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Steady-State Kinetics of Mouse DNA Polymerase β†

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ABSTRACT: DNA polymerase β from mouse myeloma has been purified to near homogeneity, and its properties have been examined. The enzyme did not catalyze a detectable level of dNTP turnover, pyrophosphate exchange, pyrophosphorolysis, 3′-exonuclease degradation, or 5′-exonuclease degradation. Steady-state kinetic studies point to an ordered bibi mechanism for the polymerization reaction. Metal activation, which is required for polymerization, did not alter the K_m for either the dNTP or the template–primer.

In subunit composition, size, and catalytic repertoire, DNA polymerase β is the simplest naturally occurring DNA polymerase known. This enzyme is a single polypeptide of approximately 40 000 daltons, and it does not degrade nucleic acids or nucleoside 5′-triphosphates [see below; Chang & Bollum (1973)]. Earlier kinetic studies have indicated that homopolymer replication by calf thymus β-polymerase involves just one binding site for dNTP and one binding site for the primer (Chang, 1973b); in addition, the enzyme can be inhibited by pyrophosphate, one of the products of the polymerization reaction (Chang & Bollum, 1973). Because of these properties and the availability of methods for preparing near homogeneous β-polymerase (Chang, 1973a; Wang et al., 1974, 1977; Stalker et al., 1976), this enzyme is a good candidate for use in steady-state kinetic studies of the DNA polymerase mechanism.

Here we describe the preparation of near homogeneous mouse β-polymerase and the characterization of its catalytic activity. Aided by the conceptual framework described by McClure & Jovin (1975), we undertook a steady-state kinetic evaluation of the mechanism of this enzyme. Substrate kinetics measured with three different template–primer/dNTP systems indicated a sequential mechanism for polymerization. These results together with product inhibition studies using pyrophosphate suggest an ordered bibi mechanism.

Materials

Chemicals and Enzymes. DNA polymerase I from *Escherichia coli* and terminal deoxynucleotidyltransferase from calf thymus were kindly provided by Drs. L. Loeb and R. L. Ratliff, respectively. Polynucleotide kinase from T4 phage infected *E. coli* was obtained from P-L Biochemicals. Unlabeled deoxynucleoside 5′-triphosphates and 5′-monophosphates were from Calbiochem and P-L Biochemicals, respectively. Labeled nucleotides were from ICN Pharma-

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ceuticals. [^{32}P]NaPP$_i$[1] was from New England Nuclear. Sodium dextran sulfate and Sephacryl S-200 were from Pharmacia Fine Chemicals. Activated charcoal and phosphocellulose were from Sigma. Polyacrylamide gel electrophoresis materials were from Bio-Rad Laboratories. Proteins used as molecular weight markers were from Miles Laboratories. Bovine serum albumin was from Reheis Chemicals. Single-stranded DNA–cellulose was kindly provided by Dr. S. Planck, and it contained 5 mg of DNA per mL of column bed volume. The 5′-end ^{32}P-labeled d(T)$_{\overline{550}}$ was prepared with polynucleotide kinase; after incubation the reaction mixture was phenol-extracted, and the labeled poly[d(T)] was collected by ethanol precipitation. The 3′-end [^{3}H]dTMP-labeled d(T)$_{\overline{17}}$ was prepared with terminal deoxynucleotidyltransferase; after incubation the reaction mixture was phenol-extracted, and the labeled oligo[d(T)] was purified by chromatography on a poly[r(A)]–agarose column.

Template–Primers. Concentrations of template–primers are expressed as micromolar total nucleotide. Calf thymus DNA was from Worthington Biochemical Corp.; it was first activated by the method described by Schlabach et al. (1971), and then it was ethanol-precipitated, dissolved in 50 mM Tris-HCl, pH 7.5, and dialyzed against this same solution. d(T)$_{\overline{14}}$ and d(T)$_{\overline{580}}$ were from Collaborative Research and Miles Laboratories, respectively. Poly[d(A)] was obtained from Miles Laboratories; the average chain length of this material was 255 nucleotides as determined by a 5′-end labeling method (Gupta et al., 1968; Flint et al., 1974) using polynucleotide kinase. For the d(A)$_{\overline{255}}$·d(T)$_{\overline{14}}$ template–primer system, the molecular ratio of template to primer was 1:10. The template and primer were mixed and incubated at 60 °C for 5 min in the presence of 10 mM KCl. This mixture was then held at 22 °C for 1 h before being mixed at 0–1 °C with other components of the DNA polymerase reaction mixture. After standing at 0–1 °C for 15 min, the β-polymerase was added and the reaction mixtures were incubated at 37 °C. To prepare a "hooked" (Chang, 1973b) template–primer, we first incubated the d(A)$_{\overline{255}}$ with dCTP and terminal deoxynucleotidyltransferase under conditions controlled for the addition of 5–7 dCMP residues per d(A)$_{\overline{255}}$ molecule. After this incubation, the reaction mixture was phenol-extracted, and the polymer was collected by ethanol precipitation. Based upon the amount of dCMP incorporated and the number of 5′ ends, an average of six dCMP residues had been added onto the d(A)$_{\overline{255}}$. dGMP was then grafted onto the d(A)$_{\overline{255}}$-d(C)$_{\overline{6}}$ in a similar fashion. The resulting d(A)$_{\overline{255}}$-d(C)$_{\overline{6}}$-d(G)$_{\overline{3}}$ was incubated with mouse DNA polymerase β and dGTP under conditions where dGMP incorporation was carried to completion. The reaction product was phenol-extracted and collected by ethanol precipitation. The ethanol precipitation was repeated once to remove traces of dGTP, and the final precipitate was dissolved in 20 mM Tris-HCl, pH 7.5, and 20 mM NaCl. Based upon the amount of dGMP that had been added and the number of 5′ ends, the final product was d(A)$_{\overline{255}}$-d(C)$_{\overline{6}}$-d(G)$_{\overline{14}}$. With this synthetic polynucleotide as the template–primer, mouse β-polymerase had relatively high activity (see Results); the rate of dTMP incorporation was the same in the presence and absence of dGTP, and the incorporation rate in reactions containing a single dNTP was 10–20 times lower with dGTP than with dTTP.

Methods

Polymerization Assay. For the kinetic studies, reaction mixtures were incubated for 5 or 10 min at 37 °C and contained, in a final volume of 10 μL, the following: 50 mM Tris-HCl, pH 8.7–8.8 at 37 °C, 100 mM NaCl, 15% glycerol, 200 μg/mL BSA, 1 mM DTT, 20 μM EDTA, 0.05 nM β-polymerase (fraction V) plus template–primer, dNTP–metal and free-metal activator as indicated. [^{3}H]DNA products were collected on nitrocellulose filters, and radioactivity was measured as described (Schrier & Wilson, 1975). The specific activity of [^{3}H]dTTP was 12000–24000 dpm/pmol. Linearity of dTMP incorporation with time of incubation was observed for the various concentrations of dNTP, template–primer, and metal. The rate of dTMP incorporation was proportional to the amount of β-polymerase with the lowest and highest concentration of each substrate tested. Incorporation of dTMP was about 20% of the total dNMP incorporation with activated DNA as template–primer. This was confirmed with the highest and lowest concentrations of DNA and dNTP tested and also with reactions containing 80 μM NaPP$_i$.

Kinetic constants were evaluated graphically and also by least-square fits of data to the appropriate equations using the interactive curve-fitting program, MLAB, described by Knott & Schrager (1972). Levels of free magnesium or manganese were calculated by using the value of 18000 or 45000 M^{-1}, respectively, as the stability constant for the metal–dNTP^{2-} complex (Khan & Martell, 1966; O'Sullivan & Perrin, 1961; Morrison et al., 1961). Any binding to Tris-HCl (O'Sullivan & Cohn, 1966) or other components of the reaction mixture was ignored.

In the purification studies reaction mixtures were incubated for 30 min at 37 °C and contained, in a final volume of 10 μL, the following: 50 mM Tris-HCl, pH 8.7, 10 mM $MgCl_2$, 100 mM KCl, 15% glycerol, 400 μg/mL BSA, 1 mM DTT, 625 μM AcDNA, 100 μM each of dATP, dGTP, and dCTP, [^{3}H]dTTP (sp act. 1130 dpm/pmol), and an enzyme fraction. One unit of enzyme activity is defined as the amount of enzyme giving 1 μmol of total dNMP incorporation per 60 min at 37 °C.

Pyrophosphate Release Assay. Reaction conditions were as described for the polymerization assay except as follows: the four dNTPs were present at 20 μM each; [^{32}P]dTTP (3000 dpm/pmol) was used; free Mg^{2+} was 2 mM and activated DNA was 290 μM. After incubation the reaction mixtures were treated according to the method described by Deutscher & Kornberg (1969), and radioactivity not adsorbed to charcoal was measured.

Pyrophosphate Exchange and Pyrophosphorolysis Assays. Reaction mixtures were incubated at 37 °C for 30 min and contained, in a final volume of 10 μL, the following: 50 mM Tris-HCl, pH 8.8 at 37 °C, 1 mM DTT, 0.02 mM EDTA, 100 mM NaCl, 10 mM $MgCl_2$, 200 μg/mL BSA, 15% glycerol, 130 μM AcDNA, 50 μM each of the four dNTPs, 90 μM [^{32}P]NaPP$_i$ (2000–3000 cpm/pmol), and 0.05 nM β-polymerase. For the pyrophosphorolysis assay the four dNTPs were omitted. Polymerization was measured in some reactions with [^{3}H]dTTP as one of the four dNTPs. After incubation the reaction mixtures were treated according to the method described by Deutscher & Kornberg (1969).

5′-Exonuclease Assay. Reaction conditions were as described for the polymerization assay except as follows: the incubation time was 90 min; 15 μg/mL 5′-end ^{32}P-labeled d(T)$_{\overline{550}}$ (4200 dpm/pmol) and 2 mM $MgCl_2$ were present; the dNTPs were omitted. After incubation, the ^{32}P-labeled

[1] Abbreviations used: AcDNA, activated DNA; BSA, bovine serum albumin; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate; KP$_i$, potassium phosphate buffer; P$_i$, phosphate; PP$_i$, pyrophosphate; dNMP, deoxynucleoside 5′-monophosphate; dNTP, deoxynucleoside 5′-triphosphate; kinetic abbreviations and nomenclature are according to Cleland (1963a,b); subscripts with a bar indicate the average chain length of polynucleotides.

Table I: Purification of Mouse DNA β-Polymerase

fraction[a] no.	description	protein (mg)	act.[b] (units)	sp act. (units/mg protein)
I	crude extract	2600		
II	DEAE-cellulose, phosphocellulose pool	80	8	0.1
III	50–80% $(NH_4)_2SO_4$ fraction	7	4	0.6
IV	Sephacryl S-200 pool	2	2	1
V	first DNA-cellulose pool	0.02	1.3	65
(IV	second DNA-cellulose pool	0.01	0.9	90)

[a] A nuclear fraction from 180 g of MOPC-104E was used. Experiments were performed as described under Methods. [b] One unit = 1 μmol of dNMP incorporated per 60 min at 37 °C.

remained was collected on a nitrocellulose filter and measured.

3′-Exonuclease Assay. Reaction conditions were as described for the polymerization assay except as follows: the incubation time was 60 min; 3.4 μg/mL 3′-end [^{3}H]-dTMP-labeled $d(T)_{\overline{17}}$ (7200 dpm/pmol) and 2 mM $MgCl_2$ were present; the dNTPs were omitted. After incubation the [^{3}H]dTMP released was separated by chromatography on DE-81 paper and measured at a counting efficiency of 4.8%.

$NaDodSO_4$–Polyacrylamide Slab Gel Electrophoresis. Each enzyme fraction was dialyzed against 0.05% $NaDodSO_4$ at room temperature for 24 h and then lyophilized. The residue was dissolved in 300 μL of 0.05% $NaDodSO_4$, and the solution was dialyzed against 0.05% $NaDodSO_4$ and then lyophilized. The residue was dissolved in 25 μL of 0.0625 M Tris-HCl, pH 6.8, 2% $NaDodSO_4$, 5% β-mercaptoethanol, and 10% glycerol. After standing for 5 min in boiling water, the sample was electrophoresed in a $NaDodSO_4$–polyacrylamide slab gel (Laemmli, 1970) with a separating zone of 10% polyacrylamide. Stained gels were scanned with an Ortec Model 4310 densitometer; the following were the protein standards: β-galactosidase, 135 000; phosphorylase *a*, 94 000; BSA, 68 000; human IgG heavy chain, 55 000; ovalbumin, 45 000; α-chymotrypsinogen, 25 000.

Buffers. All pH values refer to the final solution at 4 °C. Buffer A is 20 mM Tris-HCl, pH 7.6, 0.25 M sucrose, 4 mM magnesium acetate, 0.1 mM EDTA, and 1 mM DTT. Buffer B is 20 mM KP_i, pH 7.4, 0.1 mM EDTA, 1 mM DTT, and 10% glycerol. Buffer C is 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 10% glycerol, 0.1 mM EDTA, and 1 mM DTT. Buffer D is 20 mM Tris-HCl, pH 8.0, 20% glycerol, 0.1 mM EDTA, and 1 mM DTT. The saturated solution of ammonium sulfate also contained 1 mM DTT and 0.1 mM EDTA and was at pH 7.0.

Purification of Mouse DNA Polymerase β. β-Polymerase was purified from a nuclear fraction of myeloma MOPC-104E solid tumor (Wilson et al., 1974) by a modification (Table I) of procedures described previously (Chang, 1973a; Stalker et al., 1976). In brief, frozen tissue (180 g) was thawed, minced, and homogenized with 3 volumes of buffer A by using a motor-driven loose-fitting glass–Teflon homogenizer. The homogenate was centrifuged at 15000*g* for 15 min, and the pellet fraction (85 g) was suspended in 2 volumes (170 mL) of buffer A. The suspension was centrifuged at 15000*g* for 10 min, and the supernatant fraction was discarded. After repeating this step once, the washed pellet fraction was mixed with 215 mL of buffer B containing 0.5 M KCl, and the mixture was homogenized and then sonicated. The resulting homogenate was adjusted to a final concentration of 0.5% in Triton X-100 and held at 35 °C for 10 min. The solution was centrifuged at 15000*g* for 20 min, and the supernatant fraction was then centrifuged at 70000*g* for 2.5 h. The turbid layer at the top of the resulting supernatant fraction was discarded, and the upper four-fifth of the clear supernatant fraction was collected (fraction I; 195 mL) and mixed with an equal volume of buffer B. The solution was loaded onto an 80-mL DEAE-cellulose column, the effluent from which passed directly onto a 115-mL phosphocellulose column; the two columns had been equilibrated with buffer B containing 0.25 M KCl. After a wash of 600 mL of buffer B containing 0.25 M KCl had passed onto the DEAE-cellulose column, the phosphocellulose column was separated from the DEAE-cellulose column and then eluted with a 900-mL liner gradient of 0.25–0.8 M KCl in buffer B. The column profile contained a single sharp peak of DNA polymerase activity centered at 0.5 M KCl. The peak fractions were pooled (fraction II) and mixed with enough solid ammonium sulfate to bring the final solution to 50% saturation. Protein that precipitated was removed by centrifugation, and the supernatant fraction was mixed with saturated ammonium sulfate to adjust the solution to 80% saturation. Proteins that precipitated were collected, dissolved in buffer C (fraction III; 2.9 mL), and loaded onto a 0.9 × 90 cm column of Sephacryl S-200, which was connected in tandem to an identical column. These columns were equilibrated and developed with buffer C at a flow rate of 10 mL/h. Fractions containing the single sharp peak of polymerase activity were pooled (fraction IV; 8.4 mL), mixed with an equal volume of 20 mM Tris-HCl, pH 8.0, 30% glycerol, 0.1 mM EDTA, and 1 mM DTT, and then loaded onto a 4.6-mL column of single-stranded DNA–cellulose. This column had been equilibrated with buffer D containing 0.25 M NaCl. After the column had been washed with 18 mL of buffer D containing 0.3 M NaCl, the enzyme was eluted with a 50-mL linear gradient of 0.3–0.8 M NaCl in buffer D. The column profile contained a single sharp peak of activity centered at 0.55 M NaCl. Peak fractions were pooled (10 mL), and this was referred to as fraction V.

The activity of fraction V was stable at −20 °C in the presence of 50% glycerol and 0.5 mg/mL BSA, and the sample used in the kinetic studies to be described was stored in this way.

In order to test for cochromatography of the polymerase activity and a 40 000-M_r polypeptide in fraction V, we dialyzed fraction V against buffer D containing 0.25 M NaCl and then loaded it onto a 1.5-mL column of single-stranded DNA–cellulose. After the column had been washed with 6 mL of buffer D containing 0.25 M NaCl, the enzyme was eluted with a 20-mL linear gradient of 0–0.4 mg/mL dextran sulfate in buffer D containing 0.25 M KCl. Fractions containing the single sharp peak of activity (centered at 0.1 mg/mL dextran sulfate) were pooled (fraction VI) and stored in liquid nitrogen.

Results

Some Characteristics of Mouse β-Polymerase. The elution position of β-polymerase from the "high salt" Sephacryl S-200 column corresponded to a molecular weight of 40 000. As shown by the sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis analysis described in Figure 1, a protein band with an apparent molecular weight of 40 000 accounted for about 70 and 85%, respectively, of the total Coomassie blue stained protein in fractions V and VI. Thus, the 40 000-M_r polypeptide cofractionated with the DNA polymerase activity during the dextran sulfate elution from DNA–cellulose. In studies not shown, we found that the minor band with a M_r of 45 000 did not cofractionate with the polymerase activity. Based upon these results and the relatively high specific activities of fractions V and VI (Table I), we believe it is likely

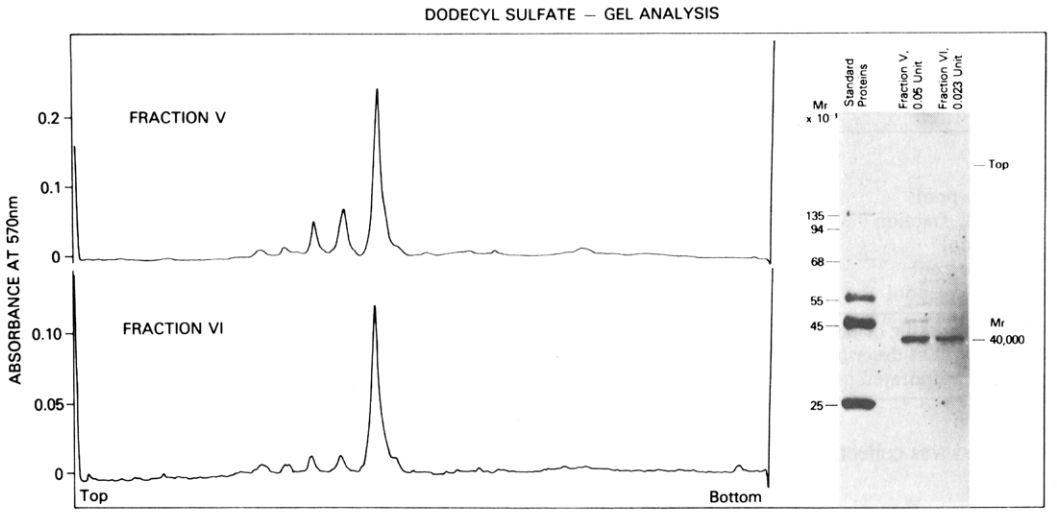


FIGURE 1: Sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis of fraction V and fraction VI. Experiments were performed as described under Methods.

that mouse β-polymerase is composed of a single polypeptide with a M_r of approximately 40000. Likewise, Chang (1973a) has presented evidence that calf thymus β-polymerase is a single polypeptide of about this same molecular weight, and similar results have been reported with β-polymerase from other mammalian sources (Wang et al., 1974, 1977). The 32000-M_r polypeptide attributed to rat Novikoff hepatoma β-polymerase (Stalker et al., 1976) is significantly smaller than the polypeptides attributed to these other β-polymerases, and we note that no band at 32000-M_r was detected in fraction V or VI.

dNMP incorporation by mouse β-polymerase was in reasonable stoichiometric agreement with pyrophosphate release, and there was no detectable degradation (turnover) of deoxynucleotide. Neither 3′-exonuclease nor 5′-exonuclease activity was detected. Pyrophosphate exchange and pyrophosphorolysis were not detected at levels of sensitivity equal to 0.5% of the dNMP incorporation activity. Also, these two latter reactions were too low to be detected when higher concentrations of $NaPP_i$ were used or when Mn^{2+} was the metal activator; further, this mouse β-polymerase did not catalyze a dNTP turnover reaction under the conditions reported for the KB cell β-polymerase dNTP turnover reaction (Wang et al., 1975).

Polymerization was inhibited both by $NaPP_i$ and by NaP_i. Much less $NaPP_i$ was required for 50% inhibition—80 μM vs. 4 mM—indicating that any contaminating NaP_i was not responsible for the $NaPP_i$ inhibition. Thus, these results on enzymatic properties of mouse β-polymerase indicate (1) that there were two products of the forward reaction, DNA_{n+1} and PP_i, (2) the forward reaction could be inhibited by one of the products, PP_i, and (3) the ratio of the forward and reverse reactions was >100, and the rate of any reverse reaction was so low that isotope exchange studies were not possible.

Metal Activation. At levels of activated DNA in the range of 5–200 μM, the dNMP polymerization rate was higher with the optimum concentration of $MgCl_2$ (~2 mM) than with the optimum concentration of MnCl (~0.5 mM). This is in contrast to the Mn^{2+} preference observed by Wang et al. (1977) with a human β-polymerase. The apparent "K_m" for free Mg^{2+} was 340 μM. The effect of various fixed concentrations of Mg^{2+} on the initial rate of dNMP incorporation was examined. With either activated DNA or Mg–$dNTP^{2-}$ as the variable substrate, linear double-reciprocal plots and a noncompetitive pattern was observed; i.e., Mg^{2+} did not affect the apparent K_m for either type of substrate (data not shown).

Table II: Kinetic Constants for Polymerization by Mouse DNA β-Polymerase

kinetic constant	template-primer/dNTP system: activated calf thymus DNA	$d(A)_{\overline{255}}$ $d(T)_{\overline{14}}$	hooked primer $d(A)_{\overline{255}}$[a]
K_m of metal–$dNTP^{2-}$ (μM)	13.7	10.3	83
K_m of template–primer (μM)	14.4	50	80
K_m of free Mg^{2+} (μM)	340		
V_{max} [(molecules/sec)/enzyme]	11	30	17

[a] Hooked primer $d(A)_{\overline{255}}$ is $d(A)_{\overline{255}}$-$d(C)_{\overline{6}}$-$d(G)_{\overline{14}}$.

Steady-State Kinetics. With activated DNA as the template–primer and 2 mM free Mg^{2+} as the metal activator, the double-reciprocal plots were linear and the patterns were intersecting with either Mg–$dNTP^{2-}$ or DNA as the varied substrate (Figure 2a). As shown in Figure 2b, replots of the slopes and intercepts were also linear.

In order to examine more simplified polymerization reactions, we used dTTP as the nucleotide and $d(A)_{\overline{255}}$ as the template, either with a covalently attached hooked primer or with $d(T)_{\overline{14}}$ as the primer. Double-reciprocal plots of substrate kinetic data from these reactions were linear, and the patterns were intersecting (data not shown). Replots of the slopes and intercepts are shown in Figure 2c,d. Some kinetic constants for the three template–primer/dNTP systems are summarized in Table II.

These findings on substrate initial velocity patterns are most consistent with a sequential mechanism, either where the enzyme binds to the DNA before the deoxynucleotide and is released after pyrophosphate or where binding of the two substrates and release of the two products is random.

Pyrophosphate Inhibition. In order to distinguish between ordered and random mechanisms, we analyzed the kinetics of $NaPP_i$ inhibition. The effect of various fixed concentrations of $NaPP_i$ on the initial velocity pattern with either Mg–$dNTP^{2-}$ or DNA as the variable substrate is shown in Figure 3. With Mg–$dNTP^{2-}$ as the variable substrate, the lines form a converging pattern with an intercept beyond the ordinate. However, as seen in Figure 3b,d, essentially parallel patterns were observed when DNA was the varied substrate. In the experiments shown in Figure 3b,d, relatively high concen-

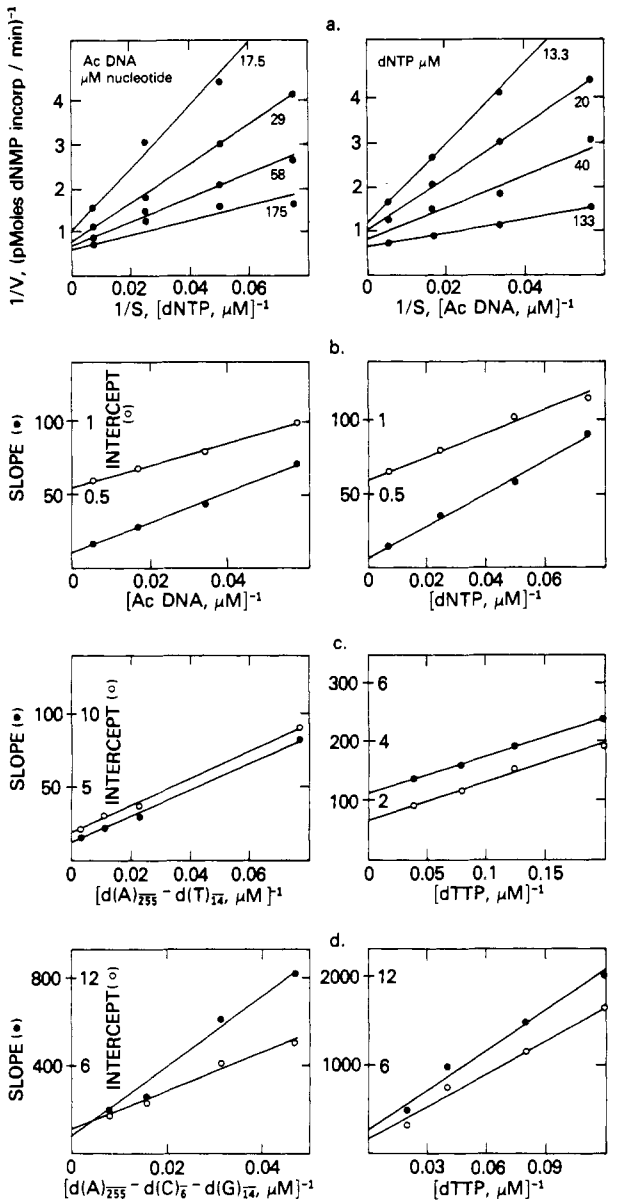
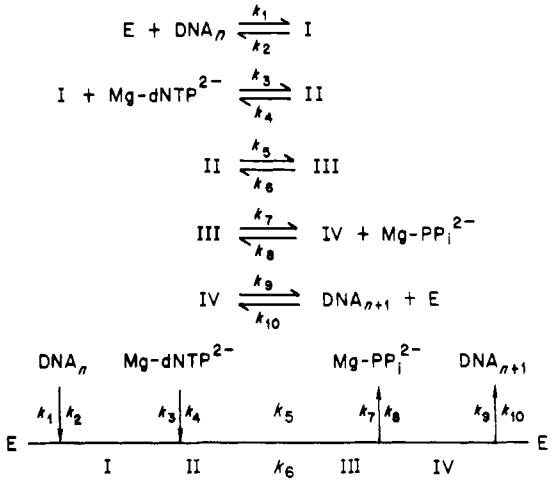


FIGURE 2: Substrate kinetics of DNA polymerase β with three different template–primers/dNTP systems. Activated DNA and Mg–dNTP^{2-}, the right-hand and left-hand portions of the panel a, show double-reciprocal plots with Mg–dNTP^{2-} or activated DNA as the varied substrate. The metal activator was Mg^{2+} at a concentration of 2 mM (free). Replots of the slopes and intercepts from panel a are shown in panel b. $d(A)_{\overline{255}}$–$d(T)_{\overline{14}}$ and Mn–dTTP^{2-}, replots of the slopes and intercepts from double-reciprocal plots, are shown in panel c. The metal activator was Mn^{2+} at a concentration of 0.5 mM (free). Hooked primer $d(A)_{\overline{255}}$ and Mg–dTTP^{2-}, replots of the slopes and intercepts from double-reciprocal plots, are shown in panel d. The metal activator was Mg^{2+} at a concentration of 0.5 mM (free).

trations of Mg–dNTP^{2-} were used, whereas in similar experiments with a very low concentration of Mg–dNTP^{2-} (2 μM), a gradually intersecting pattern was obtained. These results are consistent with an ordered mechanism but not with a random mechanism.

Discussion

The results of the kinetic experiments presented here point to an ordered bibi mechanism for polymerization by mouse β-polymerase. This mechanism, which is similar to that proposed for *E. coli* DNA polymerase I by McClure & Jovin (1975), may be written and diagramed as follows.



This scheme depicts binding of the enzyme at two different DNA sites that are designated DNA_n and DNA_{n+1}. The DNA_n site represents the microenvironment around the 3′-end hydroxyl group of the primer, whereas the DNA_{n+1} site represents the microenvironment around the newly formed phosphodiester bond. Results from the initial characterization experiments indicated that polymerization by mouse β-polymerase is a two substrate–two product reaction and is not complicated by such other reactions as deoxynucleotide degradation or DNA degradation. This system is potentially more complicated, however, than the general two substrate–two product system (Chao et al., 1969; McClure & Jovin, 1975) because the polymeric product is present at the beginning of the reaction and may compete for the enzyme to form complex IV. The rate equation for this mechanism where both DNA_n and DNA_{n+1} sites are present at the beginning of the reaction is

$$V/E_T = (V_1[DNA_n][dNTP])/\Bigg[K_{i(DNA_n)}K_{dNTP} + K_{DNA_n}[dNTP] + K_{dNTP}[DNA_n]\left[1 + \frac{K_{i(DNA_n)}}{K_{i(DNA_{n+1})}}\right] + [DNA_n][dNTP]\left[1 + \frac{K_{DNA_n}}{K_{i(DNA_{n+1})}}\right]\Bigg]$$

This equation has the same form as the general equation for a two substrate–two product system, and it becomes equivalent to the general equation if the free enzyme has a much higher affinity for the DNA_n site than the DNA_{n+1} site, i.e., if $K_{i(DNA_{n+1})}$ is relatively large. Several observations suggest that this is the case: β-polymerase did not catalyze either a measurable backward reaction or a 3′-end degradation of DNA; polymerization was not inhibited by DNA, either in the presence or in the absence of PP_i; finally, it has been shown that under a variety of reaction conditions β-polymerases from calf thymus and KB cells act in a nonprocessive fashion, i.e., the enzyme dissociates from DNA after the addition of each dNMP (Chang, 1975; Bambara et al., 1978). Therefore, if competition by the DNA_{n+1} site is ignored, the general equation for a two substrate–two product ordered sequential mechanism applies.

$$V/E_T = (V_1[DNA_n][dNTP])/[K_{i(DNA_n)}K_{dNTP} + K_{DNA_n}[dNTP] + K_{dNTP}[DNA_n] + [DNA_n][dNTP]]$$

This equation predicts linear converging patterns for the double-reciprocal plots with each substrate, and it also predicts linear secondary plots. These predictions are consistent with

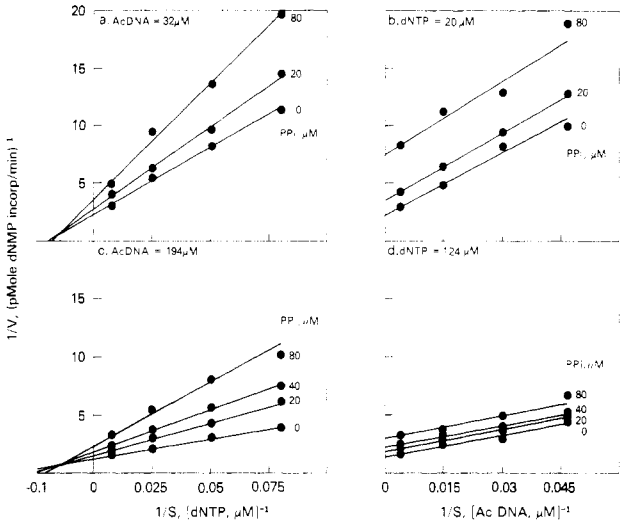
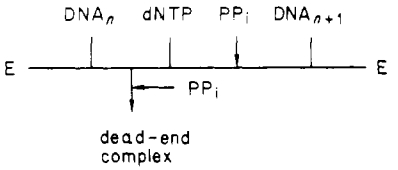


FIGURE 3: Substrate initial velocity patterns and product inhibition by pyrophosphate. The metal activator was Mg^{2+} at a concentration of 10 mM (free). Activated DNA and Mg–$dNTP^{2-}$ were at the levels shown. Other reaction conditions were as described under Methods.

the experimental results with all three template–primer/dNTP systems examined. Other possible models involving different ordered sequential mechanisms or ping pong mechanisms appear to be excluded by these results.

The distinction between an ordered and a random sequential mechanism depends upon the pyrophosphate inhibition study. It is clear from the results (Figure 3) that the PP_i inhibition patterns for the substrate kinetics with varied DNA and with varied dNTP were different and were consistent with an ordered mechanism. This interpretation is unchanged even if the PP_i inhibition observed was not simple product inhibition but rather some combination of dead-end complex formation and product inhibition. Secondary plots of the PP_i inhibition results with dNTP as the variable substrate revealed that the noncompetitive inhibition was S-parabolic I-linear with lower concentrations of DNA and S-linear I-linear with a saturating concentration of DNA (250 μM). This is consistent (Cleland, 1963b) with a model where PP_i interacts with the enzyme as a product inhibitor and also as a dead-end inhibitor.



A key feature of the polymerization mechanism proposed here is that the free enzyme must bind to the proper site on the template–primer in order for the dNTP binding site to be created. It might therefore be expected that certain template–primer analogues with which a normal enzyme–(template–primer) interaction is not possible would also be associated with higher K_m values for dNTP. Perhaps this could be the explanation for the six- to eightfold higher K_m values observed with the hooked primer poly[d(A)] system (Table II).

Metal Activation. In the present study the mechanism of metal activation of β-polymerase was not examined in detail, although we did find that metal activation does not alter the K_m for either DNA or Mg–$dNTP^{2-}$. All of the secondary plots of slopes and intercepts from these substrate kinetic experiments with different levels of Mg^{2+} revealed that the activation was S-hyperbolic I-hyperbolic. It is, therefore, likely that the mechanism is complex and involves multiple binding sites for the metal activator. Indeed, Mildvan and Loeb and co-workers (Slater et al., 1972) have found that *E. coli* DNA polymerase I has multiple binding sites for metal activator. In addition, we have found that excess Mg^{2+} or Mn^{2+} has a marked inhibitory effect on polymerization by mouse β-polymerase.[2] Also, as noted above, there are differences in the metal activation properties of various β-polymerases, perhaps depending upon the enzyme source and method of isolation [see above and Wang et al. (1977)].

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[2] Tanabe and Wilson, unpublished experiments.