Poliovirus RNA-dependent RNA Polymerase (3D<sup>pol</sup>)

ASSEMBLY OF STABLE, ELONGATION-COMPETENT COMPLEXES BY USING A SYMMETRICAL PRIMER-TEMPLATE SUBSTRATE (sym/sub)*

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Detailed studies of the kinetics and mechanism of nucleotide incorporation catalyzed by the RNA-dependent RNA polymerase from poliovirus, 3D<sup>pol</sup>, have been limited by the inability to assemble elongation complexes that permit activity to be monitored by extension of end-labeled primers. We have solved this problem by employing a short, symmetrical, heteropolymeric RNA primer-template that we refer to as "sym/sub." Formation of 3D<sup>pol</sup>-sym/sub complexes is slow owing to a slow rate of association (0.1 μM<sup>-1</sup> s<sup>-1</sup>) of 3D<sup>pol</sup> and sym/sub and a slow isomerization (0.076 s<sup>-1</sup>) of the 3D<sup>pol</sup>-sym/sub complex that is a prerequisite for catalytic competence of this complex. Complex assembly is stoichiometric under conditions in which competing reactions, such as enzyme inactivation, are eliminated. Inactivation of 3D<sup>pol</sup> occurs at a maximal rate of 0.051 s<sup>-1</sup> at 22 °C in reaction buffer lacking nucleotide. At this temperature, ATP protects 3D<sup>pol</sup> against inactivation with a K<sub>0.5</sub> of 37 μM. Once formed, 3D<sup>pol</sup>-sym/sub elongation complexes are stable (t<sub>1/2</sub> = 2 h at 22 °C) and appear to contain only a single polymerase monomer. In the presence of Mg<sup>2+</sup>, AMP, 2'-dAMP, and 3'-dAMP are incorporated into sym/sub by 3D<sup>pol</sup> at rates of 72, 0.6, and 1 s<sup>-1</sup>, respectively. After incorporation of AMP, 3D<sup>pol</sup>-sym/sub product complexes have a half-life of 8 h at 22 °C. The stability of 3D<sup>pol</sup>-sym/sub complexes is temperature-dependent. At 30 °C, there is a 2–8-fold decrease in complex stability. Complex dissociation is the rate-limiting step for primer utilization. 3D<sup>pol</sup> dissociates from the end of template at a rate 10-fold faster than from internal positions. The sym/sub system will facilitate mechanistic analysis of 3D<sup>pol</sup> and permit a direct kinetic and thermodynamic comparison of the RNA-dependent RNA polymerase to the other classes of nucleic acid polymerases.

Critical to the multiplication of all viruses are transcription and replication of the viral genome. In the case of positive-strand RNA viruses, these processes are catalyzed by the virus-encoded RNA-dependent RNA polymerase (RdRP). Specific inhibitors of the RdRP should serve as potent antiviral agents. Indeed, inhibitors of viral DNA polymerases and reverse transcriptase (RT) are used clinically to treat infection by herpesviruses and human immunodeficiency virus (1, 2). Of course, cellular polymerases are also potential targets for antiviral chemotherapeutics that inhibit viral polymerases. Therefore, target specificity is of central importance to the development of clinically useful compounds. In some cases, drug activation only occurs in virus-infected cells thereby increasing specificity and diminishing toxicity (1). In others, however, infected cell-specific, activation mechanisms are not an option, and other approaches are required (2).

A comprehensive understanding of the differences between viral and cellular polymerases facilitates the design of specific inhibitors. In the case of human immunodeficiency virus RT, a combination of detailed kinetic (3–12) and structural (13–18) information was essential to provide an understanding of the molecular basis for RT function and the mechanism of action of non-nucleoside inhibitors of RT (9, 10). In fact, a description of the nucleotide addition cycle catalyzed by all classes of polymerases studied to date has benefited from both kinetic and structural analyses (19–28). The "structural era" for the RdRP has begun with the recent solution of the x-ray crystal structure for the RdRP from poliovirus, 3D<sup>pol</sup> (29). For this reason, poliovirus 3D<sup>pol</sup> is the ideal model system to use in the study of RdRP function. Unfortunately, detailed, mechanistic information regarding 3D<sup>pol</sup>-catalyzed nucleotide incorporation is sparse. The existing gap derives from the inability to assemble stoichiometrically 3D<sup>pol</sup>-primer-template complexes that permit nucleotide incorporation to be followed by extension of end-labeled primers.

Previous studies of 3D<sup>pol</sup>-catalyzed nucleotide incorporation that utilized short, heteropolymeric RNA primer-templates as substrates demonstrated that this enzyme binds to primer-template such that some enzyme has the 3'-OH of the "primer" strand in the catalytic center ("correct" orientation) and some enzyme has the 3'-OH from the "template" strand in the catalytic center ("incorrect" orientation) (30). When bound in the "incorrect" orientation, 3D<sup>pol</sup> adds non-template nucleotides to the blunt end of primer-template (30). Of course, heterogeneous binding of 3D<sup>pol</sup> to primer-template further complicates mechanistic analysis of this enzyme. In this report, we demonstrate that the use of a symmetrical primer-template substrate (sym/sub) permits the assembly of stable, elongation-competent 3D<sup>pol</sup>-primer-template complexes. The sym/sub system will facilitate quantitative analysis of the kinetics and mechanism of 3D<sup>pol</sup>-catalyzed nucleotide incorporation.

EXPERIMENTAL PROCEDURES

Materials—[γ-<sup>32</sup>P]ATP (7,000 Ci/mmol) was from ICN; nucleoside 5'-triphosphates, 2'-deoxynucleoside 5'-triphosphates, and 2',3'-deoxyx nucleoside 5'-triphosphates (all nucleotides were ultrapure solutions) were from Amersham Pharmacia Biotech; 3'-deoxyadenosine 5'-triphosphate (cordycepin) was from Sigma; all RNA oligonucleotides

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were from Dharmacon Research, Inc. (Boulder, CO); T4 polynucleotide kinase was from New England Biolabs, Inc.; all other reagents were of the highest grade available from Sigma or Fisher.

Expression and Purification of 3Dpol—Expression and purification of 3Dpol was performed as described previously (31).

**Purification of sym/sub**—The RNA oligonucleotide was purified by denaturing PAGE. Gels consisted of 21.3% acrylamide, 1.7% bisacrylamide, 7 M urea, and 1× TBE (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA). The oligonucleotide ladder was visualized by UV shadowing. A gel slice containing only the full-length oligonucleotide was removed, and the nucleic acid was electroeluted from the gel in 1× TBE by using an Elutrap apparatus (Schleicher & Schuell). Oligonucleotides were desalted on Sep-Pak columns (Millipore) as specified by the manufacturer. Oligonucleotides were hydrolyzed to remove the 2'-OH protecting groups as specified by the manufacturer. The concentrated, hydrolyzed RNA stocks (typically 1 μm) contained 330 μM Tris acetate, pH 7.0. Oligonucleotides were diluted in T<sub>r</sub>E<sub>1</sub> (10 mM Tris, 1 mM EDTA, pH 8.0), to the indicated concentrations. Oligonucleotides were stored at −80 °C until use. Concentrations were determined by measuring the absorbance at 260 nm in 50 mM K<sub>2</sub>HPO<sub>4</sub>, pH 7.0, 6 μM guanidine by using a calculated extinction coefficient of 101,600 M<sup>−1</sup> cm<sup>−1</sup> (32).

**5<sup>−32</sup>P Labeling of sym/sub**—The RNA oligonucleotide was end-labeled by using [γ-<sup>32</sup>P]ATP and T4 polynucleotide kinase from New England Biolabs essentially as specified by the manufacturer. Reactions were initiated by adding 30 μl, contained 11 μM [γ-<sup>32</sup>P]ATP, 10 μM RNA oligonucleotide (sym/sub), 1× Kinase Buffer and 0.4 units/μl T4 polynucleotide kinase. Reactions were incubated at 37 °C for 60 min. Unincorporated nucleotide was removed by passing the sample over two consecutive 1-ml Sephadex G-25 (Sigma) spin columns. We assumed the concentration of end-labeled RNA remained constant.

**Annealing of sym/sub**—1 μl end-labeled sym/sub was mixed with 99 μl unlabeled sym/sub in T<sub>r</sub>E<sub>1</sub>, pH 8.0, and heated to 90 °C for 1 min and slowly cooled to 10 °C at a rate of approximately 5 °C/min in a Progene Thermocycler.

**3D<sup>e</sup> Assays**—Reactions contained 50 mM HEPES, pH 7.5, 10 mM 2-mercaptoethanol, 5 mM MgCl<sub>2</sub>, 60 μM ZnCl<sub>2</sub>, 500 μM NTP, sym/sub, and 3D<sup>e</sup>. Reactions were quenched by addition of EDTA to a final concentration of 50 mM. Specific concentrations of primer-template and 3D<sup>e</sup>, along with any deviations from the above, are indicated in the appropriate figure legend. 3D<sup>e</sup> was diluted immediately prior to use in 50 mM HEPES, pH 7.5, 10 mM 2-mercaptoethanol, 60 μM ZnCl<sub>2</sub>, and 20% glycerol. The volume of enzyme added to any reaction was always less than or equal to one-tenth the total volume.

**Rapid Chemical Quench-flow Experiments**—Rapid mixing/quenching experiments were performed by using a model QBF-3 chemical quench-flow apparatus (KinTek Corp., State College, PA). Experiments were performed at 30 °C by using a circulating water bath. 3D<sup>e</sup>sym/sub complex in 50 mM HEPES, pH 7.5, 10 mM 2-mercaptoethanol, 60 μM ZnCl<sub>2</sub>, and 5 mM MgCl<sub>2</sub> was rapidly mixed with the nucleotide substrate in 50 mM HEPES, pH 7.5, 10 mM 2-mercaptoethanol, 60 μM ZnCl<sub>2</sub>, and 5 mM MgCl<sub>2</sub>, and the reactions were quenched by addition of 0.5× EDTA to a final concentration of 0.25×.

**Product Analysis, Denaturing PAGE**—An equal volume of loading buffer, 10 μl (90% formamide, 0.025% bromphenol blue, and 0.025% xylene cyanol), was added to 10 μl of the quenched reaction mixtures and heated to 70 °C for 2–5 min prior to loading 5 μl on a denaturing polyacrylamide gel of the appropriate percentage containing 1× TBE and 7 μl urea. Highly cross-linked gels contained 1.7% bisacrylamide. Electrophoresis was performed in 1× TBE at 75 watts. Gels were visualized by using a PhosphorImager and quantitated by using the ImageQuant software (Molecular Dynamics). Data were fit by nonlinear regression using the program, KaleidaGraph (Synergy Software, Reading, PA).

**Kinetic Simulation**—Kinetic simulations were performed by using KinTekSim version 2.03 (KinTek Corp., State College, PA). All rate constants were determined experimentally, except where noted. The agreement between the experimental data and kinetic simulations was determined by visual inspection.

**RESULTS**

**A Symmetrical Primer-Template Substrate (sym/sub) to Study 3Dpol-catalyzed Nucleotide Incorporation**—We designed the symmetrical, heteropolymeric RNA primer-template substrate shown in Fig. 1A to study the kinetics and mechanism of 3Dpol-catalyzed nucleotide incorporation. We refer to this substrate as “sym/sub” throughout. This substrate is a 10-nt, self-complementary RNA that forms a 6-base pair duplex flanked by two, 4-nt 5′-overhangs. Each overhang is capable of templating incorporation of a unique nucleotide, thus permitting the evaluation of single and multiple cycles of nucleotide incorporation. The calculated T<sub>m</sub> value is 55 °C for this duplex when 1 μM RNA strands (0.5 μM duplex) is employed (33). Based upon previous studies of 3Dpol, this T<sub>m</sub> value is in the optimal range for maximal rates of nucleotide incorporation (34).

**Incorporation of the First Nucleotide into sym/sub by 3Dpol Is Slow**—Utilization of sym/sub by 3Dpol is shown in Fig. 1B. In this experiment, enzyme was in excess (5 μM) relative to sym/sub (0.5 μM duplex); ATP (500 μM) was supplied as the sole nucleotide substrate; and either Mn<sup>2+</sup> or Mg<sup>2+</sup> was employed as a divalent cation cofactor. In the presence of both divalent cations, AMP incorporation into sym/sub was observed. Reactions were initiated by addition of 3Dpol and incubated at 30 °C; reactions were quenched at the indicated times by addition of EDTA to a final concentration of 50 mM. Products were resolved by electrophoresis on a denaturing 20% polyacrylamide gel. C, kinetics of AMP incorporation in the presence of MnCl<sub>2</sub> fit best to a single exponential (solid line) with an observed rate of 0.041 ± 0.004 s<sup>−1</sup> and an amplitude of 0.36 ± 0.02 μM. The kinetics of AMP incorporation in the presence of MgCl<sub>2</sub> fit best to a single exponential (solid line) with an observed rate of 0.012 ± 0.001 s<sup>−1</sup>, an amplitude of 0.34 ± 0.01 μM, and a starting point of 0.52 ± 0.02 μM.

**Incorporation of Subsequent Nucleotides**—Next, incorporation of AMP was utilized at a rate of 0.012 ± 0.001 s<sup>−1</sup>. It should be noted that in order to extend greater than 0.5 μM RNA strands, the “flip side” of an extended sym/sub duplex must be utilized, thus mandating dissociation of a previously existing 3Dpol-sym/sub product complex. In the presence of Mg<sup>2+</sup>, AMP incorporation into sym/sub was monophasic; 0.36 ± 0.02 μM sym/sub was

![Fig. 1. Use of a symmetrical primer-template (sym/sub) to study 3Dpol-catalyzed nucleotide incorporation.](http://www.jbc.org/)

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extended at a rate of 0.041 ± 0.004 s⁻¹. The measured value of extended sym/sub (0.36 μM) was lower than the theoretical value (0.5 μM) for a single, stoichiometric turnover. 

**Incorporation of the Second Nucleotide into sym/sub by 3Dpol**

**Is Fast**—If the slow observed rate of nucleotide incorporation in the presence of Mg²⁺ reflected the true rate of 3Dpol-catalyzed nucleotide incorporation, then each subsequent cycle of incorporation should occur at approximately the same rate. However, if the true rate of incorporation was masked by the slow assembly of 3Dpol-sym/sub complexes, then each subsequent cycle of nucleotide incorporation may be faster. In order to determine whether a preferred order of assembly existed, the experiment shown in Fig. 3 was performed. In each experiment, the final concentrations of 3Dpol (5 μM), sym/sub (0.5 μM duplex), and ATP (500 μM) remained constant. The only difference was in the order of mixing/incubation at 30 °C. The results from a reaction initiated by addition of enzyme (Fig. 3A) were consistent with the analysis presented in Fig. 1 (kobs was 0.06 ± 0.01 s⁻¹; amplitude was 0.38 ± 0.01 μM). Incubation of 3Dpol with sym/sub for 90 s prior to addition of ATP (Fig. 3B) completely removed the slow rate of incorporation. However, a 2-fold reduction in the amplitude (0.21 μM) was noted. Incubation of 3Dpol and ATP prior to addition of sym/sub (Fig. 3C) reduced the observed rate of AMP incorporation by 3-fold (kobs was 0.02 s⁻¹) with very little effect on the end point of the reaction (amplitude was 0.33 μM). Incubation of 3Dpol at 30 °C prior to addition of sym/sub and ATP (Fig. 3D) showed significant thermal inactivation of 3Dpol. The kinetics of AMP incorporation could be fit to a single exponential with an observed rate of 0.02 s⁻¹ and an amplitude of 0.21 μM. The kinetics of AMP incorporation could be fit to a single exponential with an observed rate of 0.02 s⁻¹ and an amplitude of 0.33 μM. D. 3Dpol was incubated at 30 °C for 90 s, at which time sym/sub and ATP were added to initiate the reaction. Reactions were quenched at the indicated times by addition of EDTA (50 mM). The kinetics of AMP incorporation could be fit to a single exponential with an observed rate of 0.02 s⁻¹ and an amplitude of 0.18 μM.

![assembly of 3Dpol-sym/sub complexes](image)

**Fig. 2.** UMP incorporation into sym/sub. A, experimental design. Reactions were initiated by mixing 3Dpol (5 μM) with sym/sub (1 μM), ATP (500 μM), and MgCl₂ (5 mM) and incubated at 30 °C for 90 s at which time either buffer or UTP (500 μM) was added. Reactions proceeded for indicated times then quenched by addition of EDTA (50 mM). B, products from reactions described in A resolved by electrophoresis on a denaturing 20% polyacrylamide gel. C, kinetics of UMP incorporation into sym/sub in the reaction described in A.

determine whether a preferred order of assembly existed, the experiment shown in Fig. 3 was performed. In each experiment, the final concentrations of 3Dpol (5 μM), sym/sub (0.5 μM duplex), and ATP (500 μM) remained constant. The only difference was in the order of mixing/incubation at 30 °C. The results from a reaction initiated by addition of enzyme (Fig. 3A) were consistent with the analysis presented in Fig. 1 (kobs was 0.06 ± 0.01 s⁻¹; amplitude was 0.38 ± 0.01 μM). Incubation of 3Dpol with sym/sub for 90 s prior to addition of ATP (Fig. 3B) completely removed the slow rate of incorporation. However, a 2-fold reduction in the amplitude (0.21 μM) was noted. Incubation of 3Dpol and ATP prior to addition of sym/sub (Fig. 3C) reduced the observed rate of AMP incorporation by 3-fold (kobs was 0.02 s⁻¹) with very little effect on the end point of the reaction (amplitude was 0.33 μM). Incubation of 3Dpol at 30 °C prior to addition of sym/sub and ATP (Fig. 3D) showed significant thermal inactivation of 3Dpol. The kinetics of AMP incorporation could be fit to a single exponential with an observed rate of 0.02 s⁻¹ and an amplitude of 0.18 μM. 5331

**Assembly of 3Dpol-sym/sub Complexes Prior to Addition of the First Nucleotide Results in Fast Incorporation**—In order to
into our standard reaction buffer which lacks glycerol (see “Experimental Procedures”). 3Dpol activity was lost at a finite rate at temperatures greater than or equal to 0 °C (Fig. 4A). Consistent with previous results (35), nuclease, in this case ATP, protected 3Dpol against inactivation (Fig. 4B). Protection of 3Dpol by ATP was apparent at all temperatures analyzed (Table I) and was saturable (Fig. 4C and Table II). The maximal rates of inactivation varied over a 20-fold range between 0 and 30 °C. Interestingly, the concentration of ATP sufficient to decrease the observed rate of inactivation to 50% of the maximal value ($k_{\text{obs}}$) also showed a temperature dependence, ranging from 17 μM at 0 °C to 84 μM at 30 °C. In addition to ATP, GTP, GDP, UTP, and CTP were also capable of protecting 3Dpol against thermal inactivation (Fig. 5). As observed previously, GTP protects 3Dpol against thermal inactivation better than any other nucleotide (35). We also noted a linear, 3-fold decrease in $k_{\text{obs}}$ when the concentration of 3Dpol was varied between 2 and 20 μM (data not shown).

### Stability of 3Dpol-sym/sub Complexes—Although the rate of formation of the initial 3Dpol-sym/sub complex was slow, this complex was stable enough to incorporate quantitatively the second nucleotide. In order to determine directly the rate constant for dissociation of this complex, we performed the experiment that is illustrated in Fig. 6A. 3Dpol and 5′-[32P] labeled sym/sub were given 90 s to assemble, at which point a 1000-fold molar excess of unlabeled sym/sub was added as a trap for free enzyme. At various times after addition of trap, the amount of complex remaining was determined by following production of 11-mer (Fig. 6B). At 30 °C, the 3Dpol-sym/sub complex had a half-life of ~20 min, whereas at 22 °C, this complex had a half-life of ~2 h (Fig. 6C). A similar experiment was performed to determine the stability of the 3Dpol-sym/sub product complex (Fig. 6D); however, in this case, the ability to form 12-mer was monitored (Fig. 6E). Again, there was a temperature dependence to the stability of this complex (Fig. 6F). The product complexes had half-lives of 4.5 and 8.5 h at 30 and 22 °C, respectively.

### Kinetics of Ribo- and Deoxyribonucleotide Incorporation by 3Dpol—By using a rapid mixing/quenching device, we were able to evaluate the kinetics of AMP incorporation into sym/sub from preassembled 3Dpol-sym/sub complexes (Fig. 7). In the presence of 600 μM ATP, incorporation of AMP was fast, occurring at a rate of ~70 s⁻¹. Under the same conditions, both 2′-dAMP and 3′-dAMP were incorporated, albeit at rates ~70–120-fold slower than for AMP (Table III). The loss of each hydroxyl appeared to be additive as incorporation of 2′,3′-dAMP was reduced by 35,000-fold relative to AMP incorporation (Table III).

The steady-state kinetic analysis of AMP incorporation into sym/sub showed a burst of product formation followed by a linear accumulation of product (Fig. 8). The steady-state rate constant, $k_{\text{cat}}$, for AMP incorporation was 0.0001 s⁻¹, and the y intercept was 0.8 μM. In the presence of all four NTPs, the $k_{\text{cat}}$ value was increased by 10-fold relative to that in the presence of ATP only (Fig. 8). There was not a significant difference in the y intercept (Fig. 8). The $k_{\text{cat}}$ value for AMP incorporation did not change by increasing the concentration of 3Dpol to 2.5

### Table I: Comparison of 3Dpol inactivation rates as a function of temperature and ATP concentration

| Parameter | 0 °C | 22 °C | 30 °C |
|-----------|------|------|------|
| [ATP] (μM) | [ATP] (μM) | [ATP] (μM) |
| 0 | 5.0 ± 0.5 | 51.0 ± 5.0 | 90.0 ± 11.0 |
| 50 | 1.3 ± 0.1 | 21.0 ± 2.0 | 55.0 ± 5.0 |
| 200 | 0.31 ± 0.02 | 8.5 ± 0.9 | 24.0 ± 1.0 |
| 500 | 0.16 ± 0.01 | 4.8 ± 1.5 | 15.0 ± 1.0 |

### Table II: Temperature dependence of constants for inactivation of 3Dpol

| Parameter | 0 °C | 22 °C | 30 °C |
|-----------|------|------|------|
| $k_{\text{obs}}$ (μM) | $K_{\text{0.5}}$ (μM) | $K_{\text{0.5}}$ (μM) |
| 0.0050 ± 0.0001 | 17.0 ± 1.1 | 37.0 ± 3.1 |
| 0.051 ± 0.001 | 38.6 ± 9.2 |
Fig. 5. Incorrect nucleotides and nucleoside diphosphates protect 3Dpol against inactivation. Reactions contained 5 μM 3Dpol, 1 μM sym/sub (0.5 μM duplex), 5 mM MgCl₂, 50 μM ATP, and either 0 μM ATP (○), 450 μM ATP (□), 450 μM CTP (■), 450 μM GTP (▲), or 450 μM UTP (▲). Reactions were initiated by addition of 3Dpol and incubated at 30 °C; reactions were quenched at the indicated times by addition of EDTA to a final concentration of 50 mM. The y intercept was 2.0 μM (data not shown).

Kinetic Mechanism for Assembly of Stable, Elongation-competent 3Dpol-sym/sub Complexes—In order to understand better the kinetic mechanism for assembly of 3Dpol-sym/sub complexes, we evaluated the kinetics of assembly indirectly by monitoring AMP incorporation into sym/sub under conditions in which either the concentration of 3Dpol or sym/sub was varied. The results of this analysis are presented in Fig. 9. The data were most consistent with the mechanism presented in Scheme 1. The validity of this model was tested by kinetic simulation of this mechanism using the parameters shown in Table IV. The simulated data are represented by the solid lines through the data shown in Fig. 9. The concentration of active 3Dpol (0.8 μM) was from the y intercept of the steady-state kinetic data (Fig. 8). A Kd value for ATP of 100 μM was employed. This value is consistent with reported Km values for nucleotides (30). This Kᵥ value was inconsequential to the outcome of the simulation as this value could be varied by an order of magnitude in either direction without any effect on the simulated kinetics of assembly. The rate constant for the conformational change was constrained by the observed rate of assembly at 5 and 10 μM sym/sub. A value of 0.076 s⁻¹ was obtained from a single exponential fit of these individual time courses. Therefore, the only simulated parameters were k₋₁ and k₁. Given the constraints discussed above, values of 0.1 μM⁻¹ s⁻¹ and 0.1 s⁻¹ for k₋₁ and k₁, respectively, were shown to fit the data best. The simulated data were quite sensitive to these rate constants as a 25% change in either altered the goodness of fit to the data. A good fit of the data acquired by varying 3Dpol concentration was obtained only when the fraction of inactive enzyme (20% of the total upon initiation) was allowed to bind to sym/sub. This unproductive complex was kinetically significant at all concentrations of 3Dpol, even more so at low concentrations of sym/sub (≤1 μM). At 30 °C in the presence of sym/sub and absence of glycerol, 3Dpol concentrations in the 10–25 μM range showed evidence of precipitation; therefore, these data were not included in the analysis. Given this problem with precipitation, we did not force a fit of the mechanism to the 5 μM 3Dpol data (Fig. 9A). It should be noted, however, that at 5 μM 3Dpol we were unable to observe any precipitation even after centrifugation.

DISCUSSION

We have used a symmetrical primer-template substrate (sym/sub) to assemble stable, elongation-competent 3Dpol-sym/sub complexes suitable for analysis of the kinetics and mechanism of nucleotide incorporation catalyzed by this enzyme. Our current model for the mechanism of 3Dpol-sym/sub complex assembly is presented in Scheme 1. 3Dpol undergoes iterative cycles of binding and release of sym/sub until the complex isomerizes into a catalytically competent elongation complex. After each cycle of dissociation, enzyme inactivation can occur. 3Dpol inactivation results in substoichiometric assembly of elongation complexes. The fraction of enzyme that will be inactivated during the assembly process depends upon the partitioning between the two pathways. Maximizing the rate of 3Dpol-sym/sub complex formation and minimizing the rate of 3Dpol inactivation will permit stoichiometric assembly of elongation complexes. By using concentrations of sym/sub in a range greater than or equal to 10 μM, the rate of assembly will be maximized (Fig. 9B). By assembling complexes at lower temperatures in the presence of nucleotide, the rate of 3Dpol inactivation will be minimized (Fig. 4). It may also be possible to assemble complexes efficiently by using Mn²⁺ as the divalent cation cofactor and then diluting these mixtures into Mg²⁺-containing buffer to prevent error-prone RNA synthesis (Fig. 1 and Ref. 30). Once complexes form, they are very stable (Fig. 6A), support a fast rate of nucleotide incorporation (Fig. 7), and produce complexes that are significantly more stable than the initial complex (Fig. 6B). The rate of complex disassembly limits the rate of primer utilization (Fig. 8). The rate of complex disassembly is faster from the end of template than from internal positions (Fig. 8). Therefore, the number of primers utilized during the course of a reaction can be increased by following incorporation of all four nucleotides instead of a single nucleotide.

We were also able to fit the data by using an alternative model with a slow association step without a conformational change step. In this case, the rate constant for association was 0.035 μM⁻¹ s⁻¹, and the rate constant for dissociation was that measured directly, 0.0006 s⁻¹ (Fig. 6). However, in order to obtain a good fit, it was necessary to reduce artificially the concentration of sym/sub employed from 0.5 to 0.4 μM. We observe stoichiometric (0.5 μM) utilization of sym/sub in the presence of Mn²⁺ (Fig. 1C). This observation confirms that all of the substrate is competent for primer extension. Kinetic simulation should always be constrained by parameters determined empirically. Therefore, this model was not considered further. Attempts were also made to add a monomer-oligomer equilibrium to the mechanism in order to fit the kinetics of assembly in the presence of excess (5 μM) 3Dpol (Fig. 9A). These attempts failed owing to the lack of empirical data (and corresponding constraints) to describe this reaction. Direct analysis of 3Dpol oligomerization should provide information necessary to explore this possibility more rigorously.

Whether the mechanism of assembly described herein can be related to initiation of transcription or replication in vivo remains to be determined. These studies employ an artificial primer-template; therefore, the kinetic scheme and associated parameters may only be relevant for the model system. The kinetic properties may be different for a reaction of the polymerase with a natural template in the presence of other protein factors. Replication of the poliovirus genome initiates within the poly(A)-tract at the 3'-end of genomic RNA from a complex comprising viral factors 3AB, 3B (VPg), 3CD, and 3Dpol (Fig. 10) (36). Once 3Dpol initiates RNA synthesis from the protein primer, an elongation-competent complex is formed. As indicated in Fig. 10, a single template is thought to support multiple, continuous cycles of initiation. The nascent RNA chain is only annealed to template over a short distance, and the RNA is spooled through a channel formed by the polymerase (37). Whether or not a helicase is required to disrupt base pairing.

2 J. J. Arnold and C. E. Cameron, unpublished results.
and gives a lines represent the fit of the data to a single exponential and gives a
plexes formed during elongation of poliovirus plus- and minus-
m, respectively. D, experimental design. 3Dpol (1 μm) was incubated with 100% labeled sym/sub (0.1 μm) and ATP (1 μm) for 90 s at either 22 or 30 °C at which point trap (100 μm unlabeled sym/sub) was added to the reaction. At fixed times after the addition of trap, UTP (500 μM) was added, and the reaction was allowed to proceed for 30 s and then quenched by addition of EDTA (50 mM). E, products from reactions described in D performed at 22 °C resolved by electrophoresis on a denaturing, highly cross-linked 23% polyacrylamide gel. F, kinetics of dissociation of 3Dpol-sym/sub product complexes from the reactions described in D at 22 (●) and 30 °C (○). The solid lines represent the fit of the data to a single exponential and gives a kobs at 22 and 30 °C of 0.000001 ± 0.000005 and 0.00059 ± 0.00002 s⁻¹, respectively.

**FIG. 7.** Pre-steady-state incorporation of AMP into sym/sub. 2 μM 3Dpol was incubated with 2 μM sym/sub (1 μm duplex) and rapidly mixed with 1200 μM ATP as described under "Experimental Procedures." After mixing, reactant concentrations were reduced by 50%. Reactions were quenched at the indicated times by addition of 0.5 μM EDTA to a final concentration of 0.3 μM. The solid line represents the fit of the data to a single exponential with a kobs of 72.4 ± 4.4 s⁻¹.

between template and nascent RNA is not known.

We hypothesize that 3Dpol-sym/sub complexes mimic complexes formed during elongation of poliovirus plus- and minus-strand RNAs by 3Dpol (Fig. 10). In support of this hypothesis is the observation that a 3Dpol derivative with an amino acid substitution in conserved structural motif B that has less than a 2-fold reduction in poly(U) polymerase activity but a 15-fold reduction in the observed rate of elongation measured by using the sym/sub system produces virus with significantly impaired growth properties relative to wild-type virus. Moreover, the rate of nucleotide incorporation into sym/sub and stability of the 3Dpol-sym/sub complex predicts processive replication of the complete genome by a single polymerase molecule in approximately 100 s. This value is in agreement with the value of 45 s measured in vivo (37). As long as 3Dpol can proceed along the template unimpeded by RNA secondary structure, additional factors may not be required to assist in elongation.

Within infected cells, replication occurs in spherical compartments formed by virus-induced vesicles that are 0.5 μm in diameter (38). This diameter predicts a volume of 0.065 μm³. Enclosure of as few as 10 RNA molecules within this structure would correspond to a concentration in the micromolar range. Nonstructural proteins, such as 3Dpol, are present in a 1:1 stoichiometry with structural proteins that are required in stoichiometry with structural proteins that are required in

![Image](http://www.jbc.org/content/5334/1/5334/F6.large.jpg)

**Fig. 6.** Dissociation of 3Dpol-sym/sub and 3Dpol-sym/sub product complexes. A, experimental design. 3Dpol (1 μm) was incubated with 100% labeled sym/sub (0.1 μm) for 90 s at either 22 or 30 °C at which point trap (100 μm unlabeled sym/sub) was added to the reaction. At fixed times after the addition of trap, ATP (500 μM) was added, and the reaction was allowed to proceed for 30 s and then quenched by addition of EDTA (50 mM). B, products from reactions described in A performed at 22 °C resolved by electrophoresis on a denaturing highly cross-linked 23% polyacrylamide gel. C, kinetics of dissociation of 3Dpol-sym/sub complexes from the reactions described in A at 22 °C (●) and 30 °C (○). The solid lines represent the fit of the data to a single exponential and gives a kobs at 22 and 30 °C of 0.000011 ± 0.000005 and 0.000059 ± 0.00002 s⁻¹, respectively.
polymersase subunit can be found in precursor (P3 and 3CD) and processed forms (3Dpoly and 3D). 3D’ is a proteolytic fragment of 3D that presumably lacks polymerase activity. The ratio of P3:3CD:3Dpoly:3D’ is 2:4:1:1 after a 10-min labeling period, thus reducing the concentration of 3Dpoly available for transcription and replication. We conclude that the concentration of sym/sub and 3Dpoly employed in this study is in the biologically relevant range. In addition, the calculated $K_\text{a}$ value
channel limits and regulates the rate of initiation in vivo. An alternative possibility for the conformational change step observed kinetically comes from structural studies of the RdRP from hepatitis C virus (40). Extensive interactions between the fingers and thumb subdomains exist in this enzyme which completely encircle the catalytic center of the enzyme. Similar interactions should be possible for 3Dpol; therefore, the observed conformational change step may reflect "closing" of the enzyme. The increased stability of this complex would derive from the additional interactions between the fingers and thumb subdomains.

The steady-state kinetic experiments (Fig. 8) were performed by using excess sym/sub; therefore, the y intercept reflects the active concentration of enzyme used in the reaction. The value measured in all cases was 80% of the value expected based on the concentration determined by absorbance at 280 nm (31). The value of 80% was also corroborated by the assembly experiments shown in Fig. 9B. That 20% was inactive was supported by using excess sym/sub; therefore, the y intercept reflects the active fraction (20%) observed at 1 μM 3Dpol (Fig. 9A). Taken together, these data suggest that a 3Dpol monomer is an active form of the enzyme. Oligomerization of this enzyme clearly can occur in the presence and absence of nucleic acid (29, 39). Mutations in 3Dpol-coding sequence that alter selectivity, catalytic activity, or conformational change step may reflect "closing" of the enzyme which stabilizes this complex.

The use of the sym/sub system to study 3D pol will advance our understanding of the RdRP to the level of the other classes of nucleic acid polymerase. The sym/sub system should be useful for the analysis of other RdRPs, especially members of the supergroup I family (44). We have used this substrate to identify and characterize the active form of the feline calicivirus polymerase.4 In contrast, the RdRPs from bovine viral diarrhea virus and hepatitis C virus, members of the supergroup II family (44), do not assemble stable, elongation-competent complexes by using sym/sub.2 Whether a universal primer-template design exists that is suitable for mechanistic analysis of all RdRPs remains to be determined.

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Poliovirus RNA-dependent RNA Polymerase ($3D^{\text{pol}}$): ASSEMBLY OF STABLE, ELONGATION-COMPETENT COMPLEXES BY USING A SYMMETRICAL PRIMER-TEMPLATE SUBSTRATE (sym/sub)

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