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# NAD+ is not utilized as a co-factor for DNA ligation by human DNA ligase IV

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## ABSTRACT

**As nucleotidyl transferases, formation of a covalent enzyme-adenylate intermediate is a common first step of all DNA ligases. While it has been shown that eukaryotic DNA ligases utilize ATP as the adenylation donor, it was recently reported that human DNA ligase IV can also utilize NAD+ and, to a lesser extent ADP-ribose, as the source of the adenylate group and that NAD+, unlike ATP, enhances ligation by supporting multiple catalytic cycles. Since this unexpected finding has significant implications for our understanding of the mechanisms and regulation of DNA double strand break repair, we attempted to confirm that NAD+ and ADP-ribose can be used as co-factors by human DNA ligase IV. Here, we provide evidence that NAD+ does not enhance ligation by pre-adenylated DNA ligase IV, indicating that this co-factor is not utilized for re-adenylation and subsequent cycles of ligation. Moreover, we find that ligation by de-adenylated DNA ligase IV is dependent upon ATP not NAD+ or ADP-ribose. Thus, we conclude that human DNA ligase IV cannot use either NAD+ or ADP-ribose as adenylation donor for ligation.**

## INTRODUCTION

DNA ligases are members of the nucleotidyl transferase family of enzymes that utilize a nucleotide co-factor to form a covalent enzyme-nucleoside monophosphate reaction intermediate (Figure 1). In the initial discovery and characterization of DNA ligases, it was demonstrated that the *Escherichia coli* DNA ligase utilizes $NAD^+$ as the co-factor for adenylation whereas bacteriophage DNA ligases utilize ATP (1–4). Subsequent studies have shown that eukaryotic DNA ligases are similar to bacteriophage DNA ligases in that ATP rather than $NAD^+$ is used as the source of adenylate moiety (5,6). While sharing the same catalytic mechanism (Figure 1), the three human DNA ligases are targeted to different nuclear DNA transactions through interactions with different protein partners (5,6). For DNA ligase I, these dynamic interactions do not appear to influence protein stability, whereas stable interactions of DNA ligases IIIα and DNA ligase IV (LigIV) with partner proteins, XRCC1 and XRCC4, respectively, are required for their stability and activity in nuclear DNA repair (5,6). In contrast to DNA ligase I and XRCC1/DNA ligase IIIα, which have overlapping functions in nuclear DNA replication and excision repair (5,6), XRCC4/DNA ligase IV (X4L4) is the unique ligase in the non-homologous DNA end joining (NHEJ) pathway that repairs DNA double strand breaks (DSBs) (7,8). In addition, X4L4 is distinct from DNA ligase I and LigIIIα/XRCC1 in that the complex purified after expression in either *E. coli* or insect cells predominantly catalyses a single round of DNA ligation because of a slow rate of re-adenylation (9–12).

While the majority of the purified X4L4 complex is pre-adenylated (9–13), ATP supports the re-adenylation of de-adenylated X4L4 demonstrating that LigIV can utilize ATP as the source of the AMP moiety (12). Surprisingly, a recent publication reported that both $NAD^+$ and ADP-ribose can also be utilized as co-factors for ligation by X4L4 and that $NAD^+$ supports multiple rounds of ligation by X4L4 (14). This unexpected co-factor specificity of LigIV was dependent upon the ability of the first BRCT domain in the non-catalytic C-terminal region of LigIV to bind to $NAD^+$ (14). The utilization of $NAD^+$ and ADP-ribose as co-factors by LigIV in addition to ATP has significant implications for DSB repair given the role of poly(ADP-ribose) polymerase 1 (PARP1), which consumes $NAD^+$ to generate poly(ADP-ribose) that is subsequently degraded to ADP-ribose by poly(ADP-ribose) glycohydrolase (PARG)

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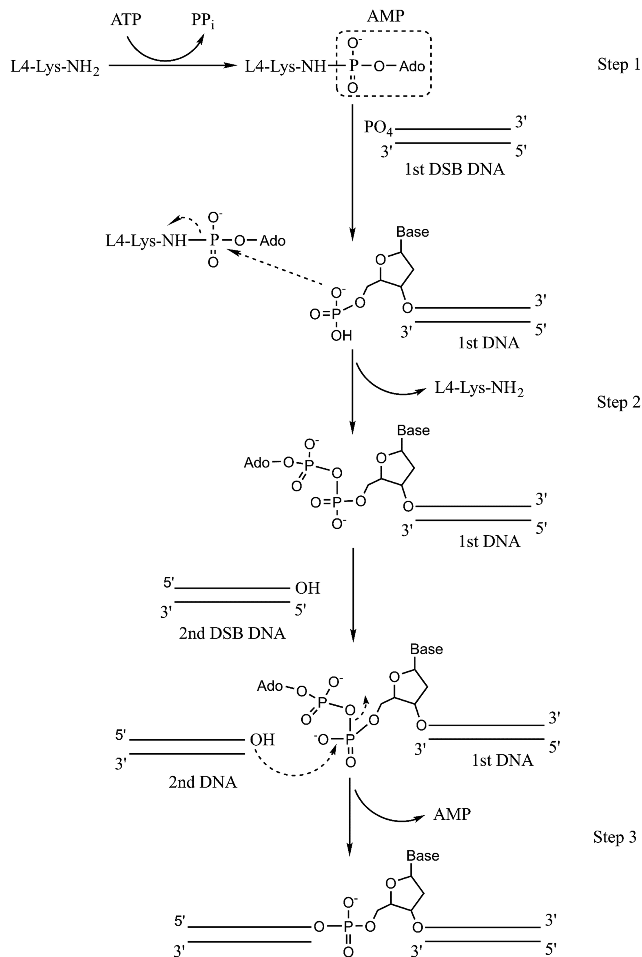


**Figure 1.** Mechanism of DSB DNA ligation mediated by LigIV. Step 1. Adenylation of LigIV (L4). Step 2. AMP moiety is transferred to the 5′ $PO_4$ at one end of a DSB, generating adenylated DNA. Step 3. The 3′ OH at the end of the second duplex at the DSB carries out a nucleophilic attack at the 5′ $PO_4$ to release the AMP from the DNA, and covalently link the two DNA duplexes. It is not known whether the joining of the second strand occurs independently or is linked to the first ligation event and whether the joining of both strands involves one or two LigIV molecules.

at DSB sites (15,16), in the regulation of DSB repair by NHEJ (17,18). This prompted us to examine the effect of $NAD^+$ and ligation by purified X4L4 in two independent laboratories. Our results show that $NAD^+$ neither stimulates DNA ligation by de-adenylated LigIV nor supports multiple rounds of ligation.

## MATERIALS AND METHODS

### Proteins

X4L4 complexes were purified independently from baculovirus-infected insect cells by the Tomkinson and Lieber laboratories. Both laboratories used purification schemes utilizing three column chromatography steps that were modifications of the detailed purification protocol described by Chen *et al.* (19). In the Tomkinson lab, C-terminally histidine-tagged LigIV and XRCC4 were co-expressed in insect cells and purified from cell lysates as a complex (Supplementary Figure S1A) using metal ion affinity chromatography (HisTrap HP, GE Healthcare), gel filtration (Superdex 200 16/60 hiload) and strong anion exchange chromatography as described (20).

Ku70/80 heterodimer (Ku) and X4L4 (Supplementary Figure S1B) are the same purified batches as used in the previous studies by the Lieber laboratory (21,22). Briefly, Ku70 and Ku80 were co-expressed in High-5 insect cells and the Ku heterodimer purified from cell lysates using metal ion affinity chromatography (Ni-NTA agarose, QIAGEN), DNA affinity chromatography (CNBr-activated dsDNA sepharose 4B), anion exchange chromatography (Mono Q, GE Healthcare) and gel filtration (Superdex 200, GE Healthcare). Similarly, X4L4 complex was purified from High-5 insect cell lysates using metal ion affinity chromatography (Ni-NTA agarose, QIAGEN), Mono Q anion exchange chromatography and Mono S cation exchange chromatography (GE Healthcare). All the recombinant proteins were documented to be functional in binding and NHEJ assays. *E. coli* DNA ligase and T4 DNA ligase were purchased from New England Biolabs Inc.

### DNA ligation substrates

DNA oligonucleotides (oligos) used in this study were synthesized by IDT. For nick ligation, oligo Top1: 5′-CAA ATC TCG AGA TCA CAG CAA CTA GAC CA-3′ was 5′ end-labeled by [$\gamma$-$^{32}$P] ATP. Labeled oligo Top1 and oligo Top2: 5′-GGT AAT TGT GAG CGC TCA CAA GCA GGT ACG TCA ACG GAA CG-3′ were then annealed to a complementary template oligo: 5′-GGT AGA ATT CCT CAC GTC ATC TTC AGG TCT AGT TGC TGT GAT CTC GAG ATT TGC GTT CCG TTG ACG TAC CTG CTT GTG AGC GCT CAC AAT TAC-3′ to generate a duplex with single ligatable nick. For duplex ligation, the following oligos were used; HC115: 5′-GAT GCC TCC AAG GTC GAC GAT GCA GAC ACT GAT ATA TGT ACA GAT TCG GTT GAT CAT AGC ACA ATG CCT GCT GAA CCC ACT ATC G-3′, HC116: /5BiosG/CGA TAG TGG GTT CAG CAG GCA TTG TGC TAT GAT CAA CCG AAT CTG TAC ATA TAT CAG TGT CTG CAT CGT CGA CCT TGG AGG CAT CGG GG-3′, HC102: 5′-GGT TCT CGA TGC GCT TGA CCA GTA GTC TAG CAC GTG ACA GGA TCC TCC ATC AAG TAA GAT GCA GAT ACT TAA CG/3Bio/, BZ18: 5′-CGT TAA GTA TCT GCA TCT TAC TTG ATG GAG GAT CCT GTC ACG TGC TAG ACT ACT GGT CAA GCG CAT CGA GAA CC CCCC-3′. DNA duplexes HC115/116 and BZ18/HC102 were obtained by annealing corresponding oligos in a buffer of 20 mM Tris-HCl, 100 mM NaCl, pH 8.0. Oligo HC115 was first radioactively labeled by [$\gamma$-$^{32}$P] ATP, then was annealed with HC116 to obtain the duplex HC$^*$115/116.

### Nick ligation assay

Labeled nicked DNA (100 nM in assays shown in Figure 2 and 250 nM in assays shown in Supplementary Figure S2) was incubated with either X4L4 (50 nM) or *E. coli* ligase (200 nM) in 20 μl of 40 mM HEPES-NaOH (pH 7.5), 50

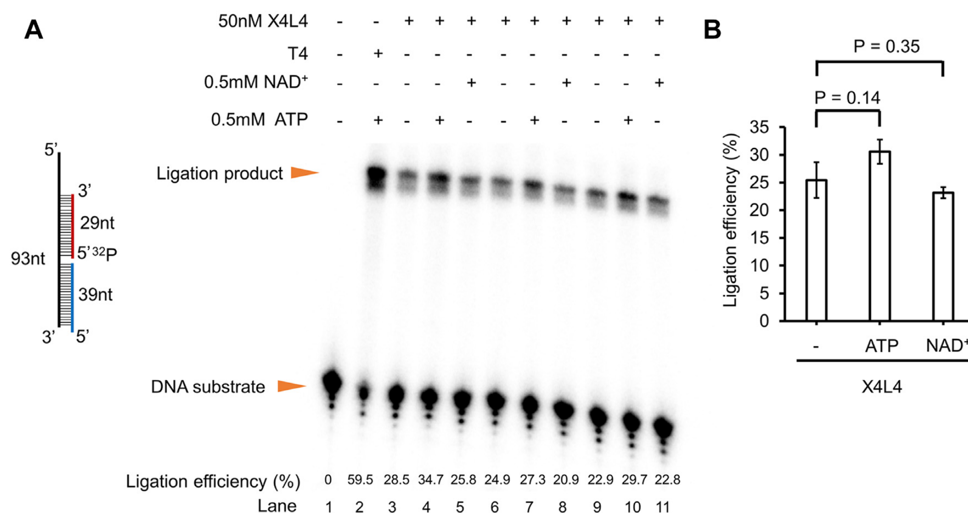


**Figure 2.** $NAD^+$ does not stimulate nick ligation by X4L4. (**A**) Representative gel of nick ligation. X4L4 (50 nM) and T4 DNA ligase (10 units, NEB) were incubated with 100 nM labeled nicked DNA duplex as described in 'Materials and Methods' section in the absence or presence of 0.5 mM $NAD^+$ and ATP as indicated. The duplex DNA substrate with a single ligatable nick is represented schematically. (**B**) Quantification of nick ligation. Data are represented as the mean ± SD of three independent replicates. Two-tailed Student's *t*–test was used for *P* value calculations.

mM NaCl, 10 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin (BSA), 2 mM dithiothreitol (DTT) in the absence or presence of a nucleotide co-factor ($NAD^+$ or ATP at 0.5 mM) for 20 min at 37°C. Reactions were stopped by the addition of 30 mM ethylenediaminetetraacetic acid (EDTA) containing formamide dye, boiled at 100°C for 5 min and then electrophoresed through a 8 M urea/15% polyacrylamide gel. Labeled oligonucleotides in the gel were detected by phosphorimager analysis on a Typhoon FLA 7000 and quantitated using ImageJ 1.52a software.

### X4L4 de-adenylation

About 48 pmol X4L4 was treated with 5 mM sodium pyrophosphate at 30°C for 20 min in 50 mM Tris-HCl (pH 7.5), 150 mM KCl, 10% glycerol, 0.05% Triton X-100 and 1 mM DTT. The reaction mixture was then loaded onto a Sephadex G25 de-salting spin column to remove the remaining pyrophosphate.

### Duplex ligation reaction

Ligation reactions were performed as previously described (23) with minor modification. Briefly, in 10 μl reaction volume, 20 nM $HC^*115/116$, 20 nM BZ18/HC102 and 0.1 mg/ml Neutravidin were first incubated at room temperature for 5 min in 20 mM Tris-HCl (pH 7.5), 75 mM KCl, 10 mM $MgCl_2$, 2 mM DTT, 10% (w/v) PEG-8000. Ku70/80 (25 nM), adenylated or de-adenylated X4L4 (50 nM) and nucleotide co-factor (1 mM ATP, 1 mM $NAD^+$ or 1 mM ADP-ribose (Millipore Sigma)) were then sequentially added into the above reaction mixture as indicated. After incubation at 37°C for 90 min, reactions were stopped by the addition of 0.1% (w/v) SDS plus 20 mM EDTA and 10 μl formamide. Samples were then heated at 100°C for 3 min and put on ice immediately prior to denaturing polyacrylamide electrophoresis.

### Quantification and statistical methods

The unpaired, two-tailed *t*-test was performed using Microsoft Excel.

## RESULTS

### $NAD^+$ cannot stimulate multiple turnover of human DNA ligase IV for DNA ligation

Since it has been reported that human X4L4 mediates multiple rounds of DNA ligation *in vitro* using $NAD^+$ as a cofactor (14), we initially examined the effect of ATP and $NAD^+$ on nick ligation by X4L4 purified from insect cells (Supplementary Figure S1A) and *E. coli* DNA ligase. As expected, the addition of $NAD^+$ but not ATP stimulated ligation by the *E. coli* enzyme above the levels due to pre-adenylated ligase (Supplementary Figure S2). In the absence of added cofactor, 1 pmol of X4L4 generated 0.5 pmol of ligated product, indicating that at least 50% of the LigIV molecules were adenylated. Under these reaction conditions, neither $NAD^+$ nor ATP significantly enhanced ligation (Figure 2A and B). These results indicate that, like ATP, $NAD^+$ is not able to support multiple rounds of DNA ligation, presumably because of the slow rate of re-adenylation (9–12).

### X4L4 is not able to use $NAD^+$ as an adenylation donor for DNA ligation

To directly check if X4L4 is able to use $NAD^+$ as an adenylation donor for DNA ligation, we de-adenylated an independently purified preparation of X4L4 (Supplementary Figure S1B) using 5 mM pyrophosphate and examined the joining of DSB DNA substrates with cohesive overhangs in the presence of the NHEJ DNA end binding protein, Ku70/80. Without de-adenylation, X4L4 joined about 90% of the duplex substrates (Figure 3A, lane 1). As expected, removal of the existing AMP moiety (de-adenylation) by incubation with pyrophosphate markedly reduced the amount

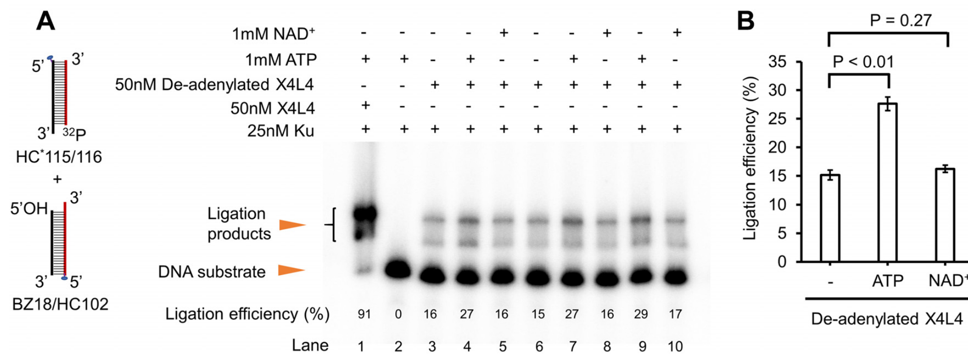


**Figure 3.** X4L4 is not able to use $NAD^+$ as adenylation donor for DNA ligation. (**A**) Representative gel of duplex ligation. Adenylated or de-adenylated X4L4 (50 nM) were incubated with 20 nM HC*115/116 and 20 nM BZ18/HC102 as described in 'Materials and Methods' section in the absence or presence of 1.0 mM $NAD^+$ and ATP. The two DNA duplexes are shown schematically with the asterisk indicating the position of the $^{32}P$ label. The low level of ligation (lanes 3 and 6) is likely due to incomplete de-adenylation of X4L4. (**B**) Quantification of duplex ligation. Data are represented as the mean ± SD of at least two independent replicates. Two-tailed Student's *t*-test was used for *P* value calculations.

of ligation to about 15% (Figure 3A, lanes 3 and 6). The inclusion of 1 mM ATP (Figure 3A, lanes 4, 7 and 9) but not 1 mM $NAD^+$ (Figure 3A, lanes 5, 8 and 10) in reactions with de-adenylated X4L4 significantly stimulated ligation (Figure 3A and B). Together these results show that, in contrast to the recently published report (14), X4L4 is not able to use $NAD^+$ as the co-factor for adenylation and $NAD^+$ does not enhance ligation by enabling X4L4 to function in a multiple turnover mode. In similar studies, we found that ADP-ribose, which was also reported to be an alternative donor for X4L4 adenylation (14), did not promote DSB DNA ligation by de-adenylated X4L4 (Supplementary Figure S3).

## DISCUSSION

Based on the extensive published literature, it has been assumed that, like all other eukaryotic DNA ligases (5), LigIV is an ATP-dependent DNA ligase (12,13). The apparent use of $NAD^+$ and, to a lesser extent ADP-ribose, in addition to ATP as sources of the AMP moiety that is covalently linked to the active site lysine residue of LigIV and then transferred to the 5′ phosphate termini of DNA to form the DNA-adenylate intermediate during the ligation reaction (Figure 1) reported by Chen and Yu (14), was very surprising. The apparent preferential use of $NAD^+$ over ATP for adenylation (14) suggests that X4L4 is competing with PARP1 for $NAD^+$. Notably, PARP1 is activated by both double and single strand breaks in DNA and rapidly depletes cellular $NAD^+$ during the synthesis of poly (ADP-ribose) in the vicinity of strand breaks (16). However, the subsequent degradation of poly (ADP-ribose) by PARG does generate high local concentrations of ADP-ribose at the break site (15). Thus, the potential use of $NAD^+$ and ADP-ribose as alternative adenylation donors by X4L4 has important implications for the mechanism and regulation of DSB repair by NHEJ. Here, we provide direct evidence that $NAD^+$ is not utilized to re-adenylate X4L4 for subsequent rounds of ligation and that de-adenylated X4L4 cannot use $NAD^+$ or ADP-ribose (Supplementary Figure S3) as the adenylation donor to carry out DSB DNA ligation.

While the reason for the discrepancy between our biochemical results and the published study (14) is not evident, it is possible that the apparent enhancement of LigIV activity by $NAD^+$ may be due to the presence of a contaminating enzyme, such as nicotinamide mononucleotide adenylyltransferase, which can convert $NAD^+$ back to ATP (24–26). Notably, our results were obtained with preparations of X4L4 from two different laboratories that have been utilized in experiments analyzing NHEJ complexes and reconstituting NHEJ *in vitro* (20–22). Moreover, our results are consistent with published studies showing that ATP is used by X4L4 to form the LigIV-adenylate complex and that X4L4 behaves as a single turnover enzyme in the presence of ATP because of the slow rate of re-adenylation (11,12). While the mechanisms by which NHEJ protein complexes are dissociated after completion of DSB repair and X4L4 is re-adenylated are poorly understood, there is evidence that XLF and the functionally homologous yeast protein Nej1 enhance ligation by X4L4 (Lif1/Dnl4 in yeast) by promoting re-adenylation for the next repair event (12,27).

Since PARP1 and poly(ADP-ribose) enhance the recruitment of X4L4 to *in vivo* damage sites and assembly of NHEJ protein complexes (28), it is conceivable that it is the interaction of the LigIV BRCT domain with poly(ADP-ribose) rather than $NAD^+$ or ADP-ribose that underlies the increased repair of DSBs by NHEJ (28). This view is supported by the observation that X4L4 cannot be directly recruited to the DSB sites by the mono-ADP-ribosyltransferase PARP3; instead, X4L4 is recruited through interactions with other repair proteins, such as the XRCC4-interacting protein APLF, which recognize the mono-ADP-ribose introduced by PARP3 (29). In addition to their direct role in the DNA damage response, PARPs and poly(ADP-ribose) may indirectly enhance NHEJ by increasing the local ATP concentration as a result of ATP synthesis from ADP-ribose, similar to the process catalyzed by the pyrophosphatase NUDIX5 for chromatin remodeling (30), thereby promoting the ligase activity of X4L4.

## DATA AVAILABILITY

All the data that support the findings of this study are available from the corresponding authors upon reasonable request.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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*Author contributions*: M.R.L. and A.E.T. conceived the study. B.Z. performed the de-adenylation related experiments. T.N. performed the DNA multiple round ligation experiments. All the authors wrote and revised the manuscript.

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