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Kinetic Characterization of Single Strand Break Ligation in Duplex DNA by T4 DNA Ligase[S]

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Background: T4 DNA ligase catalyzes the formation of phosphodiester bonds in dsDNA.
Results: Single turnover rates of all chemical reaction steps exceed steady state turnover rates by 10-fold.
Conclusion: Product release or a postligation conformational change is rate-limiting during turnover.
Significance: This study represents the first detailed analysis of the kinetic mechanism of nick ligation by T4 DNA ligase.

T4 DNA ligase catalyzes phosphodiester bond formation between juxtaposed 5′-phosphate and 3′-hydroxyl termini in duplex DNA in three steps: 1) enzyme-adenylylate formation by reaction with ATP; 2) adenylyl transfer to a 5′-phosphorylated polynucleotide to generate adenylylated DNA; and 3) phosphodiester bond formation with release of AMP. This investigation used synthetic, nicked DNA substrates possessing either a 5′-phosphate or a 5′-adenylyl phosphate. Steady state experiments with a nicked substrate containing juxtaposed dC and 5′-phosphorylated dT deoxynucleotides (substrate 1) yielded k_{cat} and k_{cat}/K_m values of 0.4 ± 0.1 s^{-1} and 150 ± 50 μM^{-1} s^{-1}, respectively. Under identical reaction conditions, turnover of an adenylylated version of this substrate (substrate 1A) yielded k_{cat} and k_{cat}/K_m values of 0.64 ± 0.08 s^{-1} and 240 ± 40 μM^{-1} s^{-1}. Single turnover experiments utilizing substrate 1 gave fits for the forward rates of Step 2 (k_2) and Step 3 (k_3) of 5.3 and 38 s^{-1}, respectively, with the slowest step ~10-fold faster than the rate of turnover seen under steady state conditions. Single turnover experiments with substrate 1A produced a Step 3 forward rate constant of 4.3 s^{-1}, also faster than the turnover rate of 1A. Enzyme self-adenylylation was confirmed to also occur on a fast time scale (~6 s^{-1}), indicating that the rate-limiting step for T4 DNA ligase nick sealing is not a chemical step but rather is most likely product release. Pre-steady state reactions displayed a clear burst phase, consistent with this conclusion.

DNA ligases are divalent metal cation-dependent enzymes that utilize ATP or NAD^+, depending on the ligase, to catalyze phosphodiester bond formation between adjacent double-stranded polynucleotide termini possessing a 3′-hydroxyl and a 5′-phosphate (1–3). DNA ligases are essential enzymes for DNA replication and repair and are found ubiquitously in eukaryotes, bacteria, archea, and many viruses. These enzymes are closely related in structure and mechanism to the other members of the nucleotidyltransferase family (RNA ligases and RNA capping enzymes), consisting of core nucleotidyltransferase and oligonucleotide binding domains flanked by variable N- and C-terminal DNA binding and protein-protein interaction domains specific to each enzyme. All known DNA ligases operate through a common, three-step mechanism (Fig. 1). In Step 1, an adenylyl group is transferred from NAD^+ or ATP, dependent on the ligase, to an active site lysine. In Step 2, the adenylylated enzyme transfers the adenylyl group to a 5′-phosphate of the duplex DNA substrate, forming an adenylylated DNA (AppDNA)[2] intermediate. Finally, in Step 3, nucleophilic attack of a 3′-hydroxyl on the App group results in phosphodiester bond formation with concomitant release of AMP. All three steps have been shown to be divalent metal cation-dependent (typically Mg^{2+}), although the number of cations required and the binding affinity vary throughout the steps of reaction (4, 5).

Support for this mechanism comes from the study of ligases from several organisms, including *Chlorella* virus, T series bacteriophages, *Escherichia coli*, and humans. Crystal structures (particularly those of *Chlorella* virus DNA ligase (6, 7), human DNA ligases I (8) and III (9), and *E. coli* DNA ligase (10) bound to DNA substrates), mutational analysis of *Chlorella* virus DNA ligase (11, 12) and *E. coli* DNA ligase (13) and numerous studies of substrate specificity and steady state kinetics have all contributed to the understanding of the ligation mechanism (4, 5, 14–18).

T4 DNA ligase was one of the first DNA ligases isolated (19, 20), and the enzyme has since seen widespread use as a tool in molecular biology applications, such as cloning, sequencing, and gene synthesis (21). T4 DNA ligase readily accepts both nicked dsDNA and double-stranded breaks with sticky ends and accepts substrates with various base pair mismatches (22, 23). Furthermore, it is unusual among the characterized DNA ligases for its ability to efficiently seal blunt-ended DNA fragments in the absence of a ligation enhancer, such as polyethylene glycol (24–27) or large excesses of enzyme over the substrate (28). Despite this property, it appears to share the general ligation mechanism of other known DNA ligases (Fig. 1) (4, 15, 29).

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5.

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[2] The abbreviations used are: AppDNA, nicked DNA substrate possessing a 5′-adenylyl phosphate; pDNA, nicked DNA substrate possessing a 5′-phosphate; FAM, 6-carboxyfluorescein; CE, capillary electrophoresis; RQF, rapid quench-flow.

Although T4 DNA ligase has seen extensive use in modern biotechnology applications, there are still many unanswered questions about its detailed mechanistic and kinetic behavior. No crystal structure of T4 DNA ligase exists, and only limited mutational analysis has been performed, which identified Lys-159 as the site of enzyme self-adenylylation in Step 1 (29). Early characterization identified its approximate K_m (1.5 nM) for internal phosphomonoesters and turnover rate for nick sealing (1.7 μmol/mg enzyme in 20 min, a turnover number of ~0.075 s^{-1}), using a heterogeneous DNA substrate (20). Detailed kinetic characterization of T4 DNA ligase exists only for Step 1 (4, 14–16). Studies have explored the rates of ATP binding (k_{+ATP} = 0.87 μM^{-1} s^{-1}), reaction to the covalent adenylyl adduct (k_1 = 12.83 s^{-1}), and cleavage of this adduct by inorganic pyrophosphate (k_{-1} = 0.5 μM^{-1} s^{-1}). These rate constants were determined based on stopped-flow measurements at 20 °C of tryptophan fluorescence quenching by adenosine. The Mg^{2+} dependence of each substep was also investigated, indicating that the true substrate for self-adenylylation is the dimagnesium-ATP complex and that pyrophosphate leaves with a single magnesium still bound (4, 5, 15).

AppDNA does not build up during turnover to a measurable degree, thus Step 2 has been hypothesized to be the rate-limiting step in DNA ligase turnover (30). Its presence as a reaction intermediate has been demonstrated through several methods. The use of low pH (31) or substrates with base pair mismatches or gaps (32, 33) causes buildup of AppDNA in the reaction. AppDNA has been shown to convert to ligated product in the absence of exogenous ATP with stoichiometric release of AMP (31). The rate of formation of AppDNA (Step 2, k_2) has not been measured under normal reaction conditions for T4 DNA ligase; nor has the rate of Step 3 phosphodiester bond formation (k_3). These rate constants have been reported very recently for *Chlorella* virus DNA ligase at 22 °C (17, 18) and a truncated human DNA ligase I at 37 °C (5). Both of these enzymes show similar rates for Step 2 adenylyl transfer (k_2 ~2.5 and 2.6 s^{-1}, respectively) and phosphodiester bond formation (k_3 = 13–24 and 12 s^{-1}).

The study described herein investigated the overall reaction rate and the detailed kinetics of adenylyl transfer and phosphodiester bond formation by T4 DNA ligase. These studies used defined dsDNA substrates that contained a single nick, facilitating data interpretation. We report the steady state and single turnover kinetics at millisecond time scales for both the 5′-phosphorylated substrate and preadenylylated DNA with deadenylylated enzyme. The results show that T4 DNA ligase has both a fast turnover rate and a large k_{cat}/K_m as compared with other known ligases. The rates of adenylyl transfer and phosphodiester bond formation are reported and show the formation of AppDNA by T4 DNA ligase to be kinetically competent. Both adenylyl transfer and phosphodiester bond formation appear to be effectively irreversible under the reaction conditions tested. Interestingly, the rates of the slowest chemical steps for reaction of both phosphorylated substrate and adenylylated substrate were found to be ~10 times faster than the steady state turnover rates for each substrate, suggesting that the true rate-limiting step during turnover is release of the ligated product or a post-product release conformational change. Pre-steady state experiments show a burst phase followed by a slower linear phase, consistent with this conclusion. To our knowledge, this study represents the first demonstration of a postligation step being rate-limiting for any known ligase.

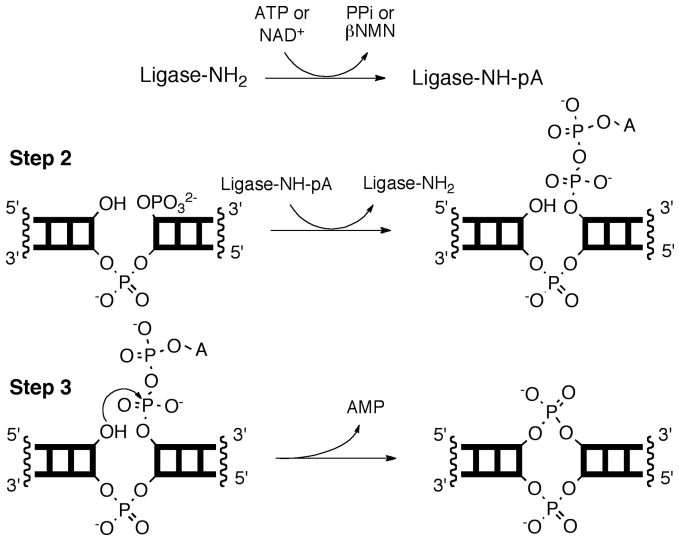

FIGURE 1. **The chemical mechanism of DNA ligases.** The ligation of 5′-phosphate and 3′-hydroxyl termini in DNA proceeds in three steps. Step 1 is the self-adenylylation of an active site lysine using ATP or NAD^+, depending on the ligase; Step 2 is the transfer of the adenylyl group to the 5′-phosphorylated terminus; Step 3 is the formation of the phosphodiester bond with loss of an AMP leaving group. *β-NMN*, β-nicotinamide mononucleotide; *P*, phosphate; *A*, 5′-adenosine.

EXPERIMENTAL PROCEDURES

Buffers and Chemicals—T4 DNA ligase buffer (50 mM Tris, pH 7.5, at 25 °C, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP) was obtained as a 10× stock from New England Biolabs, Inc. ATP-free T4 DNA ligase buffer (50 mM Tris, pH 7.5, at 25 °C, 10 mM $MgCl_2$, 10 mM DTT) was prepared from 1 M stocks of each salt from Amresco or New England Biolabs. Diluent A (10 mM Tris-HCl, pH 7.4, at 25 °C, 1 mM DTT, 0.1 mM EDTA, 50% glycerol, 200 μg/ml BSA, 50 mM KCl) was obtained from New England Biolabs. Oligonucleotide annealing buffer (10 mM Tris, pH 7.5, at 25 °C, 50 mM KCl, 0.1 mM EDTA) was prepared as a 10× stock.

Preparation of dsDNA Substrates—HPLC-purified synthetic single-stranded oligonucleotides were obtained from Integrated DNA Technologies as lyophilized solids, with the exception of 5′-adenylylated sequences, which were provided by the New England Biolabs Organic Synthesis division (34). Oligonucleotides were stored as 100 μM stocks in water. The nicked dsDNA substrates (Fig. 2) were made by combining the 6-carboxyfluorescein (FAM)-labeled sequence (10 μM) with 1.2 mol

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5′-TATAACTTTACTTCTATTGM NGATGGGACCTACAATGTACCAGAAGCGTC-FAM
3′AM-ATATTGAAATGAAGATAACX-YCTACCCTGGATGTTACATGGTCTTCGCAG
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FIGURE 2. **Sequence of nicked dsDNA substrates.** *AM*, 3′-$PO_3(CH_2)_5NH_2$. *M/X* and *N/Y*, correctly base-paired nucleotides with *N* possessing either a 5′p or 5′ App group. The remaining 5′-ends of the helix are unmodified hydroxyls.

eq of the upstream fragment and the complementary strand in DNA annealing buffer. This mixture was heated to 85 °C for 2 min and allowed to cool slowly in a heat block to room temperature over 3 h. Annealed dsDNA stocks were stored at −20 °C. The concentration of dsDNA stocks is expressed in terms of the FAM-labeled fragment concentration.

Preparation and Handling of T4 DNA Ligase—The enzyme concentration of 2,000,000 units/ml T4 DNA ligase (New England Biolabs) was determined to be 35 ± 2 μM by A280 using a calculated ϵ_{280} = 57,300 M^{-1}. The concentration of active enzyme was determined by reacting T4 DNA ligase (3–4 μM) in a 200-μl total volume of T4 DNA ligase buffer containing [α-^{32}P]ATP (specific activity 33,760 cpm/nmol) for 30 min at 25 °C. The protein was then separated from small molecules using two Centrispin-10 desalting columns in series, and the flow-through (25 μl) was counted on a PerkinElmer Life Sciences Tri-Carb 2900TR liquid scintillation counter. The nmol of ^{32}P coeluting with the protein was determined by comparison with a [^{32}P]ATP standard curve. Background was determined by using samples with no protein. The protein concentration in the flow-through and in the initial reaction volume was estimated using a Bio-Rad protein assay (Bradford), and the nmol of ^{32}P was corrected to account for the drop in concentration. A 1:1 enzyme/^{32}P reaction stoichiometry was assumed. Three samples were run for each lot of ligase and yielded a calculated concentration of active protein in the 2,000,000-unit/ml stocks of 32 ± 1 μM.

For fully adenylylated lots, the concentration of active, adenylylated enzyme was additionally checked by ligation of a FAM-labeled substrate in the absence of ATP, with the amount of ligated DNA presumed to be equal to the amount of active adenylylated enzyme. This method gave a concentration of 31 ± 0.3 μM, in agreement with the self-adenylylation results. T4 DNA ligase stocks were diluted to the desired concentration for each assay using Diluent A and stored at −20 °C.

Determination of Ligase Adenylylation State—Ligase adenylylation state was determined using mass spectrometry. Enzyme samples were diluted to 1 μM in 50 mM Tris, pH 7.5, at 25 °C and analyzed by reverse phase liquid chromatography (LC) followed by electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS) using an Agilent 1200 series nano-LC connected directly to an Agilent 6210 series ESI-TOF MS. A custom reverse phase chip (Agilent Technologies), containing an integrated trapping column (40-nl capacity), separation column, and nano-ESI emitter (75 μm × 150 mm, both packed with PLRP-S, 5-μm particles, 1000-Å pore size) was used for protein separation (35). The column was equilibrated with 0.1% formic acid in water containing 15% acetonitrile, loaded at 2–4 μl/min, and run at 500 nl/min with a 10-min linear gradient from 15 to 90% acetonitrile. The mass spectra were acquired from 150 to 3200 *m*/*z*, one cycle/s, and 10,000 transients/scan using an ionization energy of 1875 V, fragmentor of 215 V, and drying gas of 275 °C at 4.0 liters/min. The acquired spectra were extracted and deconvoluted with Agilent MassHunter Qualitative Analysis Software (with Bioconfirm) B 2.0.2 using a mass range of 10,000–100,000 daltons. The ratio of the peak area of each enzyme form was used to estimate the percentage of adenylylation. Using this method, one enzyme lot that was >95% adenylylated and one that was <5% adenylylated were identified for use in the ligation assays.

Fragment Analysis by High Throughput Capillary Electrophoresis (CE)—CE samples were prepared by dilution to 0.5–2 nM in total FAM label using double-distilled H_2O. When dilution was <100-fold, the samples were desalted using Edge-Bio Performa™ VTR 96-well plates before injection. The GeneScan™-120 LIZ™ size standard was diluted 1:40 in formamide, and 10 μl of this solution was combined with 1 μl of each sample before application to either an Applied Biosystems 3130xl Genetic Analyzer (16-capillary array) or an Applied Biosystems 3730xl Genetic Analyzer (96-capillary array) at a 36-cm capillary length with POP7 polymer. Data were collected via Applied Biosystems Data Collection software and analyzed using Applied Biosystems PeakScanner software (version 1.0). The retention times and areas of all peaks in the blue (FAM) channel were recorded. Oligonucleotides (30-mer starting material, adenylylated 30-mer, and 50-mer ligation product) were identified by coelution with synthetic standards. The fraction of each oligonucleotide in the sample was determined by dividing the peak area of each by the total peak area of all three oligonucleotides.

Steady State Ligation Assay—A typical assay mixture consisted of 70 μl of water, 10 μl of 10× T4 DNA ligase assay buffer or 10× ATP-free assay buffer, and 10 μl of a 10× stock of T4 DNA ligase in Diluent A. The components were mixed by gentle pipetting and incubated at 16 °C for 2 min. The ligation assay was initiated by the addition of a 10× solution of the nicked dsDNA substrate followed by gentle mixing and incubation at 16 °C. Final concentrations of enzyme were typically 0.1 or 1.0 nM. Final concentrations of DNA were 1 nm to 1 μM in the FAM label, with most turnover assays run at 1 μM DNA. Time points (5 μl) were removed and quenched with either 10 μl of 100 mM EDTA or 10 μl of 100 mM EDTA in 80% formamide.

Rapid Quench-Flow (RQF) Ligation Assay—Rapid quench experiments were performed using a KinTek RQF-3 instrument. Samples were prepared with T4 DNA ligase (typically 1 μM) in syringe A and the DNA substrate (typically 200 nM) in syringe B and initiated by rapid mixing. Reactions were performed at 16 °C and quenched with 1 N H_2SO_4 containing 0.25% SDS. The buffer in the drive syringes was either 1× T4 DNA ligase assay buffer or ATP-free assay buffer, depending on the experiment. After each time point was collected, 20 μl was immediately diluted into 400 μl of 100 mM Tris, pH 8.0, 10 mM EDTA to raise the sample pH to between 7 and 8. At the end of each experiment, the samples were further diluted with water and desalted as described above for CE fragment analysis.

RQF assays were alternatively initiated by the addition of Mg^{2+} to premixed substrate and enzyme. Under these reaction conditions, syringe A contained both T4 DNA ligase and the nicked DNA substrate in 50 mM Tris, pH 7.5, 10 mM DTT, and 5 mM EDTA. Syringe B contained 50 mM Tris, pH 7.5, 10 mM DTT, 25 mM $MgCl_2$, and no enzyme or substrate. Data points were collected and quenched as described above.

RQF Assay for Enzyme Self-adenylylation—The RQF was utilized as above with T4 DNA ligase (5 μM, <5% adenylyated in ATP-free buffer) in syringe A and ATP (2 mM ATP added to 1× ATP-free buffer containing 200 μCi of [α-^{32}P]ATP/ml solu-

$$E + A \underset{k_{-ATP}}{\overset{k_{+ATP}}{\rightleftharpoons}} EA \underset{k_{-1}}{\overset{k_{1}}{\rightleftharpoons}} F \quad (1)$$

$$F + B \underset{k_{-DNA}}{\overset{k_{+DNA}}{\rightleftharpoons}} FB \underset{k_{-2}}{\overset{k_{2}}{\rightleftharpoons}} EX \underset{k_{-3}}{\overset{k_{3}}{\rightleftharpoons}} EP \xrightarrow{k_{off}} E + P \quad (2)$$

tion) in syringe B. Drive syringes contained ATP-free buffer, and a quench of 250 mM EDTA plus 0.25% SDS was used. Collected time points were treated by passing 25 μl through a Centrisep® 10 spin column equilibrated in 50 mM Tris, pH 7.5, 0.1% SDS. The flow-through was mixed with 10 μl of 3× SDS loading buffer, and 20-μl aliquots were loaded onto SDS-polyacrylamide gels and run at 100 V. After staining, the protein band was cut out and counted on a PerkinElmer Life Sciences Tri-Carb 2900TR liquid scintillation counter. Background was determined by averaging a sample lacking ATP and a sample lacking enzyme. Completion (>95% reaction) was judged by the cpm present in a sample incubated for 60 s before quenching, and the amount of reaction at each time point was expressed as a fraction of maximum activity.

Determination of Turnover Number for Nicked Substrates—The turnover number (k_{obs}) for each tested substrate was determined from steady state ligation assays in the presence of ATP with 1 nM ligase and 1 μM substrate. The slope of the line of fraction product *versus* time was determined by linear regression in Microsoft Excel 2008. The determined slope was multiplied by the substrate concentration, and divided by the enzyme concentration to generate a turnover number (k_{obs}) in s^{-1}. Six repeats were performed for each substrate. The reported k_{obs} is the mean, and the reported error is one S.D. The turnover number for deadenylylated enzyme ligating AppDNA was determined in the same manner, except the assay mixture lacked ATP, and the enzyme used was <5% adenylylated.

Fitting Kinetic Data by Simulation—Data fitting was performed through direct simulation of mechanistic models to untransformed reaction data using KinTek Global Kinetic Explorer (version 2.6.1602) (36, 37). The full model used for data fitting is shown in Equations 1 and 2, where E represents T4 DNA ligase lacking an adenylyl group (apoligase); A is ATP; F is adenylylated T4 DNA ligase; B is nicked dsDNA with a 5′-phosphate flanking the nick (pDNA); X is nicked dsDNA adenylylated at the nick (AppDNA); and P is ligated dsDNA. The rates of PP_i and AMP release were assumed to be fast, and the exogenous concentrations were too low to cause significant reverse reaction rates (4, 38). In general, chemical steps were fit assuming irreversibility, based on literature assumptions (5, 17) and a lack of evidence for reverse reactions in the experimental data modeled ($k_{-2} = k_{-3} = 0$). Binding steps were typically fit as if they were irreversible second order reactions to form the bound reactive complex or as reversible reactions with the on-rate set to diffusion-limited. In most experiments, only a subset of the above steps could be fit meaningfully to the data; the specific models fit to each data set are described below.

Estimation of k_{cat} and K_m—To measure the Michaelis-Menten parameters k_{cat} and K_m, a series of steady state assays were performed using the steady state ligation protocol described above. Time points were collected for reactions of T4 DNA ligase (0.1 nM) with various nicked substrate concentrations (1, 2, 5, 10, 20, and 50 nM). Typically, reactions reached an apparent end point asymptote at 10–15% unreacted starting material remaining (>85% ligation). This material was assumed to be in a form not ligatable on the time scale of the experiments, presumably due to improper annealing. For fitting, the substrate concentration was corrected by subtracting the amount of unreacted substrate. The corrected time courses for substrate consumption were fit to a simple steady state model shown in Equation 3.

$$E + B \xrightarrow{k_{cat}/K_M} EB \xrightarrow{k_{cat}} E + P \quad (3)$$

Steps were assumed to be irreversible, and product release was assumed to be very fast relative to the chemistry. Chemistry was assumed to be irreversible because in the absence of excess AMP, T4 DNA ligase is not known to act as a nicking enzyme, and no truncated side products were observed in fragment analysis. The data were fit using KinTek Global Kinetic Explorer over the full time course and concentration series. The experiment was repeated three times for each substrate tested, and the reported values are the mean of three experiments with the reported error as one S.D. The average of the data sets is presented with a simulation using these averaged fit constants.

Determination of Rate Constants of Chemical Steps (k_2 and k_3) from Single Turnover Data—Single turnover data experiments were run using the RQF procedure described above. Each experiment used T4 DNA ligase (500 nM) and nicked dsDNA substrate (100 nM) in the absence of ATP. Ligase >95% adenylylated was used for reactions of pDNA, and ligase <5% adenylylated was used for AppDNA. Time points were collected in triplicate, and the results were averaged. Starting material remaining after 60 s was assumed to be in a non-ligatable form (typically ~10% of the starting material remained unreacted). The quantity of remaining starting material was subtracted from the substrate measured at each time point as well as from the initial concentration of DNA. The data were fit using KinTek Global Kinetic Explorer to the model described above, excluding steps related to enzyme self-adenylylation and the release of ligated product. Fits were attempted assuming both chemical steps were irreversible and the binding was fast and saturating ($k_{+DNA} > 1000\ \mu M^{-1}\ s^{-1}$).

Product Disassociation Rate (k_{off}) from Pre-steady State Burst Analysis—Pre-steady state reactions were performed using the RQF assay described above at final concentrations of 100 nM nicked dsDNA and 30 or 50 nM T4 DNA ligase in the presence of ATP. Time points were collected over 5 s. Each time point was measured in triplicate, and the results were averaged. The data were fit to the standard model fixing k_{+ATP} and k_1 to their literature values (4, 5, 15), all reverse chemistry rates to 0, and k_2 and k_3 to the values determined in the single turnover of pDNA described above. The rate of product disassociation (k_{off}) was allowed to vary. The predicted output of [product] was additionally multiplied by a scaling factor (a) that was also allowed to vary in the simulation.

TABLE 1
Nicked dsDNA substrate turnover rates
The generic version of the substrate used is shown in Fig. 2.

Substrate	M/X	N/Y[a]	k_{obs}[b]	Error
			s^{-1}	
2	A/T	pA/T	0.055	0.007
3	C/G	pA/T	0.068	0.007
4	G/C	pA/T	0.063	0.003
5	T/A	pA/T	0.065	0.013
6	A/T	pC/G	0.11	0.01
7	C/G	pC/G	0.10	0.04
8	G/C	pC/G	0.16	0.05
9	T/A	pC/G	0.085	0.005
10	A/T	pG/C	0.053	0.008
11	C/G	pG/C	0.065	0.008
12	G/C	pG/C	0.068	0.010
13	T/A	pG/C	0.057	0.008
14	A/T	pT/A	0.085	0.005
1	C/G	pT/A	0.13	0.04
15	G/C	pT/A	0.13	0.01
16	T/A	pT/A	0.10	0.02
1A	C/G	AppT/A	0.29	0.06

[a] p, 5′ phosphate; App, 5′-adenylyl phosphate.
[b] The reported k_{obs} was measured at 1 nM T4 DNA ligase and 1 μM substrate and represents the average of six repeats with the error one S.D. from the mean.

RESULTS

Estimation of Michaelis-Menten Parameters for Nicked dsDNA—To estimate kinetic parameters under multiple-turnover conditions, a concentration series was performed for one nicked DNA substrate and its adenylylated analog. The substrate used was as illustrated in Fig. 2, with M/X = C/G and N/Y = pT/A for substrate **1** and N/Y = AppT/A for substrate **1A** (where M/X and N/Y represent correctly base-paired nucleotides, and p and App represent 5′-phosphate and 5′-adenylyl phosphate, respectively). Initial studies at saturation were run with 1 μM DNA substrate and 1 nM ligase and with ATP in the standard assay buffer for substrate **1** and without ATP for **1A**. Under these conditions, the rate of conversion to ligated product was linear, and turnover numbers (k_{obs}) of 0.13 ± 0.04 and 0.29 ± 0.06 s^{-1} were measured for **1** and **1A**, respectively (Table 1).

The Michaelis-Menten parameters k_{cat} and k_{cat}/K_m were estimated by running concentration series for both substrates (supplemental Fig. S1), holding enzyme concentration constant. Reaction time courses were run at 0.1 nM T4 DNA ligase and 1, 2, 5, 10, 20, or 50 nM substrate. Each concentration series was fit as described under "Experimental Procedures" to a simple Michaelis-Menten model, and the constants found in the three fits were averaged. Over three runs, the k_{cat} was determined to be 0.4 ± 0.1 s^{-1}, and k_{cat}/K_m was determined to be 150 ± 60 μM^{-1} s^{-1} (representing a K_m of ~2.5 nM). A similar reaction series run on substrate **11** (see Table 1) (data not shown) found similar constants, with a k_{cat} of 0.25 s^{-1} and an apparent K_m of 0.9 nM.

It was noted that the k_{cat} measured for substrate **1** appeared slightly higher in this experiment than the k_{obs} measured at 1 μM substrate (0.4 s^{-1} *versus* 0.13 s^{-1} at 1 μM). Additionally, if the time course for each substrate concentration was fit separately, the rate in terms of k_{cat} decreased slightly as the concentration of substrate increased, with the largest effect at 20 and 50 nM **1**. This result is suggestive of substrate inhibition, quite likely due to the nonspecific interaction of the ligase with the DNA. Indeed, reaction of 100 nM substrate **1** with 0.1 nM T4 DNA ligase in the presence of added carrier DNA (100 μg/ml pUC19 vector, preincubated 10 min with ligase, ~150 μM in base pairs) gave a k_{obs} value that was half that found when reacting 100 nM substrate **1** in the absence of added carrier (5 μM in base pairs), giving confidence that the small decrease in turnover rate seen at 1 μM substrate (50 μM base pairs) can be explained primarily by nonspecific inhibition by binding to dsDNA.

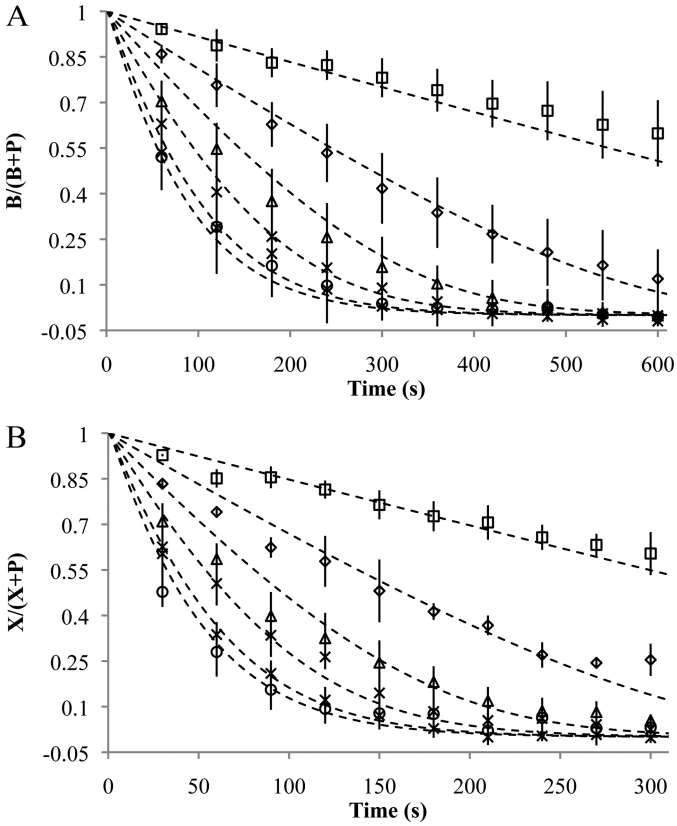


FIGURE 3. **Determination of k_{cat} and k_{cat}/K_m for T4 DNA ligase and nicked substrates.** Shown is reaction of 1 nM T4 DNA ligase with 1 nM (○), 2 nM (*), 5 nM (×), 10 nM (△), 20 nM (◇), and 50 nM (□) substrate **1** in standard assay buffer at 16 °C (*A*) and 1 nM T4 DNA ligase (<5% adenylylated) with 1 nM (○), 2 nM (*), 5 nM (×), 10 nM (△), 20 nM (◇), and 50 nM (□) substrate **1A** in standard assay buffer without ATP at 16 °C (*B*). The plotted points are corrected to account for non-reactive starting material as described under "Experimental Procedures." The *dashed lines* are the fit by simulation of all six concentrations to a single Michaelis-Menten model in KinTek Global Kinetic Explorer. The displayed plots correspond to kinetic parameters of k_{cat} = 0.34 ± 0.02 s^{-1}, k_{cat}/K_m = 210 ± 30 μM^{-1} s^{-1} (*A*) and k_{cat} = 0.62 ± 0.04 s^{-1}, k_{cat}/K_m = 250 ± 30 μM^{-1} s^{-1} (*B*). *B*, CE peak area of nicked dsDNA; *X*, CE peak area of AppDNA; *P*, CE peak area of ligated product.

Similar results were obtained for the adenylylated substrate **1A** (Fig. 3*B*). The same model was fit to concentration series using the adenylated substrate with deadenylylated enzyme in the absence of ATP. Using this method, a k_{cat} of 0.64 ± 0.08 s^{-1} and a k_{cat}/K_m of 240 ± 40 μM^{-1} s^{-1} (a K_m of ~3 nM) were measured, very similar to the constants measured for substrate **1**. The data recorded both appeared noisier and showed more obvious deviations from the simple Michaelis-Menten model than seen for substrate **1**, particularly at long times for the highest two concentrations (supplemental Fig. S1, *D–F*); thus, the fits were limited to the first 5 min of data. Enzyme stability

studies (New England Biolabs)[3] showed that T4 DNA ligase retained >80% of its activity when incubated in assay buffer for 24 h at 16 °C. Thus, the declines in rate at long time points were unlikely to be due to instability of the enzyme over the time course of the experiment. A likely explanation would be that a small amount of the reverse reaction of Step 2 resulted in adenylylated enzyme that was unable to bind the adenylylated substrate, apparently inactivating a portion of the ligase as the reaction progressed.

Turnover Rate Dependence on the Sequence at the Nick—Experiments were performed on 16 different nicked dsDNA substrates under saturating steady state turnover conditions. All 16 substrates had identical sequences except for the bases directly flanking the nick (Fig. 2). Each substrate was tested at 1 nM T4 DNA ligase and 1 μM substrate, and the initial rate of enzyme turnover was determined by fitting a line to the linear region of the time course (generally the first 50–80% conversion, depending on substrate). Six repeats were performed for each substrate, in two sets of three reactions with a fresh enzyme dilution used for each set of three. The full list of substrates tested and the enzyme turnover numbers (reactions/active site/s) measured are listed in Table 1.

Under these conditions, the ligase turned over nicked DNA substrate at a constant rate for the majority of the time course, indicating that at 1 μM substrate, the reaction was saturating and greatly over the K_m and that the ligase itself was not losing activity as the reaction progressed. The method of quenching did not affect the result; 100 mM EDTA or 80% formamide with 100 mM EDTA showed no significant differences in the curve shapes or slopes (data not shown). The results showed only modest differences in k_{obs} as the identities of the bases flanking the nick were varied. The only significant difference (*t* test, $p < 0.05$) noted was that when the 5′-phosphorylated base was C or T, ligation rates were roughly twice that of nicks where the 5′-phosphorylated base was A or G. The identity of the 3′-OH acceptor appeared to have no effect on the rate of turnover.

Rate Constant Determination under Single Turnover Conditions—In order to gain insight into the rates of the individual chemical steps of ligation, single turnover reaction time courses were measured for both the phosphorylated substrate **1** and the preadenylylated substrate **1A**. Substrate concentrations of 100 nM were used because these were between 10- and 100-fold higher than the apparent K_m estimated under turnover conditions. A 5-fold excess of enzyme in the appropriate adenylylation state was used (>95% adenylylated enzyme for substrate **1** and <5% adenylylated enzyme for substrate **1A**). ATP was excluded from the buffer for both substrates. Initial attempts at data collection were frustrated by apparent slow quench times relative to reaction times using either 250 mM EDTA or 100 mM EDTA, 80% formamide. Although similar quenches have been used successfully for the rapid quench of other ligases (5, 17, 18) and appeared sufficient to quickly quench T4 DNA ligase in steady state assays, single turnover reactions with T4 DNA ligase consistently showed significant reaction progress at even very short (<10-ms) quench times, indicating that the reaction continued after the addition of EDTA. The addition of 0.25% SDS to the quench buffers greatly improved the results, as did acid quench with 1 N H_2SO_4. The most consistent results were obtained with the acidic quench, and both the phosphorylated and adenylylated DNA were found to be stable under the quench conditions if the pH was brought to 7–8 within 5 min. SDS (0.25%) was also included in the acid quench to maintain solubility of denatured reaction components.

In order to ensure that the measured reaction curves represented the actual single turnover rates as closely as possible, reactions were also run at 1.5 μM enzyme at both 100 and 300 nM substrate, and, for **1A**, an additional reaction time course was run at 6 μM ligase and 100 nM substrate. In all cases, no significant change in the reaction curve under alternate reaction conditions was noted (data not shown). These results indicated that initial binding was rapid and that the substrate concentration was saturating for both **1** and **1A**. An alternative mode of initiation was also attempted, in which the substrate and ligase were preincubated together in the absence of magnesium, and the reaction was initiated by the addition of $MgCl_2$. This initiation method did not change the observed time course for substrate **1A**. However, the absence of added $MgCl_2$ and inclusion of 5 mM EDTA in the buffer did not fully prevent ligation of substrate **1** by adenylylated ligase. Slow reaction of the substrate to ligated product was observed in the syringe containing ligase and substrate **1** over the course of the experiment, suggesting that Mg^{2+} was tightly bound to the purified ligase and not fully removed by 5 mM EDTA (data not shown).

Fig. 4*A* shows the results of single turnover reactions involving substrate **1**, and Fig. 4*B* shows the results for substrate **1A** using the optimized quenching and mixing approach. The reaction of the phosphorylated nick with adenylylated enzyme shows reaction progress similar to that recently reported for *Chlorella* virus DNA ligase (17, 18) and truncated human DNA ligase I (5). Only a modest buildup of AppDNA was observed, peaking at 10% of total FAM label at ~75 ms. This intermediate was observed to be rapidly converted to final ligated product. The overall rate of ligation was determined by fitting a single exponential to the plot of product *versus* time, giving a $k_{obs} = 6 \pm 1\ s^{-1}$ for the first ligation turnover. To determine the individual rate constants, k_2 and k_3, the data were fit by simulation, assuming the irreversibility of the chemical steps, as described under "Experimental Procedures." The best fit was obtained with a rate constant of $5.3 \pm 0.4\ s^{-1}$ for the Step 2 transfer of the adenylyl group from the enzyme to the DNA (k_2) and a rate constant of $38 \pm 5\ s^{-1}$ for the Step 3 phosphodiester bond formation (k_3). The measured rates were very similar for a tested second substrate (substrate **11**, M/X = C/G and N/Y = pG/C, $k_2 = 4.8 \pm 0.4$ and $k_3 = 32 \pm 3$) (data not shown) and appeared unaffected by increasing either DNA or enzyme concentration, or by the addition of ATP to the buffer. A plot of the residuals showed that the deviance of the fit from the model is small but regular. The amount of formed product is slightly underestimated at short times and overestimated at long times.

The results of the reaction of preadenylylated substrate (Fig. 4*B*) showed an apparent first order decay of AppDNA to ligated product. The k_{obs} for single turnover ligation was $5.7 \pm 1.3\ s^{-1}$,

[3] G. J. S. Lohman, L. Chen, and T. C. Evans, Jr., unpublished data.

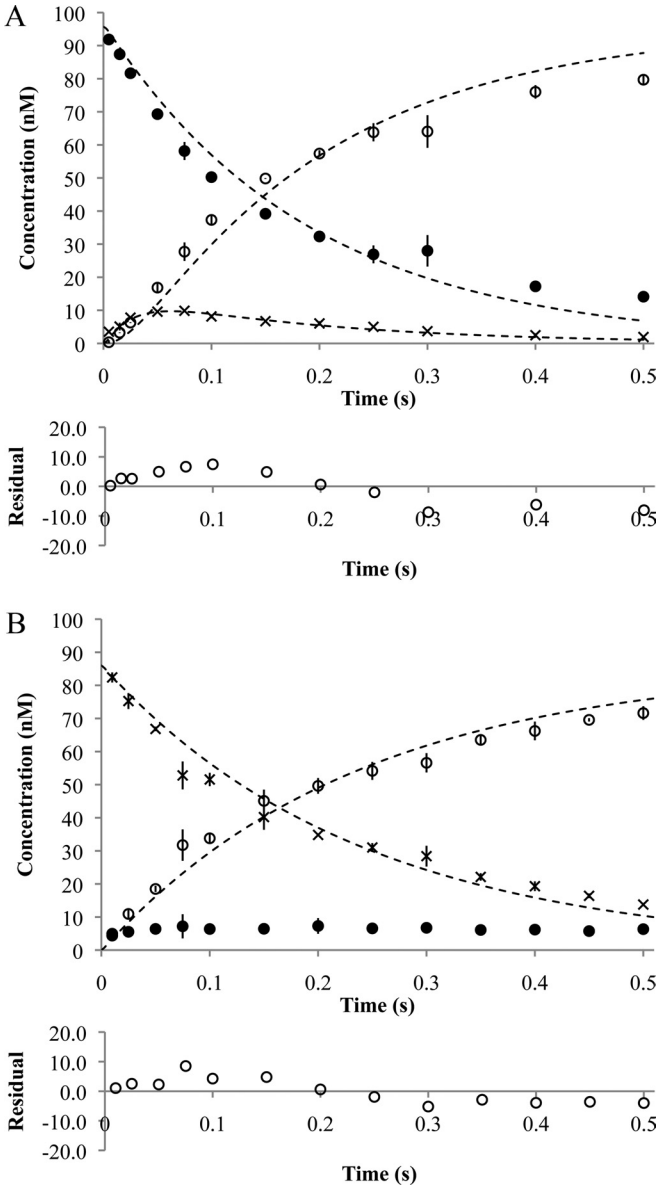


FIGURE 4. **Reaction of T4 DNA ligase with substrate 1 (*A*) and adenylylated substrate 1A (*B*) under single turnover conditions.** Each reaction was run with 500 nM ligase and 100 nM substrate in the standard ATP-free assay buffer. Ligase that was >95% adenylylated was used for *A*, and <5% adenylylated ligase was used for *B*. The measured quantity of nicked pDNA (●), nicked AppDNA (×), and ligated product (○) is shown for both reactions. The *marked points* represent the average of three experiments, and the *error bars* show one S.D. The *dashed lines* represent the output of fitting by simulation in Kinetic Explorer to models as described above. In both cases, the data have been corrected by subtracting the amount of unreacted substrate at 60 s from the substrate detected at each time point. For *B*, the amount of pDNA seen represents contaminating material in the substrate, and its concentration did not appear to change over the time course of the reaction. The residual plots show the difference between the fit and the data for the ligated product at each time point.

as determined by fitting a single exponential to the plot of ligated product *versus* time, very similar to the k_{obs} for single turnover ligation of **1**. The substrate used was contaminated with ~5% phosphorylated (unadenylylated) nicked DNA that does not react in the absence of ATP. A very slight increase in the phosphorylated material was seen to ~7% of the total FAM label, but this increase was close to the error measured in most of the data points (±1% for several points). Consequently, the fit shown fixed $k_{-2} = 0$, and the concentration of pDNA was not included in the simulation. A rough limit was set on the reverse rate by fitting the data to a model allowing reversible exchange of the adenylyl group with the enzyme, arbitrarily fixing k_2 to 5, 50, or 200 s^{-1} and increasing k_{-2} until a buildup of >2 nmol of the deadenylylated DNA was observed in the simulation (2% of the starting FAM label). At all values for k_2 tested, the reverse rate was estimated to be at a minimum 50-fold smaller than the forward adenylyl transfer rate to account for the data. The time course was thus fit to a model assuming irreversible chemistry, as described under "Experimental Procedures." For this experiment, a k_3 of 4.3 ± 0.3 s^{-1} was shown to give the best fit.

Evidence for a Postligation Rate-limiting Step—It is clear that the rates of chemistry (k_2 and k_3) determined under single turnover conditions for substrates **1** and **1A** are too large by more than an order of magnitude to be the actual rate-limiting step measured by steady state experiments (k_{cat}) for each substrate. For **1**, the slowest chemical step (k_2) was 5.3 s^{-1}, whereas k_{cat} = 0.4 s^{-1}. For the adenylylated intermediate **1A**, the slowest chemical step (k_3) was 4.2 s^{-1}, whereas k_{cat} = 0.64 s^{-1} under turnover conditions. Previously reported rates for self-adenylylation show that Step 1 occurs at a rate >10 times the rate of turnover measured in this study (4). To demonstrate that the rate of enzyme self-adenylylation was fast in our system, the single turnover experiment shown in Fig. 4*A* was repeated using deadenylated enzyme and placing 2 mM ATP in syringe B with the phosphorylated substrate. Thus, reaction of substrate required the enzyme to first adenylylate, introducing a lag phase to turnover. The results (supplemental Fig. S2) showed only a short lag consistent with the literature rate constants for ATP binding and Step 1 chemistry. In the absence of ATP, <5% ligation is seen after 24 h with this enzyme lot. Additionally, a direct measure of enzyme self-adenylation in our system with [α-^{32}P]ATP was completed as described under "Experimental Procedures." The results (supplemental Fig. S3) show a single-exponential rise reaching a maximum in radioactivity coeluting with protein at ~300–400 ms, corresponding to a Step 1 rate constant of 6 ± 1 s^{-1} when these data were fit with a simulation assuming saturating ATP binding.

To confirm that the k_{obs} for the first turnover was truly ~10-fold faster than the k_{cat} under turnover conditions and not an artifact of the experimental setup, a pre-steady state experiment was run. In this experiment, an excess of substrate over enzyme was used such that 2–4 turnovers could be observed over the course of the reaction. Fig. 5 shows the reaction progress over 5 s when mixing 100 nM substrate **1** with 30 nM or 50 nM T4 DNA ligase. Both display a short burst phase followed by a slow linear phase with k_{obs} of ~0.25 s^{-1}. This value agrees very well with the steady state k_{cat} of 0.4 ± 0.1 s^{-1} described above. It is also clear that the amplitude of the burst, as well as the slope of the linear phase, is dependent on the quantity of enzyme added. This curve is consistent with a reaction in which turnover rate is limited by a postligation step, such as product release.

To confirm that these results were consistent with the single turnover rate constants, reaction curves were simulated using the constants for k_2 and k_3 determined from the single turnover

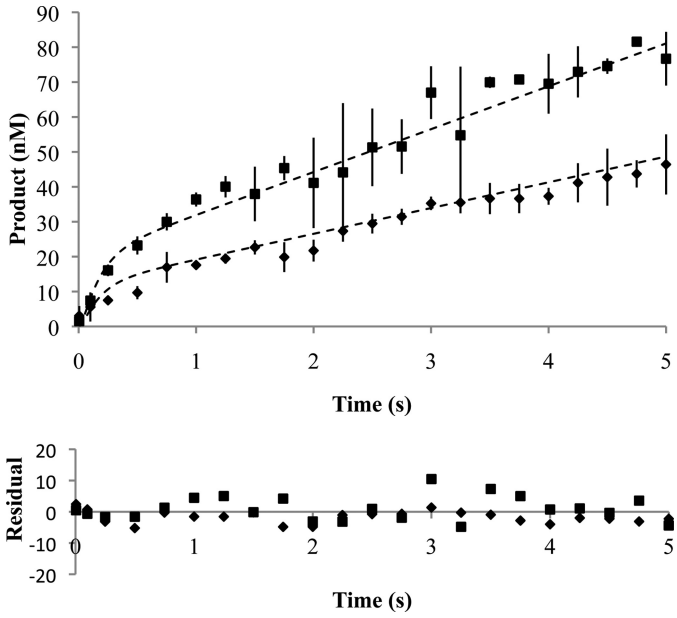


FIGURE 5. **Pre-steady state reactions of 30 nM (♦) and 50 nM (■) T4 DNA ligase with 100 nM substrate 1.** Reactions were run in the standard assay buffer. Each time point represents the average of three experiments, and the *error bars* represent one S.D. The *dashed lines* represent fits by simulation using the chemical rates determined from single turnover reaction of substrate **1**, literature values for Step 1 rates, and diffusion-limited binding of DNA and allowing the rate of product release (k_{off}) and the amplitude (a) to vary. The best fit was obtained with $a = 0.51$ and $k_{off} = 0.58\ s^{-1}$.

of substrate **1** above and the literature values for ATP binding and enzyme self-adenylylation (4). DNA binding was set to diffusion-limited, and the rate of product release (k_{off}) was allowed to vary. The outputs were additionally multiplied by a scale factor, a, which was allowed to vary in the simulation. If all enzyme present in the system was active, a scale factor of 1 would be observed. The simulated curves showed (Fig. 5, *dashed lines*) that the burst observed has a duration consistent with the previously determined k_2 and k_3, but the amplitude is lower than predicted for the amount of enzyme added (a = 0.51). The concentration of active enzyme in the stock used in this experiment was determined by two separate assays as described under "Experimental Procedures," giving high confidence that the concentration of enzyme in the reaction (30 and 50 nM) was accurately known. Nevertheless, the burst amplitude indicates that only half of the enzyme was active in the single turnover burst phase. This result could be explained by nonspecific binding to DNA (substrate inhibition) reducing the apparent amount of active enzyme available, as suggested by the steady state studies and discussed above.

DISCUSSION

At saturation (1 μM nicked dsDNA substrate), T4 DNA ligase will execute hundreds to thousands of turnovers with a steady state rate of 0.05–0.16 s^{-1}. The turnover rate varied only 2–3-fold across the substrates tested, with all combinations of natural, correctly base-paired nucleotides at the nick. This rate compared favorably with the turnover rate reported for the ligation of a heterogeneous substrate with many nicks per molecule during early work on T4 DNA ligase (k_{cat} ~0.075 s^{-1}) (39). The concentration series run for substrates **1** and **1A** further allowed for estimation of the Michaelis-Menten constants for our substrate system. Although the limitations of the Michaelis-Menten model did not permit the description of the detailed mechanism of DNA ligase activity, the fits gave estimates of the composite rate constants for the system under turnover conditions. The measured value for the k_{cat}/K_m of 150 ± 50 $\mu M^{-1}\ s^{-1}$ for substrate **1** corresponds to an apparent K_m of 2.5 nM, also very close to the previously reported value of 1.5 nM for internal nicks in the heterogeneous substrate (20). Only modest differences were seen in the k_{cat} and k_{cat}/K_m between substrates **1** and **11** (<2-fold). Together, these data indicated that T4 DNA ligase largely does not discriminate based on the base sequence at single-stranded breaks under multiple-turnover conditions.

The rate constants measured for single turnover ligation of substrate **1**, $k_2 = 5.3\ s^{-1}$ and $k_3 = 38\ s^{-1}$, compared favorably with those measured under single turnover conditions in recent reports for *Chlorella* virus DNA ligase (17, 18) and human DNA ligase I (5), suggesting that very similar chemical mechanisms operate in all of these ligases despite the ability of T4 DNA ligase to efficiently ligate blunt end substrates. These results conclusively show that AppDNA is a kinetically competent intermediate in ligation by T4 DNA ligase. However, the rate-limiting step for single turnover of substrate **1** is 5.3 s^{-1}, whereas the rate of turnover under steady state conditions is ~0.4 s^{-1}. Similarly, the rate-limiting step in the single turnover of AppDNA appears to be 4.2 s^{-1}, whereas the rate of turnover under steady state conditions is ~0.6 s^{-1}. In both cases, the rate of the first turnover is ~10 times faster than the turnover rate. A fast single turnover rate in contrast to a slower multiple turnover rate is indicative of product release being the true rate-limiting step.

Previous work has not considered a postligation, pre-enzyme readenylylation rate-limiting step likely for ligases. Upon ligation, the 5′-phosphate and the flexible nick are structurally lost, destroying the ligase binding site. T4 DNA ligase is known to have only a weak affinity for non-nicked DNA (29); thus, it might have been expected that the ligase would rapidly disassociate from the generated product. However, the results of the pre-steady state burst analysis clearly demonstrated that the rates measured in the separate steady state and single turnover systems do accurately describe the overall behavior of the enzyme. The pre-steady state result showed that, in fact, the rate-limiting step occurs after ligation and is most likely product release or a post-product release conformational change. Similar pre-steady state results have been reported for DNA ligation by the human DNA ligase IV-XRCC4 complex (40), but, although the authors concluded that the rate-limiting step must be after Step 3, they identified Step 1 enzyme self-adenylylation as the rate-limiting step. Product release was discounted due to the weak affinity of ligases for non-nicked DNA described above. However, enzyme adenylylation is known to be fast for T4 DNA ligase and was confirmed to occur at a rate compatible with the literature values in our system (supplemental Figs. S2 and S3). Product release is further indicated as the rate-limiting step by the results of AppDNA ligation. It has been previously proposed that there is an additional rate-limiting step present during the ligation of AppDNA, slowing for-

mation of the active complex and accounting for the often seen slower turnover of the intermediate as compared with the phosphorylated substrate (12, 41). This additional step must take place before initial binding, meaning both single turnover and steady state rates should be affected. However, in our hands, the rate of single turnover of **1A** was again ~10 times faster than the rate of turnover, indicating that the rate-limiting step under turnover conditions must be occurring after ligation.

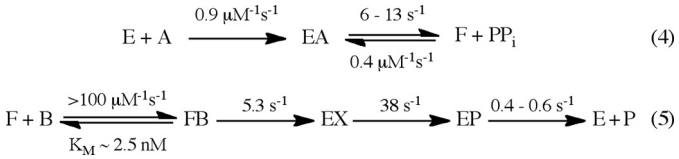
Given past reports of a weak affinity for non-nicked DNA by T4 DNA ligase, this result may seem surprising. It must be considered that the weak affinity of ligase for non-nicked DNA is reported from gel shift experiments (K_D), not as a measure of on/off rates (29). Furthermore, immediately after ligation, the DNA-ligase-AMP complex may be in a very different state from the ligase binding nonspecifically to non-nicked dsDNA; for example, the observed rate may well be the relaxation of the "S-complex" seen by Rossi *et al.* (29) in the interaction of deadenylylated enzyme with nicked DNA. Future studies directly investigating DNA binding and disassociation kinetics will allow elucidation of this mechanism.

In the single turnover studies, the rate constants k_2 and k_3 were determined based on a simulation that did not allow for reverse reactions. Previous reports show that high concentrations of PP_i or AMP are needed to observe the reverse reactions under turnover conditions (4, 42, 43). It is possible, however, that the reactions may be reversible on a single turnover scale before products have disassociated from the active site. It is the case that the residuals seen in Fig. 4*A* can be all but eliminated in a model allowing both k_{-2} and k_{-3} to vary (supplemental Fig. S4*A*). The simulation requires a value for k_{-2} 3 times larger than the forward rate of k_2, however, and we consider this to be very unlikely, given that the single turnover reaction of AppDNA indicates that k_{-2} must be at least 50 times smaller than the forward rate. Allowing k_{-3} to vary while Step 2 is irreversible allows only a modest improvement in the fit (supplemental Fig. S4*B*) and indicates that k_{-3} must be at least 25 times slower than the forward rate of Step 3. Altogether, the single turnover rates indicated that the forward rates of Steps 2 and 3 are substantially faster than the reverse rates. Additional experiments will be needed to accurately determine k_{-2} and k_{-3}.

It is evident from the reaction curves in Fig. 4 that the rate constants for Step 3 determined from the single turnover conversion of substrate **1** and the adenylylated substrate **1A** do not agree. The apparent rate of phosphodiester bond formation is only 4.3 s^{-1} when reacting AppDNA to ligated product, compared with an apparent rate of 38 s^{-1} when substrate **1** is used. The simplest model that would allow these two experiments to be reconciled is the case of T4 DNA ligase binding slowly to the AppDNA. However, the single turnover experiments at higher ligase concentrations give confidence that binding is fast because a slow binding substrate should show an increased reaction rate when the ratio of enzyme to substrate is increased. Similarly, the experiments using a higher concentration of substrate show no significant change in the reaction progress curve, giving confidence that 100 nM substrate is truly saturating. The analogous experiments performed with substrate **1** indicate that the measured rates are the true single turnover rates for both substrates.

Two major possibilities exist to explain the rate constant discrepancy. First, it is possible that the binding of AppDNA by the apoenzyme produces an active site that differs from the adenylylated enzyme binding phosphorylated nicks, resulting in a rate for the phosphodiester bond formation (k_3) that is simply slower. The second possibility is that there is an additional reaction step not considered by the model used in the data fitting of this study. The two single turnover experiments could not be adequately reconciled using the model proposed for the ligation of nicks by *Chlorella* virus DNA ligase (17), which adds a reversible conversion of the enzyme intermediate complex EX to an inactive complex (GX). Adding such a step to our model did not allow a single model to simultaneously fit both single turnover reactions. Simulations were also performed adding a two-step binding process with an initial fast binding step followed by a slow conversion to an active complex, similar to the additional rate-limiting step model proposed previously for reaction of AppDNA (12, 41). If the conformational change step occurred with a rate of ~5 s^{-1}, both experiments could be simultaneously fit reasonably well, but this model predicts a lag phase in the formation of product from **1A** not observed in the data collected (supplemental Fig. S5*A*). Another possibility proposed by the same authors was a rate-limiting conformational change in the AppDNA substrate before binding could occur; this model led to nearly identical fits, including a predicted lag phase, as are shown in supplemental Fig. S5*A*. The best fits were obtained with a model allowing substrate to bind reversibly in an inactive form as well as directly forming the catalytically active complex (supplemental Fig. S5*B*). If AppDNA is much more likely to initially bind in an inactive complex than the phosphorylated nicked substrate, both experiments can be fit to a single model. The constants for the binding rates of DNA are not well constrained by the data available, however, and further experiments into the kinetics of substrate binding will be required to determine which model most accurately reflects the mechanism of T4 DNA ligase.

It was noted that the measured k_{cat} for reaction of substrate **1** at low concentrations (1–50 nM **1**, k_{cat} = 0.4 s^{-1}) is ~3-fold larger than the k_{obs} at 1 μM substrate. This result is suggestive that there is some form of substrate inhibition, such as nonspecific DNA binding, taking place. The lower than expected burst amplitude seen in the pre-steady state experiments may likewise be due to substrate inhibition by nonproductive or nonspecific DNA binding. Preliminary simulations allowing inhibition by reversible, nonspecific binding to the DNA indeed showed that both the drop off in turnover rates as DNA concentration increased and the reduced burst amplitude can be accounted for by this sort of substrate inhibition. It is important to note that substrate inhibition cannot account for the presence of the burst phase itself except in the limiting case where the rate of enzyme disassociation from all non-nicked dsDNA is <0.5 s^{-1}. Because this would imply that release of the ligated product DNA would also take place with a rate of 0.5 s^{-1}, the details of inhibition do not impact the key conclusion that a postligation step must be rate-limiting.



This study represents the first detailed analysis of the kinetic mechanism of nick ligation by T4 DNA ligase. Equations 4 and 5 show the model for the ligation pathway, including all constants determined in this study and those previously reported in the literature (4). We have determined the turnover rates of multiple singly nicked substrates, including one preadenylylated substrate. Furthermore, both the Michaelis-Menten parameters and the single turnover rates of chemistry for one nick and its preadenylylated counterpart have been determined. Finally, we have demonstrated that chemistry is significantly faster than the steady state turnover rate, and, as evidenced by the pre-steady state burst experiment, the most likely candidate for the rate-limiting step of nick sealing by T4 DNA ligase is product release.

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