Kinetic Mechanism of Human DNA Ligase I Reveals
Magnesium-dependent Changes in the Rate-limiting Step
That Compromise Ligation Efficiency

Mark R. Taylor, John A. Conrad, Daniel Wahl, and Patrick J. O’Brien

From the Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109-0600

DNA ligase I (LIG1) catalyzes the ligation of single-strand breaks to complete DNA replication and repair. The energy of ATP is used to form a new phosphodiester bond in DNA via a reaction mechanism that involves three distinct chemical steps: enzyme adenylylation, adenylyl transfer to DNA, and nick sealing. We used steady state and pre-steady state kinetics to characterize the minimal mechanism for DNA ligation catalyzed by human LIG1. The ATP dependence of the reaction indicates that LIG1 requires multiple Mg²⁺ ions for catalysis and that an essential Mg²⁺ ion binds more tightly to ATP than to the enzyme. Further dissection of the magnesium ion dependence of individual reaction steps revealed that the affinity for Mg²⁺ changes along the reaction coordinate. At saturating concentrations of ATP and Mg²⁺ ions, the three chemical steps occur at similar rates, and the efficiency of ligation is high. However, under conditions of limiting Mg²⁺ ions, the nick-sealing step becomes rate-limiting, and the adenylylated DNA intermediate is prematurely released into solution. Subsequent adenylylation of enzyme prevents rebinding to the adenylylated DNA intermediate comprising an Achilles’ heel of LIG1. These ligase-generated 5’-adenylylated nicks constitute persistent breaks that are a threat to genomic stability if they are not repaired. The kinetic and thermodynamic framework that we have determined for LIG1 provides a starting point for understanding the mechanism and specificity of mammalian DNA ligases.

Breaks in DNA result from spontaneous hydrolysis of the phosphodiester backbone and are formed as transient intermediates during DNA replication and repair pathways. Mammals have three genes encoding DNA ligases that seal these breaks and restore the continuous nature of chromosomes. DNA ligase I (LIG1)² is essential for ligation of single-strand breaks in the nucleus, including the ligation of Okazaki fragments during discontinuous DNA replication (1). DNA ligase III (LIG3) is required for mitochondrial DNA replication and repair. Although LIG3 has been assumed to play essential roles in nuclear DNA repair, it was recently shown to be dispensable for nuclear genomic maintenance (2, 3). DNA ligase IV (LIG4) is specialized for repair of nuclear double-strand breaks and is required for nonhomologous end joining and V(D)J recombination (4, 5).

The overall reaction catalyzed by DNA ligases involves the formation of a phosphodiester bond between an adjacent 3’-hydroxyl and a 5’-phosphate in DNA. The reaction proceeds via a universally conserved pathway (Fig. 1) (6–8). First, the apoenzyme catalyzes transfer of the AMP group from a nucleotide cofactor to an active site lysine, forming an adenylylated enzyme intermediate. Eukaryotic ligases use ATP as the adenyl group donor, whereas bacterial ligases utilize either ATP or NAD⁺. After binding a nicked DNA substrate, the adenylylated enzyme catalyzes transfer of the adenylyl group to the 5’-phosphate present at the nick, forming an adenylylated DNA intermediate. In the final step of the reaction, DNA ligase catalyzes the nuclease attack of the 3’-hydroxyl on the adenylylated 5’-phosphate to form a new phosphodiester bond and to release AMP. Although enzyme adenylylation and nick sealing are known to be reversible in the presence of a large excess of pyrophosphate or AMP, these steps are effectively irreversible in their absence. All of the chemical steps catalyzed by ligase have been shown to require magnesium, but the number of magnesium ions and their affinities during each step have not been determined (9).

The crystal structure of human LIG1 in complex with an adenylylated DNA intermediate revealed that three distinct domains of the enzyme encircle the DNA substrate (10). Extensive interactions distort the DNA and allow the two ends of the nick to be juxtaposed in the active site of the enzyme. The overall structure of the LIG1 complex is expected to be similar for both the adenylyl transfer and the nick-sealing steps, although the active site may require rearrangement to reposition the reactive phosphoryl group between these two steps. In contrast, the enzyme must undergo a large conformational change to allow for enzyme adenylylation (10–12). The crystallized ligase lacks the N-terminal 232 amino acids that contain the nuclear localization signal and contribute to protein-protein interactions (7, 13, 14). We chose to use the same form that was crystallized for our biochemical studies because this truncated enzyme is fully active as a ligase in vitro (10) and is able to complement ligase-deficient bacteria (15).

We have determined the minimal kinetic mechanism for ligation of a single-strand break by human LIG1 and have examined the magnesium dependence of the individual steps.

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1 To whom correspondence should be addressed: 1150 W. Medical Center Dr., 5301 MSRB III, Ann Arbor, MI 48109-0600. Tel.: 734-647-5821; Fax: 734-764-3509; E-mail: pjobrien@umich.edu.

2 The abbreviations used are: LIG1, DNA ligase I; nDNA, nicked DNA.
These experiments revealed that the rate-limiting step changes as a function of magnesium concentration and exposed an Achilles’ heel of LIG1, whereby low magnesium concentrations cause release of the adenylylated DNA intermediate to form a persistent DNA break. This observation provides a rationale for the existence of the aprataxin pathway for repair of adenylylated DNA intermediates and implies that magnesium deficiency could cause defects in DNA repair and replication.

**EXPERIMENTAL PROCEDURES**

**Recombinant DNA Ligase I**—The catalytic domain of human LIG1 (residues 232–919) was expressed in *Escherichia coli* and purified as described previously (10). Cells were lysed in the presence of 1 mM EDTA to preserve the adenylylated form of the enzyme and subsequently purified over a phosphocellulose column and a nickel-nitrilotriacetic acid column. The His tag was removed with PreScission protease, and LIG1 was further purified with Q-Sepharose. The final purified fractions were combined and dialyzed into storage buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM DTT, and 0.1 mM EDTA). Aliquots were snap-frozen and stored at −80 °C. Purity was greater than 95% as judged by SDS-PAGE. Initial concentrations were estimated from the absorbance at 280 nm, using the calculated extinction coefficient. The concentration of active, adenylylated enzyme was determined by titration with nicked DNA substrate, as described below, and this active concentration is reported throughout.

**DNA Substrates**—Oligonucleotides were synthesized by Integrated DNA Technologies or by the Keck Center at Yale University and were purified on denaturing polyacrylamide gels. The portion of the gel containing the full-length oligonucleotide was excised and crushed and extracted by soaking overnight in 500 mM NaCl and 1 mM EDTA. The extracted oligonucleotides were desalted by binding to a C18 reverse phase column (Sep-Pak, Waters) and eluted with 30% (v/v) acetonitrile. Concentrations were obtained from the absorbance at 260 nm using the calculated extinction coefficients. The three oligonucleotides used in this study had sequences 5′-CCGAATCAGTCCGACGACGCATCAGCAC, 5′-GTGCTGATGCCTG, and 5′-P-GTCCGGACTGATTGG-FAM (P indicates 5′ phosphorylation, and FAM indicates the presence of 3′-fluorescein). The nicked, double-stranded DNA substrate (nDNA) was formed by mixing equimolar amounts of the three oligonucleotides in 10 mM NaMOPS, pH 6.5, and 50 mM NaCl and cooling the mixture from 90 °C to 4 °C at a rate of 3 °C per minute.

**Gel-based Ligation Assay**—Ligation reactions were performed at 37 °C. Unless otherwise indicated, the standard buffer contained 50 mM NaMOPS, pH 7.5 (measured at 25 °C), 1 mM dithiothreitol, 0.05 mg/ml BSA, and sufficient NaCl to maintain a constant ionic strength of 150 mM. The amounts of ATP, MgCl₂, nDNA, and LIG1 varied, as indicated below. Preincubation controls established that LIG1 retains 100% of its activity after 1 h in this standard buffer, and all of the kinetic data were collected within this window of time (supplemental Fig. S4). Reactions were quenched in formamide/EDTA (30 mM EDTA in 94% formamide), heated to 95 °C for 5 min to denature the DNA, and resolved on 20% (w/v) denaturing polyacrylamide gels containing 8 M urea. Fluorescein-labeled oligonucleotides were detected with a Typhoon Trio⁺ imager (GE Healthcare) with excitation at 488 nm and emission through a 520-nm band-pass filter. The images were analyzed using ImageQuant TL (GE Healthcare). The intensity of the individual DNA species was corrected for background fluorescence, and the fraction of the total fluorescence was determined by dividing the fluorescence intensity for the desired species by the signal for all other species in the sample. When necessary, this fraction was converted into its concentration by multiplying by the total concentration of DNA in the reaction.

**Rapid Quench Experiments**—Rapid mixing experiments were performed in a KinTek RFQ-3 quench-flow apparatus. One sample loop contained LIG1 and the other contained nDNA, each at double the reaction concentration. The samples were in 1× reaction buffer with the desired magnesium concentration, and the drive syringes contained the same solution. Loaded reactants were allowed to equilibrate for 90 s to reach the correct temperature. Reactions were initiated by mixing of...
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20 μl of each sample and were quenched at the desired times by mixing with 20 μl of 50 mM EDTA in 90% formamide. Samples were analyzed with the standard gel-based ligation assay. Unless otherwise indicated, the typical concentrations of DNA and LIG1 were 80 and 600 nM, respectively. The data were imported into the program Berkeley Madonna, and the curve-fitting function was used to globally fit the levels of intermediate and product by the scheme represented in Fig. 1 (see supplemental material for additional details). Fitting of the data to a model including reversible chemical steps did not produce significantly different results (data not shown). Fits of reactions from individual experiments provided values for the rate constants for adenylyl transfer and nick sealing, and the reported values reflect the average rates determined from at least three independent experiments at each concentration of magnesium.

These rate constants were plotted versus the concentration of magnesium ions and fit by a hyperbolic binding curve (Equation 1), in which \( v_{\text{obs}} \) is the observed rate constant at a given concentration of Mg\(^{2+} \), \( k_{\text{max}} \) is the maximal rate constant at saturating Mg\(^{2+} \), and \( K_{\text{Mg}} \) is the concentration of Mg\(^{2+} \) at which \( v_{\text{obs}} \) is equal to one-half \( k_{\text{max}} \) (KaleidaGraph, Synergy Software).

\[
\frac{v_{\text{obs}}}{k_{\text{max}}} = \frac{k_{\text{max}}}{K_{\text{Mg}} + [\text{Mg}^{2+}]} \quad \text{(Eq. 1)}
\]

**Multiple-turnover Ligation Assays**—Steady state kinetic analysis was performed in the standard ligation buffer, and the temperature was maintained at 37 °C in a circulating water bath. Reaction mixtures were preincubated for 5 min prior to the addition of enzyme. Reactions were arrested by quenching a 4-μl aliquot in 15 μl of a quench solution (50 mM EDTA in 95% formamide), and the extent of ligation was determined as described above (see supplemental Figs. S5–S7 for representative data). The initial rates were determined from the linear rate of substrate disappearance within the first 10% of the reaction. When both intermediate and product were observed, the rate of formation of each species was determined. Values reported represent the average of at least three experiments. The dependence on substrate concentration was fit by the Michaelis-Menten equation (Equation 2), and the magnesium concentration dependence was fit by Equation 3, in which \( K_{\text{Mg}} \) is the concentration of Mg\(^{2+} \) required to reach half of the maximal rate of reaction. The full ATP dependence at 0.2 and 1 mM Mg\(^{2+} \) was fit by the equation for a random, bireactant system that accounts for the depletion of one substrate by the other (see supplemental material).

\[
V_{\text{max}}/|E| = \frac{V_{\text{max}}}[S] (K_{\text{Mg}} + [\text{Mg}^{2+}]) \quad \text{(Eq. 2)}
\]

\[
V_{\text{int}}/|E| = \frac{V_{\text{int}}[\text{Mg}^{2+}]}{(K_{\text{Mg}} + [\text{Mg}^{2+}])} \quad \text{(Eq. 3)}
\]

The efficiency of ligation was defined as the partitioning between nick sealing and release of adenylylated DNA and was determined by dividing the steady state rate of product formation by the sum of the rates of formation of product and intermediate (Equation 4). See the supplemental material for additional information including the equation used for fitting the Mg\(^{2+} \) dependence for the efficiency of ligation.

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\text{Efficiency} = \frac{V_{\text{prod}}}{V_{\text{prod}} + V_{\text{int}}} \quad \text{(Eq. 4)}
\]

**RESULTS**

Gel-based Ligation Assay—We characterized the enzymatic activity of LIG1 on a synthetic 28-mer oligonucleotide duplex that contained a nick with a 3'-hydroxyl and a 5'-phosphate (Fig. 2A). This substrate is identical to the oligonucleotide that was crystallized in complex with LIG1 (10) except for the addition of a fluorescein label at the 3’-end of the downstream 15-mer. The fluorescein label enables detection of the AMP-DNA intermediate and is not expected to alter reaction kinetics because the downstream end of this DNA does not contact LIG1 in the crystal structure. To measure the pre-steady state kinetics for the LIG1-catalyzed reaction, we used a rapid-quench apparatus. Under optimal conditions, DNA ligation occurs on the second time scale, requiring a rapid and efficient quench that traps all of the intermediates. Therefore, we tested
several chemical quenches, including EDTA/formamide, urea, and concentrated sodium hydroxide, and found that EDTA/formamide and sodium hydroxide are equally effective in stopping the ligation reaction (supplemental Fig. S1). In contrast, a urea quench solution takes significantly longer to inactivate LIG1 and results in greater amounts of intermediate and product than either the EDTA or the hydroxide quench solutions. Samples quenched in EDTA/formamide can be directly analyzed by gel electrophoresis, making this a convenient quench. A representative time course for a single-turnover ligation reaction is shown in Fig. 2. Greater than 95% of the nicked DNA is ligated within 2 s, and the buildup and breakdown of the adenylylated DNA intermediate can be readily quantified. The concentration of LIG1 was varied in excess over the concentration of DNA to establish that the concentrations employed were far above the dissociation constant for DNA binding and that the maximal single-turnover rates were determined (supplemental Fig. S2). This gel-based assay allows both pre-steady state and steady state kinetics of the LIG1-catalyzed reaction to be monitored.

Pre-steady State Burst and Active Site Titration of Ligase—Recombinant LIG1 was purified from E. coli in the fully adenylylated form (10, 16). Therefore, we titrated LIG1 against a fixed concentration of DNA in the absence of ATP and allowed the ligation reaction to proceed to completion. The amount of active enzyme is indicated by the amount of DNA that was ligated because each enzyme molecule can turn over only once (supplemental Fig. S3). This analysis assumes that 100% of the enzyme is in the adenylylated form, which was confirmed by carrying out burst experiments in which enzyme was preincubated with ATP to allow for enzyme adenylylation. The identical burst amplitude observed for multiple-turnover and single-turnover reactions indicates that all of the active ligase is already adenylylated. At saturating ATP, the steady state rate is only slightly slower than the pre-steady state rate, and the burst is poorly defined. This demonstrates that enzyme adenylylation and adenyl transfer occur at similar rates when the enzyme is saturated with Mg$^{2+}$ ions and ATP and DNA substrates. The steady state level of adenylylated DNA intermediate is at almost 20% the level of total enzyme when ATP is saturating (150 μM, ○), providing additional evidence that the nick-sealing step is partially rate-limiting under these conditions. The error bars indicate one standard deviation from the mean.

FIGURE 3. Pre-steady state ligation reaction under burst conditions. Formation of DNA product (A) and adenylylated intermediate (B) was monitored during single- and multiple-turnover reactions containing 50 nM ligase, 500 nM nDNA, and 10 mM MgCl$_2$ in the absence of ATP (○) or in the presence of 4 μM (□) or 150 μM ATP (●). In reactions containing ATP, LIG1 was preincubated with ATP to allow for enzyme adenylylation. The identical burst amplitude observed for multiple-turnover and single-turnover reactions indicates that all of the active ligase is already adenylylated. At saturating ATP, the steady state rate is only slightly slower than the pre-steady state rate, and the burst is poorly defined. This demonstrates that enzyme adenylylation and adenyl transfer occur at similar rates when the enzyme is saturated with Mg$^{2+}$ ions and ATP and DNA substrates. The steady state level of adenylylated DNA intermediate is at almost 20% the level of total enzyme when ATP is saturating (150 μM, ○), providing additional evidence that the nick-sealing step is partially rate-limiting under these conditions. The error bars indicate one standard deviation from the mean.
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all conditions, the initial rate portion of the reaction progress curves is linear (supplemental Fig. S7). At a high concentration of Mg$^{2+}$ (30 mM), the ATP dependence follows Michaelis-Menten behavior with a $K_m$ value of 12 $\mu$M and a $k_{cat}$ value of 0.74 s$^{-1}$ (Fig. 5A). In contrast, at 1 mM Mg$^{2+}$, the velocity shows a biphasic dependence on the concentration of ATP (Fig. 5B).

The expected Michaelis-Menten behavior is observed at low concentrations of ATP, but strong inhibition is observed at higher concentrations of ATP. A similar biphasic ATP dependence is observed in reactions with 0.2 mM Mg$^{2+}$, but the inhibitory phase is shifted to a lower concentration of ATP than in the reactions performed at 1 mM Mg$^{2+}$ (Fig. 5C). The inhibition can be explained by the model in which LIG1 requires two Mg$^{2+}$ ions for enzyme adenylylation, with one metal coming from ATP-Mg$^{2+}$ and the other coming from solution (Fig. 5D).

The dissociation constant for ATP binding to Mg$^{2+}$ under similar conditions has been reported to be 10–30 $\mu$M (17, 18), and thus, the concentration of free Mg$^{2+}$ is predicted to be dramatically decreased by the presence of stoichiometric ATP (supplemental Fig. S8). Similar inhibition has been observed for other ATP-dependent enzymes (19, 20). Although the ATP concentration dependence could be readily fit by the requirement for two Mg$^{2+}$ ions at both 1 mM and 0.2 mM MgCl$_2$ (Fig. 5, B and C), these fits gave slightly different estimates for the Mg$^{2+}$ affinity and the maximal rate of ligation (supplemental Fig. S9). The discrepancy between different data sets could be due to experimental error or to additional complexities such as inhibition by free ATP. Therefore, we independently measured the Mg$^{2+}$ dependence of $k_{cat}/K_m$ and $k_{cat}$ under conditions in which magnesium was always in excess over ATP.

Mg$^{2+}$ Dependence of Steady State Ligation—The magnesium dependence for $k_{cat}$ was determined with saturating DNA and

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**FIGURE 4. Magnesium dependence of single-turnover ligation.** Reactions containing 80 nM nDNA, 600 nM LIG1, and Mg$^{2+}$ concentrations that ranged from 0.02 to 19 mM were monitored by quenched flow. The time-dependent changes in the concentration of DNA product and intermediate were fit by the minimal kinetic scheme to obtain the rate constants for adenylyl transfer (○) and nick sealing (□). Both rates increase as a function of Mg$^{2+}$ concentration, and the curves shown are fits to a binding hyperbola. These hyperbolic fits yield maximal rate constants of 2.6 ± 0.6 s$^{-1}$ for adenylyl transfer and 12 ± 2 s$^{-1}$ for nick sealing. The $K_m$ value for the adenylyl transfer step (0.15 ± 0.06 mM) is much lower than the value observed for nick sealing (2.6 ± 0.9 mM). Due to the difference in affinity, adenylyl transfer is mostly rate-limiting at high concentrations of MgCl$_2$, but the two steps become more closely matched at low concentrations of MgCl$_2$. The error bars indicate one standard deviation from the mean.

**FIGURE 5. ATP dependence of LIG1.** Multiple-turnover ligation assays were performed with saturating nDNA (1 $\mu$M) and with varying concentrations of ATP and Mg$^{2+}$. A, the initial rates determined with 30 mM MgCl$_2$ are plotted as a function of ATP concentration. These data were fit by the Michaelis-Menten equation, which yields a $k_{cat}$ value of 0.74 ± 0.09 s$^{-1}$ and a $K_m$ for ATP of 11 ± 3 $\mu$M. B, the ATP dependence with 1 mM MgCl$_2$. The low concentrations of ATP can be fit by the Michaelis-Menten equation (not shown) to yield a $k_{cat}$ value of 0.4 ± 0.06 s$^{-1}$ and a $K_m$ value of 13 ± 4 $\mu$M. C, the ATP dependence at 0.2 mM MgCl$_2$. D, the kinetic model describing the requirement of LIG1 for two Mg$^{2+}$ ions in the enzyme adenylylation step. The biphasic concentration dependence shown in panels B and C was fit by the model in panel D and takes into consideration the depletion of free Mg$^{2+}$ due to the presence of excess ATP (see supplemental material). In panels A–C, the error bars indicate one standard deviation from the mean.
ATP substrates. To account for the binding of Mg$^{2+}$ ions to ATP, which decreases the concentration of free Mg$^{2+}$ available for binding to the second site on LIG1, we calculated the free Mg$^{2+}$ ion concentration for each steady state reaction (Fig. 6A). The steady state $k_{cat}$ value showed a simple hyperbolic dependence on Mg$^{2+}$ ions, with an apparent dissociation constant for Mg$^{2+}$ ($K_M$) of 0.71 mM. The metal binding affinity cannot be ascribed to any single step because the rate-limiting step changes as a function of Mg$^{2+}$ concentration. The maximal turnover number ($k_{cat}$) of 0.81 s$^{-1}$ was used along with the independently determined microscopic rate constants for adenylyl transfer and nick sealing to calculate the microscopic rate constant for enzyme adenylylation of 1.3 s$^{-1}$ (see supplemental material). This rate constant is very similar to the rate constant for adenylyl transfer of 2.6 s$^{-1}$ and explains why the burst phase is poorly defined in pre-steady state reactions with saturating ATP (Fig. 3).

To determine the affinity of LIG1 for Mg$^{2+}$ during enzyme adenylylation, we monitored the steady state reaction with saturating DNA but subsaturating ATP ($k_{cat}/K_m$)ATP. This apparent second-order rate constant for the utilization of ATP can be limited by steps up to and including enzyme adenylylation. To avoid the problem of Mg$^{2+}$ ion chelation that occurs at high concentrations of ATP, Mg$^{2+}$ was in excess of ATP. The dependence of the steady state rate on the concentration of ATP is linear (supplemental Fig. S10). The resulting $k_{cat}/K_m$ values show a simple hyperbolic dependence on the concentration of Mg$^{2+}$ and yield a $K_M$ value of 1.4 mM for an essential Mg$^{2+}$ ion in the enzyme adenylylation step (Fig. 6B). As discussed above, this metal ion is in addition to the Mg$^{2+}$ ion that is already associated with the ATP molecule that is bound from solution (Fig. 5D). At saturating Mg$^{2+}$, the $k_{cat}/K_m$ value for ATP is $6.2 \times 10^4$ M$^{-1}$s$^{-1}$, which is in reasonable agreement with the $k_{cat}/K_m$ value of $6.7 \times 10^4$ M$^{-1}$s$^{-1}$ determined by varying ATP at saturating Mg$^{2+}$ (Fig. 5A). This number is significantly lower than the $k_{cat}/K_m$ value for the DNA substrate of $3 \times 10^7$ M$^{-1}$s$^{-1}$ (supplemental Fig. S5B).

Dead-end Ligation Intermediates—To investigate whether the long lifetime of the adenylylated DNA intermediate that is observed in single-turnover reactions at very low Mg$^{2+}$ concentrations results in off-pathway events, we looked more closely at multiple-turnover reactions performed under similar conditions. The dissociation of LIG1 from an adenylylated DNA intermediate is expected to be irreversible under multiple-turnover conditions because the free enzyme can adenylylate itself and is unable to rebind the adenylylated DNA intermediate. Indeed, multiple-turnover reactions containing 0.2 mM MgCl$_2$ show a significant buildup of adenylylated DNA intermediate (data not shown). Given the complex interplay between ATP and free magnesium concentrations, we expected that a similar situation would occur even at higher concentrations of total magnesium. To test this possibility, we followed steady state ligation reactions with saturating DNA, 1 mM MgCl$_2$, and high levels of ATP. Under these conditions, the depletion of free Mg$^{2+}$ results in significant off-pathway release of the adenylylated DNA intermediate (Fig. 7A, right panel). The level of intermediate that is formed greatly exceeds the enzyme concentration, indicating multiple-turnover release of the intermediate. In contrast, similar reactions with less than 200 μM ATP that proceed to equivalent levels of product formation produce no detectable intermediate (Fig. 7A, left panel). We measured the initial rates for steady state formation of adenylylated DNA intermediate and ligated product to quantify the efficiency of ligation. As ligase can take either the productive pathway of nick sealing or the nonproductive pathway of release of adenylylated DNA (Fig. 7B), this efficiency is calculated from the ratio of the rate for product formation divided by the sum of the rates for product and intermediate formation (Equation 4). Over the range of ATP concentrations tested, the efficiency of ligation falls from near 100% under optimal conditions to 60% with 1 mM Mg$^{2+}$ and 2 mM ATP (Fig. 7B). Although the truncated enzyme that lacks the amino-terminal 232 amino acids appears to be fully active as a ligase, we were concerned that the removal of this portion of the protein might affect the rate of dissociation from the adenylylated DNA intermediate. Therefore, we prepared full-length LIG1 and measured the efficiency of ligation with 1 mM Mg$^{2+}$ and 2 mM ATP. We observed that the full-length enzyme had essentially the same efficiency of ligation as the truncated enzyme under these conditions (supplemental Fig. S11).
DISCUSSION

This work constitutes the first comprehensive kinetic analysis of a human DNA ligase and provides new insight into the multistep reaction. We report the pre-steady state and steady state kinetic parameters for the ligation of a single-strand DNA break by human LIG1. Furthermore, we have investigated the Mg$^{2+}$ dependence of individual steps in the ligase reaction. These experiments revealed that the affinity of LIG1 for Mg$^{2+}$ ions varies along the reaction coordinate and that the rate-limiting step and the efficiency of ligation depend on the concentration of Mg$^{2+}$.

**Minimal Kinetic Framework for LIG1**—The minimal kinetic mechanism for LIG1 is presented in Fig. 8A. The three chemical steps of ligase are essentially irreversible in the presence of excess ATP. The rates of enzyme adenylylation and adenylyl transfer are evenly matched when the nicked DNA substrate is saturating. The multiple-turnover rate constant ($k_{cat}$) is 0.8 s$^{-1}$ in this case. However, it is important to note that DNA ligation occurring in excision repair pathways may not require multiple-turnover ligation. We found that the recombinant LIG1 that we purified from *E. coli* is fully adenylylated and extremely stable. The pool of adenylylated ligase may be sufficient for ligation of low numbers of single-strand DNA breaks, and there may not be a strong driving force to increase the rate of enzyme activation. When Mg$^{2+}$ is saturating, the nick-sealing step is significantly faster than the DNA adenylylation step, which would help to ensure efficient coupling between nick recognition and nick sealing. However, the lower affinity of Mg$^{2+}$ in the nick-sealing step leaves LIG1 vulnerable to uncoupling of these two chemical steps, which has biological implications (see below).

Although the catalytic core is universally conserved in all ligases, there are additional domains that vary between different isoforms in a given organism and between ligases from different organisms. Therefore, it is interesting to compare the ligation activity of human LIG1, which is one of the largest ligases, with the ligase from *Chlorella* virus, which is one of the smallest ligases. Recent pre-steady state analysis of the *Chlorella* virus DNA ligase revealed almost identical rate constants for adenylyl transfer and nick sealing as we have observed for human LIG1 (21). Consistent with their similar kinetic para-
eters in vitro, both enzymes are able to rescue the growth of yeast lacking cdc9, the replicative DNA ligase (22, 23).

DNA ligases require divalent metal ions for catalysis; therefore, we have investigated the magnesium dependence of the different steps in the LIG1-catalyzed reaction. The complex behavior observed when ATP and Mg$^{2+}$ concentrations are similar strongly suggests that LIG1 requires two Mg$^{2+}$ ions for adenylylation (Fig. 5D). One Mg$^{2+}$ ion is expected to be bound along with the ATP substrate, but a second is required that has much weaker affinity ($K_{Mg} = 1.4 \text{ mM}$). A similar conclusion was reached in a study of the T4 bacteriophage DNA and RNA ligases (24). The use of more than one metal ion for phosphoryl and nucleotidyl transfer appears to be quite common, with examples ranging from protein kinases (19) to DNA polymerases and nucleases (25). It is likely that subsequent steps also involve more than one metal ion, and there is evidence of two metal ion-binding sites in the structure of the adenylylated DNA intermediate (10). The magnesium dependences for adenylyl transfer and nick sealing can be explained by a single catalytic metal ion, but they are also consistent with two metal ions that have different affinity. In this case, the value of $K_{Mg}$ that we have determined corresponds to the binding of the weakest essential Mg$^{2+}$ ion. Our experiments reveal that the affinity for this metal ion changes significantly between different steps of the ligation reaction, increasing by an order of magnitude between the enzyme adenylylation step and the adenylyl transfer step and then decreasing by a similar degree in the nick-sealing step (Fig. 8B). These changes in Mg$^{2+}$ affinity could be explained by conformational changes in the substrate or enzyme or could indicate that unique metal-binding sites are involved in different steps of the reaction. Additional work is required to determine the identity and catalytic roles of these Mg$^{2+}$ ions. In many phosphoryl transfer enzymes, Mg$^{2+}$ ions play roles in activating the nucleophile and stabilizing the development of negative charge.

**Biological Implications of the LIG1 Magnesium Dependence**—The decreased affinity of LIG1 for Mg$^{2+}$ ions in the final nick-sealing step of the reaction renders LIG1 susceptible to changes in the concentration of free Mg$^{2+}$ ions. LIG1 faithfully completes ligation at high concentrations of magnesium; however, low concentrations of magnesium cause the enzyme to abort ligation. The released adenylylated DNA intermediate cannot be ligated because LIG1 reacts quickly with ATP and the occupancy of the AMP-binding pocket precludes rebinding. Thus, repair pathways are needed to remove the 5’-blocking adenylyl group to allow another opportunity for ligation. In the context of oxidative DNA damage, it has been suggested that mammalian DNA ligases attempt to ligate nicks lacking 3’-hydroxyl groups (26). Our finding that low Mg$^{2+}$ or high ATP concentration can lead to significant abortive ligation on a normal DNA nick provides an additional possibility. If a DNA break is repaired under conditions of imbalanced Mg$^{2+}$ or Mg$^{2+}$-binding metabolites, then there is a significant risk that LIG1 will fail to complete ligation. The abundance of Mg$^{2+}$ (total concentration 10–40 mM) and similarity to other metal ions have made it difficult to directly measure free Mg$^{2+}$ concentration in the cell; however, the data that are available for several different cell types suggest that the majority of Mg$^{2+}$ is bound and the free Mg$^{2+}$ is in the range of 0.2–3 mM (27). The in vitro ligation experiments show that this range of Mg$^{2+}$ ion concentration is where LIG1 becomes susceptible to abortive ligation and that even slight decreases in the availability of free Mg$^{2+}$ will further increase the burden (Fig. 7). Thus, abortive ligation intermediates could be frequently generated in a wide variety of cellular contexts.

Although this abortive ligation was observed for a truncated form of LIG1 that lacks the first 232 amino acids, we observed that the amino terminus of LIG1 does not change the efficiency of ligation under conditions of limiting Mg$^{2+}$ in vitro (supplemental Fig. S11). However, this portion of the protein is important for nuclear localization and for interactions with other proteins, including the sliding clamp proliferating cell nuclear antigen (7, 13, 14). It remains to be tested whether protein-protein interactions alter the efficiency of LIG1 in the cell, but the existence of dedicated repair pathways for adenylylated DNA suggest that abortive ligation does occur (26).

There are two pathways that would enable the repair of adenylylated DNA intermediates (Fig. 9). The first pathway is the direct repair catalyzed by aprataxin. Aprataxin is a hydrolase that catalyzes the removal of the AMP group from adenylylated DNA nicks to regenerate the 5’-phosphate (26, 28). Defects in aprataxin cause the neurodegenerative disease ataxia with oculomotor apraxia 1, which is characterized by the loss of specific neuronal cells. It has been suggested that the affected neuronal cells either experience higher levels of oxidative damage or lack alternative repair pathways (26). It is expected that long patch base excision repair would also be an effective way of repairing an abortive ligation intermediate (Fig. 9). In this pathway, strand displacement by a replicative polymerase would be followed by flap cleavage by FEN1 to generate a new 5’-phosphate that would be a substrate of DNA ligase (Fig. 9). In support of this proposed pathway, strand displacement and FEN1 cleavage have been shown to accept flap substrates with 5’-blocking lesions (29).

**Kinetic Mechanism of DNA Ligase**

![Kinetic Mechanism of DNA Ligase](image-url)

**FIGURE 9. Pathways for repair of 5’-adenylated DNA resulting from abortive ligation.** Direct repair of a 5’-adenylated nick is catalyzed by aprataxin (APTX). Alternatively, strand displacement by a replicative DNA polymerase (pol/e) generates a 5’ flap structure that can be processed by FEN1 to regenerate the adjacent 3’-hydroxyl (OH) and 5’-phosphate (P) required by DNA ligase.
Our findings indicate that 3’-blocking lesions are not the only pathway for abortive ligation by human ligases and suggest that there are multiple cellular perturbations that could impact the frequency with which 5’-adenylylated DNA breaks are formed. As persistent DNA breaks are expected to block transcription and to be converted into double-strand breaks upon DNA replication, the formation and repair of 5’-adenylylated DNA are expected to be critical to cellular viability and genomic stability. Additional work is needed to understand the physiological conditions and molecular pathways important for the formation and repair of 5’-adenylylated DNA breaks.

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