of these alternatives have been specifically developed to offer increased throughput of assays [68–70], different time resolution or are more cost-effective. In some cases the developments take advantage of novel forms of relevant molecules, such as a fluorescent derivative of  $\beta$ -NAD<sup>+</sup> that can be processed by different  $\beta$ -NAD<sup>+</sup>-consuming enzymes, including NDLs [71].

A wide range of biophysical chemistry approaches have been used to detect the joining of DNA strand breaks. Those applied to NDLs or ADLs from bacteria or archaea include use of fluorescently-labelled probes [66,70,72,73], FRET [69,74–77],

fluorescence quenching and molecular beacon-based approaches [78–81], electrochemical methods [67,79,81–84], a nanoparticle-based sensor [85] and surface plasmon resonance [86]. Biochemical analyses of the ligation reaction are facilitated by assays that do not require ‘labelling’ of the nucleic acid, such as label-free electrochemical approaches using mercury-based electrodes and nicked plasmid DNA substrates [67]. Combinations of techniques can also be powerful, as observed with the electrochemistry alternative that uses a digoxigenin-labelled probe and detects ligation via a ‘double-surface’ enzyme-linked electrochemical assay [84]. Such techniques are fast and inherently versatile due to their easy incorporation of synthetic oligonucleotide substrates, allowing the DNA breaks to be situated in diverse sequence contexts and/or structure perturbations.

As highlighted in many of these assays, oligonucleotide templates that are able to form stem-loop structures are popular since they can be immobilized on different surfaces through biotin–streptavidin interactions or direct linkage to gold surfaces. If the other end of the stem-loop contains a label, then the absence or presence of the label can be used to detect alteration to the backbone structure of DNA. By inclusion of a nick in DNA stem-loop structures, then retention of the signal indicates DNA ligation and such approaches have been monitored using fluorescence [66,78,79] or electrochemical [81,82] techniques. Interestingly, use of appropriate substrates in these assays allows study of the combined action of nucleases and ligases [66], which allows extension of these assays to assess links between DNA ligases and other proteins involved in nucleic acid metabolism, as observed in DNA repair processes.

## INHIBITORS AND NON-NATURAL SUBSTRATES OF DNA LIGASES

Detailed characterization of DNA ligases at a biochemical and molecular level has generated therapeutic interest, with the essential NDLs of bacteria being a suggested target for novel antibiotics candidates [2,7,8,11,12]. The starting point for many studies that aim to inhibit such enzymes is to assess the effects of modified derivatives of the co-factors, either  $\beta$ -NAD<sup>+</sup> or ATP in the case of DNA ligases. In assessing such compounds, it is important to recognize that they may result in non-natural substrate activity and, thus, may not inhibit the activity of the enzyme [71,87]. Therefore, it is often necessary to test the effects of many different types of modifications to the co-factors. In the case of DNA ligases, modified co-factors have produced a range of effects and it is not straightforward to predict their effects, as we discuss below. Furthermore, an influence on the enzyme activity does not necessarily require major alterations to the chemical groups of the co-factor, as demonstrated recently for T4 DNA ligase exhibiting stereo-selective properties towards modified ATP compounds [88].

The first DNA ligase inhibitors that looked promising for drug discovery purposes were discovered by screening libraries of classes of compounds, such as alkaloids, flavonoids,

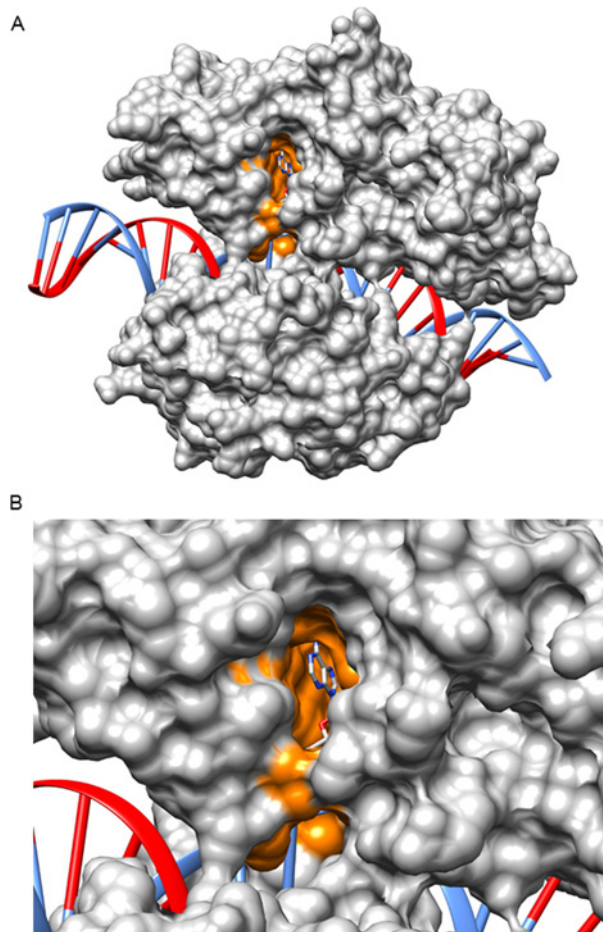
pyridochromanones, quinoline and quinacrine derivatives [8,89–91]. These compounds were then refined to generate analogues that had improved activity, selectivity, solubility and other factors that make the compounds more ‘druggable’ [53,74–76,92–98]. Importantly, some of these studies also highlighted the challenges associated with developing such compounds as antimicrobial drugs [96].

As described above, a variety of high-resolution structures of NDLs have been resolved in the last decade, providing a better understanding of the  $\beta$ -NAD<sup>+</sup> binding site [7,55]. The substantial conformational changes of the enzymes that occur during the reaction – and the conformational flexibility of the enzymes in general – makes rational design of inhibitors challenging. By their very nature, structures from crystallographic studies capture only a particular snapshot of the enzyme, which may or may not be relevant for inhibitor binding. Several studies have undertaken basic modelling of the structures of NDLs and virtual screening of molecules that they interact with [8,53,55,61,63,87,91,98–100]. However, it is clear that detailed molecular dynamics studies of NDLs would assist with structure-based design of inhibitors. Information derived from the combination of different structures has highlighted some useful approaches, particularly in relation to the challenges and potential advantages of targeting the co-factor binding site [3–5,7,15]. In particular, identification of the existence of a hydrophobic tunnel (Figure 3) in proximity to N-1, C-2 and N-3 of the adenine ring [7] has opened the opportunity for the development of inhibitors that can fit into this tunnel (Figure 4). Moreover, the tunnel is not present in ADLs, therefore potentially allowing for the generation of inhibitors that are more specific towards NDLs.

Building on previous studies [52], crystallographic structures were obtained for known inhibitors in complex with *E. faecalis* LigA (PDB accession numbers 3BA8, 3BA9, 3BAA, 3BAB; Pinko, Borchardt, Nikulin, Su, unpublished data). These structures showed the inhibitors fitting into the hydrophobic tunnel. Intuitively, adenosine-based compounds substituted on C-2 of the adenine ring fit perfectly into the active site positioning the substituent into the tunnel; consequently, they have become a prominent class of inhibitors (Table 2), extensively studied and optimized to meet optimal drug criteria [92,95]. The scaffold similarity to  $\beta$ -NAD<sup>+</sup> and the lack of interference of substrate activity make adenosine derivatives very promising inhibitors, with IC<sub>50</sub> in the low micromolar range.

Other potential inhibitors that have been assessed include 6-azaindazoles, 2-amino-[1,8]-naphthyridine-3-carboxamides (ANCs) and aminoalkoxypyrimidine carboxamide (AAPCs) (Figure 4). These compounds were modelled and synthesized to resemble the orientation of adenine, ribose and C-2 substituents into the active site with comparable inhibitory activity. Phosphorylated derivatives of adenosine, such as AMP, cAMP, ADP and ATP substituted on C-2 of the adenine ring have also been tested as inhibitors against NDLs with some promising results (Table 3).

Despite this progress, there have been relatively few reports of  $\beta$ -NAD<sup>+</sup> derivatives as inhibitors of NDLs (Table 3). The reasons for this include the difficult synthesis of  $\beta$ -NAD<sup>+</sup> de-

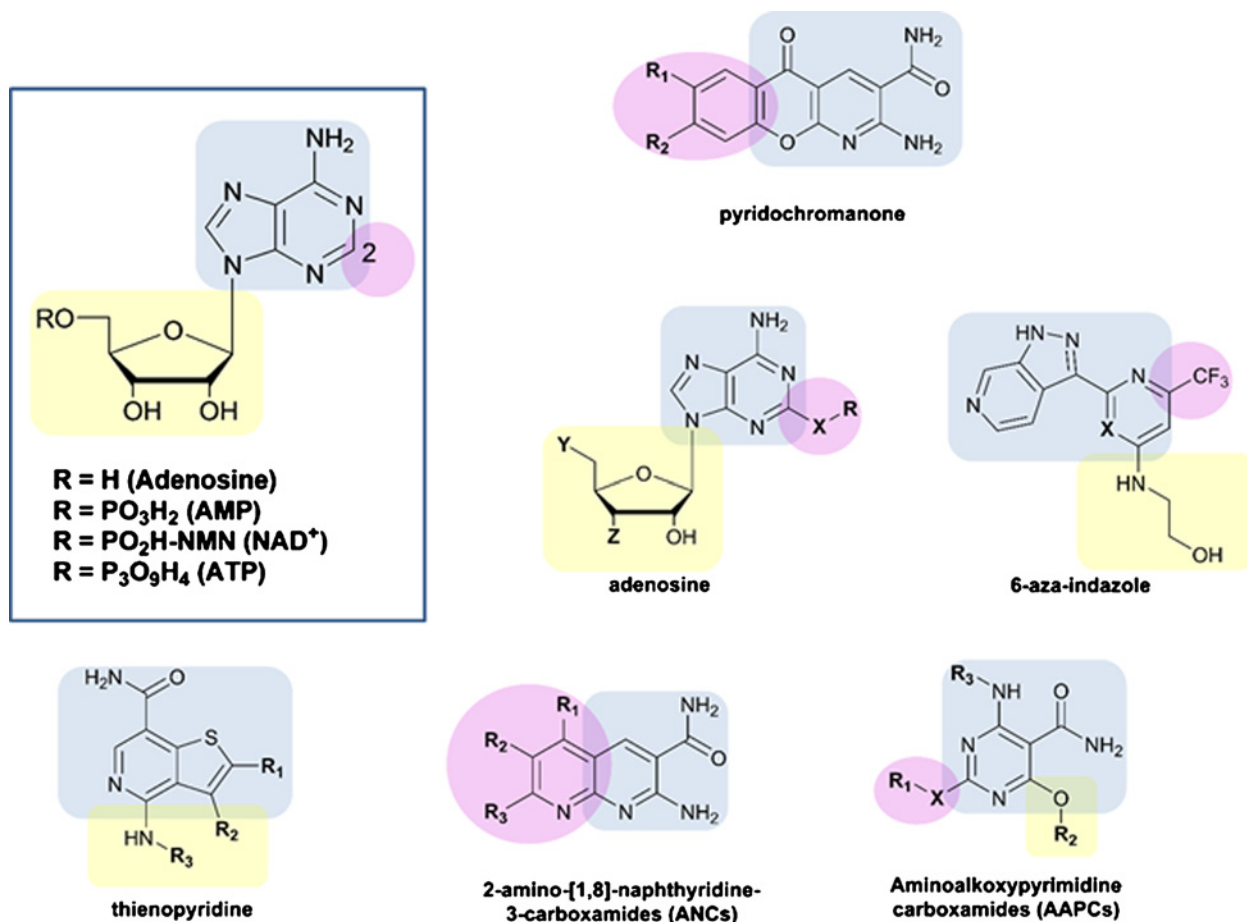


**Figure 3** NAD<sup>+</sup>-dependent DNA ligases from bacteria contain a conserved hydrophobic tunnel

All NAD<sup>+</sup>-dependent DNA ligases from bacteria contain a hydrophobic tunnel (shaded in orange). The structure is of *E. coli* LigA bound to adenylylated DNA from PDB 2OWO [55] and is visualized by UCSF Chimera. The image in (A) shows the full structure, with image (B) zooming in to give a clearer view of the location of the co-factor in the hydrophobic tunnel. Note that the C-2 of the adenine ring of  $\beta$ -NAD<sup>+</sup> points in the direction of the hydrophobic tunnel.

derivatives, their relatively limited and modest stability, and their flexible/multifunctional structure, which makes  $\beta$ -NAD<sup>+</sup> binding quite unspecific. Such modified derivatives of  $\beta$ -NAD<sup>+</sup> may also result in non-natural substrate activity, though this property can be exploited to provide useful insight into the catalytic mechanisms of the enzymes [71,87].

Recent studies have assessed the biological activity of the  $\beta$ -NAD<sup>+</sup> derivatives and some of their corresponding AMP and AMP-morpholidate derivatives. For example, using EcLigA and MtLigA, the enzymes from *E. coli* and *M. tuberculosis* that are among the best characterized NDLs, compounds modified in position 2, 6 or 8 of the adenine ring were tested as inhibitors and/or non-natural substrates [87]. The different substitution patterns on derivatives of  $\beta$ -NAD<sup>+</sup> and AMP had a pronounced effect on the biochemical activity of EcLigA and MtLigA. The



**Figure 4** Classes of NAD<sup>+</sup>-dependent DNA ligase inhibitors and their position characteristics compared with the natural substrate

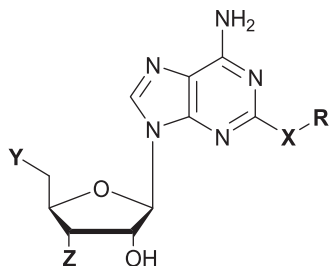
The blue box identifies the part of the molecule that binds in the position of the adenine nucleobase; the purple box identifies the part of the molecule that sits within the hydrophobic tunnel of NAD<sup>+</sup>-dependent DNA ligases; the yellow box identifies the sugar moiety binding site.

6-substituted derivatives were neither co-substrates (in the case of the  $\beta$ -NAD<sup>+</sup> derivatives) nor inhibitors (in the case of both  $\beta$ -NAD<sup>+</sup> and AMP derivatives) [87]. These results suggest that the presence of an additional substituent in position 6 is not tolerated by either EcLigA or MlLigA, and is detrimental for co-substrate binding. None of the 8-substituted  $\beta$ -NAD<sup>+</sup> derivatives were utilized as co-substrates by EcLigA either, but two were weak inhibitors [87]. This pattern of behaviour suggests that 8-substituted  $\beta$ -NAD<sup>+</sup> derivatives can bind at the active site of NDLS, but do so in a non-productive orientation. Due to the proximity of the protein backbone and position 8 of the adenine ring, there is not enough space within the active site to accommodate an additional substituent at this position without changing the original position of the adenine. Thus, the binding of 8-substituted  $\beta$ -NAD<sup>+</sup> derivatives at the active site probably occurs in a different orientation from that of natural  $\beta$ -NAD<sup>+</sup> and, consequently, precludes establishment of key interactions within the active site and, therefore, their co-substrate activity [87]. By

contrast, co-substrate and/or inhibitory activities were observed in the series of 2-substituted derivatives highlighting that an additional substituent at the 2-position of  $\beta$ -NAD<sup>+</sup>/AMP can be accommodated by NDLS and may be involved in direct interactions with the target DNA ligase [87]. This hypothesis is supported by results from molecular docking experiments taking advantage of the structure of EcLigA (PDB: 2OWO [55]), which suggest that 2-substituted AMP/ $\beta$ -NAD<sup>+</sup> derivatives can be accommodated at the active site in an orientation that is nearly identical with that of natural AMP/ $\beta$ -NAD<sup>+</sup> [87]. In this position, the adenine ring of the compounds maintains all key interactions with active site residues, whereas the substituent in position 2 fits into the hydrophobic tunnel adjacent to the adenine binding site (Figure 3). However, different substituents may interact differently with the residues in the tunnel; thus, although the 2-iodo substituent may electrostatically interact with Lys-290 at the entrance of the hydrophobic tunnel, a phenyl substituent does not appear to make any specific contacts with residues in the tunnel, explaining the

**Table 2** IC<sub>50</sub> values for nucleoside-based inhibitors of NAD<sup>+</sup>-dependent DNA ligases from bacteria

Spn: *Streptococcus pneumoniae*; Sau: *Staphylococcus aureus*; Hin: *Haemophilus influenzae*; Ec: *Escherichia coli*; Mpn: *Mycoplasma pneumoniae*. n.d.: not determined. <sup>a</sup> Details from [75]; <sup>b</sup> details from [53]; <sup>c</sup> details from [76]; <sup>d</sup> details from [74]; <sup>e</sup> details from [94].



compd	X	Y	Z	R	IC <sub>50</sub> Spn [μM]	IC <sub>50</sub> Sau [μM]	IC <sub>50</sub> Hin [μM]	IC <sub>50</sub> Spn L75F [μM]	IC <sub>50</sub> Ec [μM]	IC <sub>50</sub> Mpn [μM]
1 <sup>a,c,d</sup>	S	OH	OH	<i>n</i> -Butyl	0.14	0.08	0.51	10	1.27	0.07
2 <sup>a</sup>	S	H	OH	<i>n</i> -Butyl	0.13	0.17	n.d.	n.d.	n.d.	n.d.
3 <sup>a</sup>	S	OH	OH	<i>c</i> -Pentyl	0.44	0.38	n.d.	n.d.	n.d.	n.d.
4 <sup>a,b,c</sup>	O	OH	OH	<i>c</i> -Pentyl	0.15–0.21	0.43	0.51	30–33	n.d.	n.d.
5 <sup>a</sup>	O	F	OH	<i>c</i> -Pentyl	0.12	0.12	n.d.	n.d.	n.d.	n.d.
6 <sup>a,d</sup>	O	H	OH	<i>c</i> -Pentyl	0.08	0.12	0.11	n.d.	0.37	0.02
7 <sup>a</sup>	O	H	Cl	<i>c</i> -Pentyl	0.04	0.04	n.d.	n.d.	n.d.	n.d.
8 <sup>a</sup>	O	F	N3	<i>c</i> -Pentyl	0.07	0.09	n.d.	n.d.	n.d.	n.d.
9 <sup>a</sup>	O	OH	Cl	<i>c</i> -Pentyl	0.08	0.11	n.d.	n.d.	n.d.	n.d.
10 <sup>a</sup>	O	H	dichlorobenzyl	<i>c</i> -Pentyl	<0.01	0.13	n.d.	n.d.	n.d.	n.d.
11 <sup>a,d</sup>	O	F	OH	Cyclobutyl methyl	0.05	0.06	0.08	n.d.	0.16	0.02
12 <sup>a,c,d</sup>	O	H	OH	( <i>trans</i> -4-Methylcyclohexyl)	0.05	0.16	0.11	12	0.32	<0.01
13 <sup>a,d</sup>	O	H	OH	Spiro[2.2]pent-1-ylmethyl	0.04	0.06	0.08	n.d.	0.18	0.06
14 <sup>a</sup>	O	H	Cl	Spiro[2.2]pent-1-ylmethyl	0.02	0.03	n.d.	n.d.	n.d.	n.d.
15 <sup>a</sup>	O	H	OH	Bicyclo[3.1.0]hex-3-yl	0.06	0.13	n.d.	n.d.	n.d.	n.d.
16 <sup>a</sup>	O	F	OH	(1-Hydroxymethyl) cyclopropyl methyl	0.40	0.37	n.d.	n.d.	n.d.	n.d.
17 <sup>a</sup>	O	H	OH	Phenyl	2.67	0.14	n.d.	n.d.	n.d.	n.d.
18 <sup>a</sup>	O	F	OH	1,3-Thiazol-2-ylmethyl	0.87	0.91	n.d.	n.d.	n.d.	n.d.
19 <sup>e</sup>	O	H	OH	Decalin	0.04	n.d.	0.26	n.d.	n.d.	n.d.
20 <sup>e</sup>	O	OH	OH	Decalin	0.08	n.d.	0.40	n.d.	n.d.	n.d.
21 <sup>e</sup>	O	H	OH	Adamantane methyl	0.20	n.d.	1.7	n.d.	n.d.	n.d.

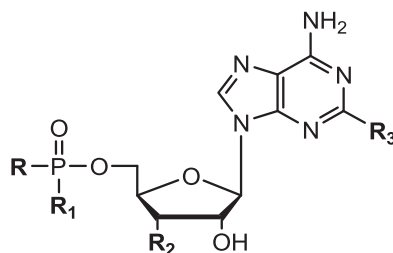
superior inhibitory activity of 2-iodo derivatives against 2-phenyl derivatives toward EcLigA (Table 3).

Moreover, the availability of the two pairs of AMP/ $\beta$ -NAD<sup>+</sup> derivatives with the same 2-substituent (2-iodo or 2-phenyl) enables a detailed assessment of the role of the NMN fragment for DNA ligase inhibition by base-modified adenine nucleotides. The presence of the complete  $\beta$ -NAD<sup>+</sup> scaffold, although not an absolute prerequisite for inhibitory activity, altered the profile of inhibition, leading to synergistic effects with  $\beta$ -NAD<sup>+</sup> or overcoming the time-dependence of inhibition. Interestingly, this inhibitory profile was again dependent on the nature of the 2-substituent suggesting that it has an important role for orienting the NAD<sup>+</sup> scaffold in the active site, and consequently for subdomain closure [87]. In addition, a different general trend for inhibition in the NAD<sup>+</sup> derivatives against EcLigA and MtLigA highlight that subtle differences in the shape of the hydrophobic

tunnel between EcLigA and MtLigA critically influence the binding of different 2-substituents and, consequently, the orientation of 2-substituted  $\beta$ -NAD<sup>+</sup> derivatives in the active site of the respective DNA ligase. The results described for 2-substituted derivatives of  $\beta$ -NAD<sup>+</sup> [87] suggest that their effects are influenced by the large conformational changes required for full activity of NDLS, highlighting the involvement of two distinct nucleotide binding sites (see Figure 5, below). Additional, detailed studies are required to identify which particular steps in the reaction mechanism are inhibited by the variously modified derivatives of  $\beta$ -NAD<sup>+</sup> and to assess whether any of them have any effects on bacteria.

Recently, the potential usefulness of adenosine-based inhibitors carrying a substituent on C-2 of the adenine ring has been questioned with the discovery of a ligase mutant, L75F, which restricts the tunnel with >10-fold loss in antibacterial activity

**Table 3** IC<sub>50</sub> values for nucleotide/dinucleotide-based inhibitors of NAD<sup>+</sup>-dependent DNA ligases from bacteria

 cAMPS: cyclic adenosine monophosphothioate; *Ec*: *Escherichia coli*; *Mt*: *Mycobacterium tuberculosis*. n.d.: not determined. <sup>a</sup> Details from [87]; <sup>b</sup> details from [68].


cmpd	R	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	Scaffold	IC <sub>50</sub> <i>Ec</i> [μM]	IC <sub>50</sub> <i>Mt</i> [μM]
22 <sup>a</sup>	OH	OH	OH	Lodo	AMP	120 ± 23	16 ± 8
23 <sup>a</sup>	NMN	OH	OH	Lodo	NAD	138 ± 18	>200
24 <sup>a</sup>	NMN	OH	OH	Phenyl	NAD	n.d.	73 ± 15
25 <sup>b</sup>	P <sub>2</sub> O <sub>7</sub> H <sub>3</sub>	OH	OH	S-Methyl	ATP	1.1	n.d.
26 <sup>b</sup>	PO <sub>4</sub> H <sub>2</sub>	OH	OH	S-Methyl	ADP	4.1	n.d.
27 <sup>b</sup>	S	R <sub>1</sub> = R <sub>2</sub>	O	S-Methyl	cAMPS	1.5 (S)120 (R)	n.d.

and >100-fold loss in target binding [74,76]. A more recent class of inhibitors based on a thienopyridine scaffold (Figure 4) avoids the presence of substituents fitting into the tunnel and resembles only the orientation of adenine and ribose within the binding site.

## MECHANISM OF DNA LIGATION BY NAD<sup>+</sup>-DEPENDENT DNA LIGASES

Biochemical studies of DNA ligases began with discovery of the enzymes in the 1960s. The most intensively studied NDL is EcLigA from *E. coli*, which is unsurprising since it was the first bacterial DNA ligase to be characterized biochemically [1–3,13]. Studies of EcLigA continue to give useful insights into the reaction mechanism of NDLs, as observed with a recent kinetic analysis that examined the way in which base-pairing at the nick in the DNA influences the activity of the enzyme [101].

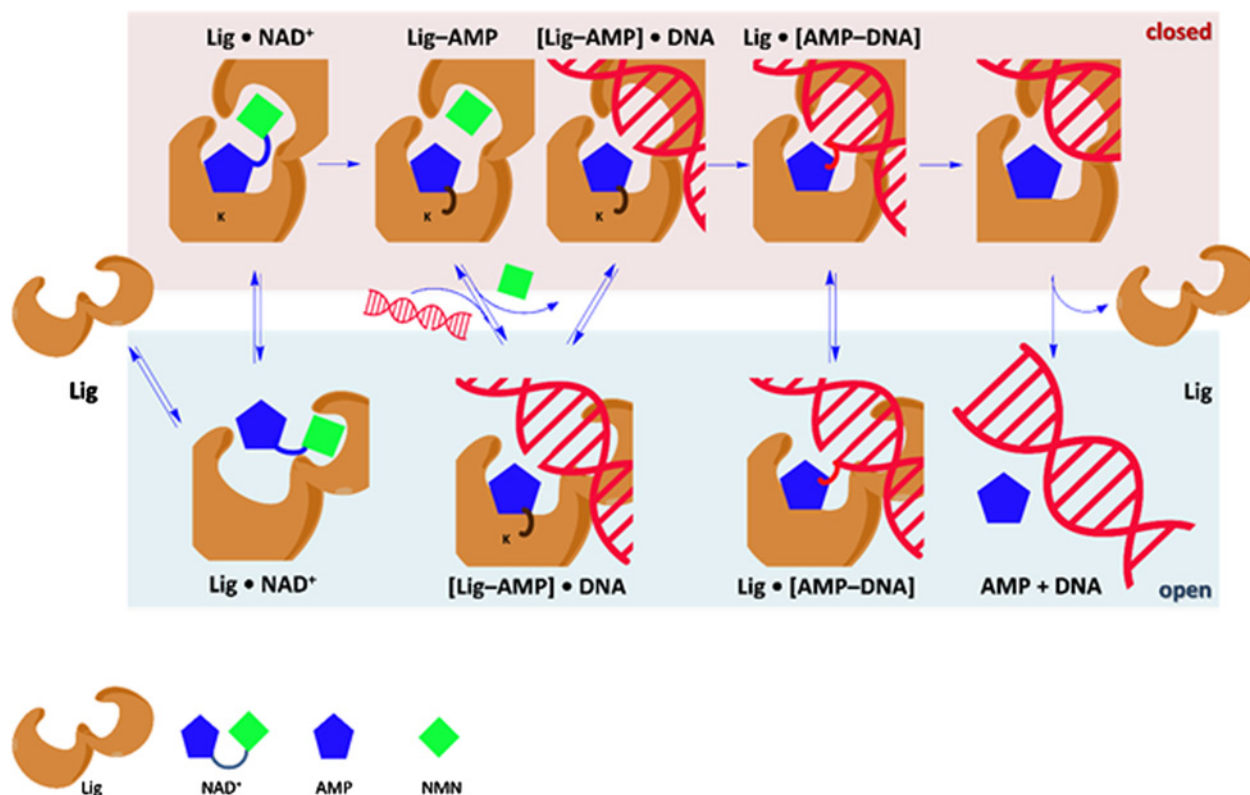
As already highlighted, NDLs follow a ping-pong mechanism (Figure 5), with β-NAD<sup>+</sup> leading to an adenylated intermediate (E-AMP) at a conserved nucleophilic lysine residue (Lys-115 in EcLigA). Throughout these reaction steps, both the DNA ligase and the bound β-NAD<sup>+</sup> co-factor undergo significant conformational changes. The two subdomains 1a and 1b of the N-terminal adenylation domain of DNA ligases include binding sites for, respectively, the NMN (subdomain 1a) and AMP (subdomain 1b) moieties of β-NAD<sup>+</sup>. The binding of the β-NAD<sup>+</sup> co-factor triggers the closure of subdomain 1a on to subdomain 1b, and the transition from an ‘open’ to a ‘closed’ conformation (Figure 5), which is critical for DNA ligase activity [52,57]. Note that progress from one ‘open’ intermediate to the next can only occur by passing through a ‘closed’ intermediate, meaning that multiple, large-scale changes take place during the overall reaction. Stud-

ies with *H. influenzae* LigA suggest that the initial recognition of β-NAD<sup>+</sup> occurs at the NMN binding site on subdomain 1a, providing an explanation for the selectivity of bacterial DNA ligases for β-NAD<sup>+</sup> over ATP [57]. In the closed conformation, the adenine of β-NAD<sup>+</sup> is bound tightly to the active site by several key interactions with conserved residues, such as Lys-290 (H-bonding to N1), Glu-113 (H-bonding to N6) and Tyr-225 (π–π stacking) in EcLigA; in particular, the interaction with Lys-290 is critical for enzymatic activity [55]. Taken together, these interactions hold the adenine ring in place, although the conformation of the AMP moiety changes, through rotation of the ribose about the N-glycosidic bond, from *syn* in non-covalently bound β-NAD<sup>+</sup>, to *anti* in the adenylate-ligase intermediate, and back to *syn* in the adenylated-DNA intermediate [55].

## CONCLUSIONS

DNA ligases are essential enzymes for all cells because they seal breaks in DNA backbones and, thus, ensure that its structural integrity is maintained. Recognition that the essential DNA ligases in bacteria use a different co-factor (β-NAD<sup>+</sup>) compared with the ATP co-factor that is used by the eukaryotic enzymes raised hopes that they could be targeted by novel antibiotics. Until recently, progress in this aim has been hampered due to the difficult synthesis of β-NAD<sup>+</sup> derivatives and their relatively limited stability. However, the complex molecular structure of β-NAD<sup>+</sup> affords multiple opportunities for chemical modification and several recent studies have synthesized promising novel derivatives.

The biological activity of these compounds have been evaluated as inhibitors for drug discovery and such modified



**Figure 5** Reaction mechanism for DNA ligation by  $\text{NAD}^+$ -dependent DNA ligases

The enzyme uses a ping-pong mechanism, with  $\beta\text{-NAD}^+$  leading to an adenylated intermediate (Lig:AMP) at a conserved nucleophilic lysine residue. The N-terminal adenylation domain of DNA ligases include binding sites for the NMN (subdomain 1a) and AMP (subdomain 1b) moieties of  $\beta\text{-NAD}^+$ . The binding of the  $\beta\text{-NAD}^+$  co-factor triggers the closure of subdomain 1a on to subdomain 1b, and the transition from an 'open' to a 'closed' conformation, which is critical for DNA ligase activity. In the closed conformation, the adenine of  $\beta\text{-NAD}^+$  is bound tightly to the active site by several key interactions with conserved residues. These interactions hold the adenine ring in place although the conformation of the AMP moiety changes from *syn* in non-covalently bound  $\beta\text{-NAD}^+$  to *anti* in the adenylate-ligase intermediate, and back to *syn* in the adenylated-DNA intermediate. Progress from one 'open' intermediate to the next can only occur by passing through a 'closed' intermediate, meaning that multiple, large-scale changes take place during the overall reaction; see text for further details. Similar large-scale conformational changes occur during ligation by ATP-dependent DNA ligases, but the specific details vary for each version of those enzymes.

derivatives of  $\beta\text{-NAD}^+$  also result in non-natural substrate activity that can be exploited to provide useful insight into the catalytic mechanisms of DNA ligases. Summarizing recent results, it is clear that aryl/heteroaryl substitutions on different positions of the adenine ring generate  $\beta\text{-NAD}^+$  derivatives with biological activities toward  $\beta\text{-NAD}^+$ -consuming enzymes. The preferential action of aryl/heteroaryl adenine-modified  $\beta\text{-NAD}^+$  derivatives as inhibitors and/or non-natural substrates toward specific  $\beta\text{-NAD}^+$ -consuming enzymes offers the prospect of their use for *in vitro/in vivo* studies. Such a step forward in their biological evaluation will require consideration of their activity and stability within cells, such as their ability to resist degradation by ecto  $\beta\text{-NAD}^+$ -consuming enzymes or intracellular nucleases. Therefore, chemical modifications on the  $\beta\text{-NAD}^+$  scaffold or alternative methods of delivery will need to be developed with a focus on the use for which they are designed. However, the validity of the use of such compounds as biochemical probes is

already a useful place to widen the knowledge of fundamental biological processes involving  $\beta\text{-NAD}^+$ -consuming enzymes.

Biochemical and structural studies have made significant progress in identifying compounds that inhibit NDLs. However, the prospects of developing such compounds as antimicrobial drugs remains challenging, particularly since it is likely that effective drugs will have to inhibit NDLs very efficiently, with very good discrimination against ADLs [21,96]. Recent comparative analysis of co-factor and inhibition experiments raises the possibility that useful inhibition may be obtained due to targeting of the significant conformational changes that occur within DNA ligases during their catalytic cycle. Importantly, such findings may provide a promising starting point for the rational design of new classes of inhibitors against bacterial DNA ligases. It is also notable that most studies of potential inhibitors have assessed the effects of compounds that bind reversibly to DNA ligases. Compounds that bind to NDLs through covalent binding could



offer novel solutions to the need to inhibit the enzymes very efficiently in the cell. Detailed molecular dynamics studies are likely to offer valuable insights into useful avenues to explore in the development of improved inhibitors of NDLs.

In summary, although progress to identify useful compounds that target DNA ligases has been rather frustrating, the recent development of modified derivatives of nucleotides offers encouragement for such studies. The continued combination of structural, biochemical and biophysical techniques will be useful in targeting these essential cellular enzymes.

#### AUTHOR CONTRIBUTION

All authors developed the ideas that led to this review and were involved in writing and editing of the manuscript.

#### ACKNOWLEDGEMENTS

We thank Prof Julea Butt, University of East Anglia, for her support with this work and for providing comments on this manuscript.

#### FUNDING

This work was supported by the University of East Anglia, Norwich, UK and King's College London, UK.

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Received 16 January 2016/28 August 2016; accepted 30 August 2016

Accepted Manuscript online 31 August 2016, doi 10.1042/BSR20160003

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