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Kinetic Analysis of *Escherichia coli* Deoxyribonucleic Acid Polymerase I*

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The kinetic properties of *Escherichia coli* DNA polymerase I were simplified to those of a 1 deoxynucleotide substrate reaction by the use of polynucleotide templates. With poly(dA)·oligo(dT) as the template·primer complex, Mg^{2+} decreases the K_m of the substrate dTTP but has little or no effect on the K_m of the substrate Mg-dTTP, suggesting that multiple pathways involving the binding of Mg^{2+}, dTTP, and Mg-dTTP are operative in forming the active complex. The K_m of free Mg^{2+}, extrapolated to zero concentration of substrate (830 ± 62 μM), agrees within a factor of 2 with the dissociation constant of magnesium from 4 ± 1 sites on the enzyme determined previously by binding studies (Slater, J. P., Tamir, I., Loeb, L. A., and Mildvan, A. S. (1972) *J. Biol. Chem.* **247,** 6784–6794). The maximal turnover number with poly(dA) as template is 5.7 ± 0.7 s^{-1}. Changing the nature of the base in the polydeoxynucleotide template alters the maximal rate of polydeoxynucleotide synthesis by an overall factor of 31 with poly(dC) > poly(dT) > poly(dA) ≫ poly(dG), indicating that pyrimidine templates are copied faster than purine templates. Changing the sugar structure from poly(dA) to poly(rA) causes a 3-fold increase in the rate of template copying.

A study of the kinetic effects of all noncomplementary deoxynucleotides with all deoxynucleotide templates, as well as with poly(rA)·oligo(dT), yields complex patterns of activation and inhibition requiring from 1 to 2 additional binding sites for the noncomplementary nucleotides. The kinetically determined affinities of the active site of the enzyme·template·primer complex for the complementary free nucleotide (as measured by K_m) generally exceed those for the noncomplementary nucleotides (as measured by K_I slope) by 1 to more than 3 orders of magnitude. Similarly, the apparent affinity of a given nucleotide or its magnesium complex as a substrate (as determined by its K_m) generally exceeds its affinity as a noncomplementary competitive inhibitor (as measured by K_I slope) by 1 to more than 3 orders of magnitude, suggesting 1 to 2 additional hydrogen bonds stabilizing the complementary interaction. Hence, the kinetic data indicate that the presence of the template facilitates the rejection of noncomplementary nucleotides at the sites of polymerization. In most but not all cases, the kinetically determined free energy of discrimination between the binding of complementary and noncomplementary nucleotides at the active site is insufficient to account for the known fidelity of DNA synthesis *in vitro*. Hence, additional orientational effects are required. The kinetic data also establish the existence of multiple nucleotide binding sites on DNA polymerase which influence the rate of polymerization and are therefore consistent with the prealignment of substrates on the enzyme prior to polymerization.

Recent evidence suggests that DNA polymerases have a common catalytic mechanism; they are zinc metalloenzymes and have identical cofactor requirements (1–3). The binding of substrates and of the required divalent cation activator (Mg^{2+} or Mn^{2+}) to *Escherichia coli* DNA polymerase I (Pol I) has been studied by several methods (4, 5). However, these methods suffer from the necessary absence of a template·primer complex which might profoundly influence the dissociation constants of the substrates. Dissociation constants of nucleotides from their ternary complexes with enzyme and Mg^{2+} have previously been determined by equilibrium dialysis (4). Dissociation constants of Mn^{2+} and nucleotides from their binary and ternary enzyme complexes have been determined by electron paramagnetic resonance and water proton relaxation rates (5). The magnetic resonance studies established the existence of 5 ± 1 binding sites on monomeric DNA polymerase I for Mn^{2+} or Mg^{2+}, and the interaction of 1 molecule of either dTTP or dTMP with each of the bound Mn^{2+} ions. Also, in these studies, dissociation constants of dTTP and its manganese complex have been compared with average K_m values of all 4 nucleotide substrates using DNA templates.

A proper kinetic analysis of this catalytic mechanism has been difficult to carry out because of the multicomponent

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nature of the system. The availability of polynucleotides of known composition has made it possible to simplify the polymerization reaction so that it can be analyzed kinetically. In this paper, we have undertaken to study the initial kinetics of DNA synthesis using homopolymer templates. We have chosen Pol I as a prototype enzyme because it has been the most intensively studied of the DNA polymerases (3).

We have studied the effects of the chemical structure of the template·primer complex on the kinetic parameters of complementary and noncomplementary nucleotides and their Mg^{2+} complexes. This was accomplished by using a template which consisted in each case of a homopolymer containing a single nucleotide moiety and a primer containing the complementary nucleotide (*i.e.* poly(dA)·oligo(dT)). In such a reaction, the deoxynucleotide triphosphate complementary to the nucleotide moiety of the template is the only deoxynucleotide substrate. Thus, a 4 nucleotide substrate reaction is reduced to a 1 nucleotide substrate reaction. Moreover, it becomes possible to study inhibition using the 2 deoxynucleoside triphosphates which are not complementary either to the nucleotide moiety of the template or to the primer.

EXPERIMENTAL PROCEDURE

Materials

Escherichia coli Polymerase I

The enzyme was purified by the method of Jovin *et al.* (6); this includes phosphocellulose chromatography and Sephadex G-100 gel filtration. The fraction used in these experiments was that used by Springgate *et al.* (7). The enzyme was estimated to be at least 99% pure by electrophoresis on polyacrylamide gels under a variety of conditions and has a specific activity with maximally "activated" calf thymus DNA (8) of 24,000 units/mg of protein (6). The molecular weight of Pol I is 1.09×10^5 (6); 1 μg of homogeneous Pol I is equivalent to 9.17 pmol.

Templates and Primers

Homopolymers and oligonucleotides were purchased from P-L Biochemicals, Inc. The homopolymers used were as follows: poly(dA), MW 2.5×10^5; poly(dT), MW 3.7×10^5; poly(dG), MW 0.7×10^5; poly(dC), MW 2.5×10^5; and poly(rA), MW 3×10^5. The oligomers were as follows: $oligo(dT)_{12\text{-}18}$; $oligo(dA)_{10}$; $oligo(dC)_{12\text{-}18}$; and $oligo(dG)_{12\text{-}18}$. The purity of the homopolymer templates was determined by measuring the simultaneous incorporation of complementary and noncomplementary nucleotides with Pol I (9).

Deoxynucleoside Triphosphates

Unlabeled deoxynucleoside triphosphates were purchased from Calbiochem. Labeled deoxynucleoside triphosphates were obtained from New England Nuclear Corp. Each substrate was determined to be >98% pure by thin layer chromatography (10) and by the method of simultaneous incorporation which is described below. The purity of each ^3H-labeled nucleoside triphosphate substrate was tested by comparing its incorporation into DNA with the incorporation of the same nucleotide, labeled with ^{14}C or α-^{32}P of known purity, in the same reaction mixture. For example, 10 nmol of [^3H]dTTP were added to a reaction mixture, which contained in a total volume of 0.3 ml: 10 nmol of [^{14}C]dTTP; 20 nmol of dATP, dCTP, and dGTP; 25 μmol of Tris-maleate buffer, pH 8.0; 4.5 μmol of $MgCl_2$, 0.5 μmol of KCl, 0.3 μmol of β-mercaptoethanol; 177 nmol of activated calf thymus DNA; and 0.1 units of sea urchin nuclear DNA polymerase (11). After incubation for 15 min at 37° the amount of [^3H]dTTP and [^{14}C]dTTP incorporated in an acid-insoluble precipitate was determined. When both labeled substrates are of identical purity, the ratio of ^3H to ^{14}C in the product will be the same as that present in the reaction mixture.

Methods

Polymerase Assays

The conditions of the reaction for each template studied are detailed below. Each reaction was incubated at 37° and was stopped by precipitating the product with 0.1 ml of 1 M $HClO_4$ containing 0.02 M sodium pyrophosphate using 0.1 ml of DNA (1 mg/ml) as carrier. Then, the precipitate, dissolved in 0.5 ml of 0.2 M NaOH/0.05 M sodium pyrophosphate, was reprecipitated with acid, collected on glass fiber filters, and counted by liquid scintillation spectroscopy. Activity is expressed as moles of nucleotide incorporated/mol of enzyme/min.

Components of the reaction mixture (0.05 ml) are as follows.

Buffer—throughout these studies, Tris-HCl, pH 7.4, was used at a concentration of 50 mM.

$MgCl_2$—the [Mg^{2+}] necessary for maximum incorporation of substrate was found to vary with the homopolymer used as template. The [Mg^{2+}] used in the nucleotide competition studies was at an activating concentration for each template·primer complex: 2 mM for poly(dA)·oligo(dT), poly(rA)·oligo(dT), and poly(dC)·oligo(dG) and 0.6 mM for poly(dT)·oligo(dA) and poly(dG)·oligo(dC).

Substrates—^3H-labeled deoxynucleotide triphosphates were serially diluted; the initial solution in each case was 10 mM (specific activity, 0.1 mCi/mM). Assays were done in duplicate at five different substrate concentrations (5, 10, 20, 50, and 100 μM) for each inhibitor concentration (0, 50, 100, 200, and 300 μM).

Template and Primer Concentrations—the saturating template concentration necessary to measure substrate incorporation for initial kinetics was found to be 400 nM based on the molecular weight of the template. With the template concentration kept at 400 nM, the ratio of primer to template was varied. A ratio of primer to template molecules greater than 2 was found to be inhibitory to the reaction; however, the rate of substrate incorporation was not affected by decreasing the ratio from 2 to 0.2. Since we were concerned that most of the template molecules should not be bound to more than 1 primer molecule, we chose a primer to template ratio of 0.5. Assuming a Poisson distribution, 60.7% of the template molecules are not bound to a primer molecule, 30.3% have 1 primer molecule, and 9.0% have 2 or more. Under these conditions, the enzymes were found to be saturated by the template·primer complex. It was not found necessary to preincubate the template and primer molecules at elevated temperatures.

Enzyme Concentrations—the concentration of enzyme needed to obtain a reaction rate, linear with time and proportional to enzyme concentration, was found to vary with the type of template involved. With poly(rA) as a template at too low a concentration of enzyme for a given template, a lag period was observed; this observation was similar to that of Cavalieri and Carroll (12). The concentrations of enzyme used per assay were as follows for each template·primer complex: poly(dA)·oligo(dT), 0.18 pmol/assay; poly(rA)·oligo(dT), 0.36 pmol/assay; poly(dC)·oligo(dG), 0.36 pmol/assay; poly(dT)·oligo(dA), 0.72 pmol/assay; and poly(dG)·oligo(dC), 0.9 pmol/assay. Enzyme concentrations were made by diluting a concentrated stock solution (2 mg/ml) with buffer (0.2 M Tris-HCl, pH 7.8; 1 mM β-mercaptoethanol; 20% glycerol) just prior to use.

Time of Incubation—because the duration of constant rates differed considerably for each template, the times used to obtain linear kinetics were as follows: for poly(dA), poly(rA), and poly(dC), 2 min at 37°; for poly(dT) and poly(dG), 5 min at 37°. All assay tubes were preincubated at 37° for 5 min prior to the addition of enzyme. At the extremes of substrate and inhibitor concentrations, control experiments were carried out to show that constant rates of DNA synthesis were occurring during the time intervals used. Each assay was run in duplicate.

Calculations

To calculate the amounts of free and Mg^{2+}-bound deoxynucleoside triphosphates, the dissociation constant of 61.1 μM for the Mg·ATP-complex under comparable conditions was used for all of the deoxynucleoside triphosphates (13). The assumption that all nucleoside triphosphates have equal affinity for metal ions is based on thermodynamic (14) and structural studies (15). Under our experimental conditions, negligible binding of Mg^{2+} to the template·primer complex (5) or to the Tris-HCl buffer took place (16) and such binding could be ignored. The kinetic data with the noncomplementary nucleotides were analyzed by weighted least square fits to the primary double reciprocal plots and unweighted least square fits to the secondary plots using the methods described by Cleland (17).

RESULTS

Metal Activation—With poly(dA)·oligo(dT) used as the template·primer complex at saturating concentrations, the

concentrations of the complementary substrate and the activator, Mg^{2+}, were systematically varied. As illustrated in Fig. 1, with Tris-HCl as buffer, the maximal activity was 3-fold greater and the optimum Mg^{2+} concentration was 10-fold lower than with phosphate buffer, presumably due to the interaction of phosphate with DNA polymerase, and with Mg^{2+}. Hence, all of the kinetic studies were done in Tris buffer and at optimum Mg^{2+} levels. Fig. 2*A* illustrates determinations of the K_m of free dTTP in the presence of varying amounts of total Mg^{2+}. From such an analysis, the K_m values for free dTTP were extrapolated to zero and infinite levels of magnesium (Table I). The data also permitted the determination of the K_m for Mg-dTTP as a function of total Mg^{2+} concentration (Fig. 2*B* and Table I), and the K_m for free Mg^{2+} as a function of total dTTP concentration (Fig. 2*C* and Table I). When the Mg^{2+} level is extrapolated to infinite concentration, the K_m of the free nucleotide is lowered (Fig. 2*A* and Table I), while that of the Mg·nucleotide complex is essentially unaffected (Table I). Even at saturating levels of Mg-dTTP, additional Mg^{2+} activates the enzyme (Fig. 2*B*), which suggests additional binding sites for Mg^{2+}. Increasing the total nucleotide concentration (*i.e.* both free and Mg^{2+}-nucleotide) produces a small but significant increase in the K_m of free Mg^{2+} (Fig. 2*C* and Table I).

The K_m of free Mg^{2+} extrapolated to zero substrate concentration (Fig. 2*C* and Table I) yields an activator constant for Mg^{2+} (830 ± 62 μM) which is in reasonable agreement with the dissociation constant for Mg^{2+} (466 ± 112 μM) from 4 ± 1 intermediate sites on the enzyme as previously determined by Mg^{2+} binding studies at 16° (5). Apparent substrate inhibition,

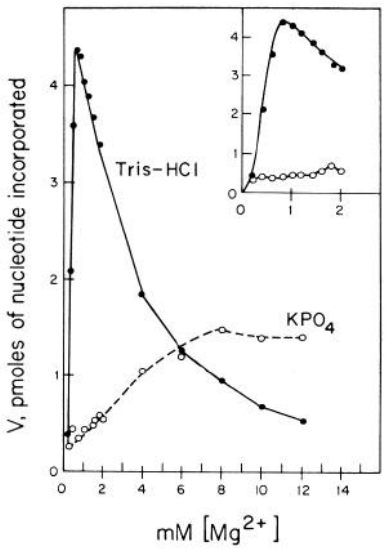

FIG. 1. Effect of total Mg^{2+} concentration on the rate of DNA replication in two buffer systems. Poly(dA)·oligo(dT) was used as the template primer complex. The assay procedures are described under "Methods." (●——●) indicates the rate of incorporation of [^{3}H]dTTP when the reaction is carried out in 50 mM Tris-HCl, pH 7.4; (O- - -O) indicates the rate of reaction in 50 mM potassium phosphate buffer, pH 7.4. *Inset* expands [Mg^{2+}] axis.

TABLE I

Kinetically determined Michaelis constants for polymerization

Component	K_m			
	[Mg]→0	[Mg]→∞	[dTTP]→0	[dTTP]→∞
		μM		
$dTTP_f$	2.9 ± 0.1	0.26 ± 0.24		
Mg-dTTP	9.4 ± 2.8	9.0 ± 2.8		
Mg_f			830 ± 62	2740 ± 89

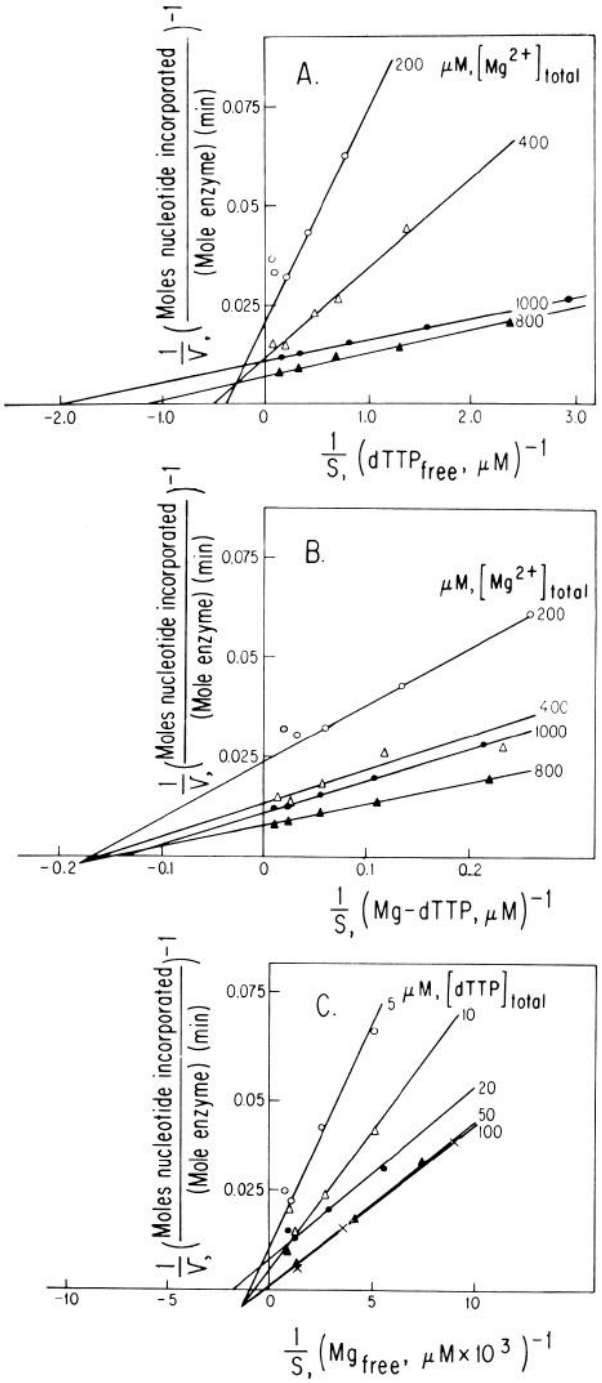

FIG. 2. Kinetic analysis of the effects of Mg^{2+} on Pol I using poly(dA)·oligo(dT) as the template·primer complex. Double reciprocal plots of the rate of incorporation of [^{3}H]dTTP into poly(dT): *A*, as a function of the concentration of free dTTP at the indicated concentrations of total Mg^{2+}; *B*, as a function of the concentration of Mg-dTTP at the indicated concentrations of total Mg^{2+}; *C*, as a function of the concentration of free Mg^{2+} at the indicated concentrations of total dTTP. The concentrations of reactants are as follows: 400 nM poly(dA); 200 nM oligo(dT); 0.9 nM enzyme; the substrate, dTTP, and $MgCl_2$ concentrations are varied as shown. Incubation was for 2 min at 37°. Further details are described under "Methods." The *lines* represent unweighted least square fits to the data points.

TABLE II

Effects of different templates on substrate kinetics of Escherichia coli DNA polymerase I

Experiment No.	Template·primer	Nucleotide substrate	K_m	V_{max}[a]
			μM	s^{-1}
1				
a	Poly(dA)·oligo(dT)	$dTTP_f$	0.10 ± 0.03	1.32 ± 0.07
b		Mg-dTTP	2.9 ± 0.6	1.28 ± 0.07
2				
a	Poly(dT)·oligo(dA)	$dATP_f$	3.7 ± 0.6	2.5 ± 0.2
b		Mg-dATP	38 ± 7	2.7 ± 0.2
3				
a	Poly(dG)·oligo(dC)	$dCTP_f$	1.4 ± 0.2	0.11 ± 0.01
b		Mg-dCTP	14 ± 2	0.11 ± 0.01
4				
a	Poly(dC)·oligo(dG)	$dGTP_f$	0.39 ± 0.01	3.4 ± 0.2
b		Mg-dGTP	11 ± 2	3.4 ± 0.1
5				
a	Poly(rA)·oligo(dT)	$dTTP_f$	0.18 ± 0.04	3.8 ± 0.2
b		Mg-dTTP	5 ± 1	3.8 ± 0.2

[a] The optimum concentration of Mg^{2+} was 2 mM for Experiments 1, 4, and 5 and 0.6 mM for Experiments 2 and 3 as described under "Methods."

which may be due in part to removal of free magnesium, is noted at the highest levels of dTTP or Mg-dTTP and at the lowest levels of Mg^{2+}.

By extrapolation of the rate to infinite levels of substrate, dTTP, and activator, Mg^{2+}, the maximal turnover number for the poly(dA)·oligo(dT) template·primer complex is calculated to be 5.7 ± 0.7 s^{-1}.

Effect of Template on Kinetic Parameters of DNA Polymerization—A comparison was made of the rates of polymerization with different polydeoxynucleotide templates at varying levels of the complementary substrate and at optimum levels of Mg^{2+}. The data (Table II) indicate that the rate of template copying decreases by a factor of 31 in the order poly(dC) > poly(dT) > poly(dA) ≫ poly(dG), *i.e.* the polypyrimidines are better templates than the polypurines. Varying the sugar structure of the template from poly(dA) to poly(rA) causes a 3-fold increase in the rate of copying (Table II).

Also shown in Table II is the K_m of each substrate, both free and complexed with Mg^{2+}. The K_m of $dTTP_f$ with poly(dA) as template is ~30-fold lower than the K_m of $dATP_f$ with poly(dT) as template; this indicates that the interaction of the substrate with a nucleotide on the template does not solely control the value of the K_m which could have kinetic components as well. Moreover, the inherent affinity of the enzyme for the nucleotide does not control the value of the K_m, since dATP binds more tightly to the enzyme than dTTP as determined by equilibrium dialysis in the presence of 50 mM phosphate buffer (4), while the K_m of dTTP is lower than that of dATP.

Effect of Noncomplementary Nucleotides on Polymerization of dCTP with Poly(dG) as Template—For each of the four possible deoxynucleotide template·primer systems a kinetic analysis has been made of the effects of the 2 noncomplementary nucleotides.[1] A relatively uncomplicated set of results from among the eight combinations studied was obtained with poly(dG) as template and dTTP as the noncomplementary nucleotide (Fig. 3). The noncomplementary nucleotide, dTTP, is a linear competitive inhibitor with respect to free dCTP (K_I = 282 μM) (Fig. 3*C* and Table III, Experiment 1, a and b) but a hyperbolic competitive inhibitor (K_I = 50 μM) with respect to Mg-dCTP. In addition, a slight activation may exist ($K_A \geq 1$ mM) with respect to free dCTP (Fig. 3*B*) and Mg-dCTP as detected by an intercept effect (Table III, Experiment 1, a and b).

With the noncomplementary purine nucleotide, dATP, parabolic inhibitory effects on slope and intercept with similar K_I values were detected with respect to dCTP and its Mg^{2+} complex (Table III, Experiment 1, c and d). The intercept effect and the parabolic nature of the inhibition require 2 sites in addition to the substrate site for the binding of the inhibitory nucleotide.

Effect of Noncomplementary Nucleotides with Other Polydeoxynucleotide Templates—With poly(dT)·oligo(dA) as template and dATP or its Mg^{2+} complex as substrate, parabolic inhibitory effects on slope and intercept were found for the noncomplementary pyrimidine nucleotide, dCTP (Fig. 4). Because of the similarity of the K_I values (Table III, Experiment 2, a and b), a minimum number of 2 additional binding sites for dCTP is required. With dGTP, parabolic slope effects

[1] Under the conditions of the kinetic studies with noncomplementary nucleotides at least 97% of the noncomplementary nucleotides were present as their Mg^{2+} complexes. Hence, all effects of noncomplementary nucleotides, although expressed in terms of total nucleotides, reflect primarily those of the Mg·nucleotide complexes.

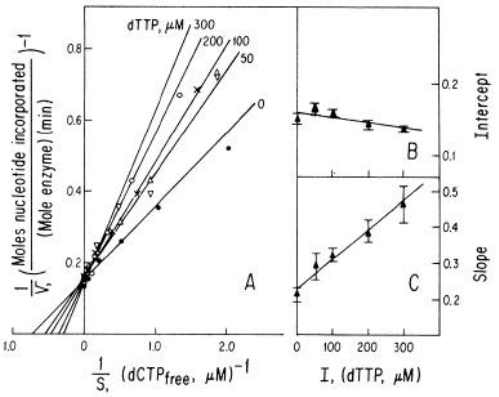

FIG. 3. Kinetic analysis of the effects of an incorrect deoxynucleotide triphosphate on Pol I using poly(dG)·oligo(dC) as the template·primer complex. *A*, a double reciprocal plot of the rate of incorporation of [^{3}H]dCTP into poly(dC) as a function of the concentration of free dCTP at the indicated concentrations of the incorrect nucleotide dTTP; *B* and *C*, secondary plots of the data illustrated in *A*. The concentrations of reactants are as follows: 0.6 mM $MgCl_2$; 400 nM poly(dG); 200 nM oligo(dC); 18.6 nM enzyme, 5, 10, 50, and 100 μM dCTP substrate, and 0, 50, 100, 200, and 300 μM dTTP inhibitor. Incubation was for 5 min at 37°. Further details are as described under "Methods." The *lines* represented in the double reciprocal plot are weighted least square fits to the data points while those in the secondary plots are unweighted least square fits (17).

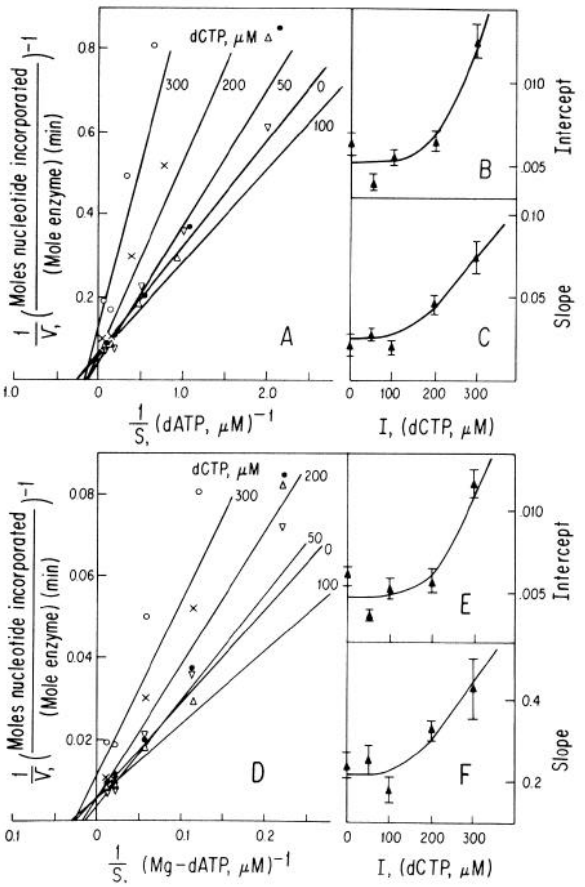

FIG. 4. Kinetic analysis of the effect of an incorrect deoxynucleotide triphosphate on Pol I using poly(dT)·oligo(dA) as the template·primer complex. *A*, a double reciprocal plot of the rate of incorporation of [^{3}H]dATP into poly(dA) as a function of the concentration of free dATP at the indicated concentrations of the incorrect nucleotide dCTP; *B* and *C*, secondary plots of the data illustrated in *A*. *D*, a double reciprocal plot similar to that of *A* but incorporation is measured as a function of the concentration of Mg-dATP at the indicated concentrations of dCTP. *E* and *F*, secondary plots of the data illustrated in *D*. The concentrations of reactants are as follows: 2 mM $MgCl_2$; 400 nM poly(dA); 200 nM oligo(dT); 3.6 nM enzyme; 5, 10, 50, and 100 μM dATP substrate; and 0, 50, 100, 200, and 300 μM dCTP inhibitor. Incubation was for 2 min at 37°. Further details are as described under "Methods." The data are analyzed as described for Fig. 3.

but hyperbolic intercept effects are observed (Table III, Experiment 2, c and d) again requiring at least 2 sites. With poly(dA)·oligo(dT) and poly(dC)·oligo(dG) as templates, the effects of noncomplementary nucleotides in most cases required 2 sites (Table III, Experiment 3, a to d; Experiment 4, a to d).

Effect of Template-Primer on Apparent Affinity for Nucleotides—With the template poly(dG), the apparent affinity of the active site of the enzyme for the substrate dCTP as measured by its K_m (1.4 μM, Table II, Experiment 3a) is 201-fold greater than its affinity for the competitive noncomplementary pyrimidine nucleotide dTTP (K_I = 282 μM, Table III, Experiment 1a). With Mg-dCTP (K_m = 14 μM, Table II, Experiment 3b) such a direct comparison cannot be made since the inhibition by dTTP is now hyperbolic competitive rather than linear or parabolic competitive (Table III, experiment 1b). Nevertheless, from the kinetic data, if linear competitive inhibition by dTTP were occurring, the K_I value would have to be >300 μM to avoid being detected. Hence, the affinity of the active site for the Mg-dCTP substrate is >21-fold greater than for the noncomplementary nucleotide. All such comparisons are summarized in Table IV.

A different comparison of dTTP as a competitive inhibitor (K_I slope = 282 μM, Table III, Experiment 1a) with free dTTP as a substrate (K_m = 0.10 ± 0.03 μM, Table II, Experiment 1a) indicates that a change in the template from poly(dG) to poly(dA) raises the apparent affinity of the enzyme for dTTP by a factor of ~2820. An analogous comparison with Mg-dTTP (Tables II, Experiment 1b, and III, Experiment 1b) indicates a 103-fold increase in apparent affinity produced by the complementary template (see "Discussion"). Similarly, dGTP as a noncomplementary competitive inhibitor has a K_I value of 72 μM (Table III, Experiment 3c) which is 185-fold greater than its K_m as a complementary substrate (0.39 μM, Table II, Experiment 4a). All such comparisons are summarized in Table V.

Effect of Noncomplementary Nucleotides in Presence of a Polyriboadenylic Acid as Template—Pol I is also able to copy efficiently polyribonucleotide templates (18–20). In fact, the V_{max}, using poly(rA) as a template, is 3-fold greater than that obtained with poly(dA) (Table II). With the noncomplementary purine nucleotide dGTP (Fig. 5) parabolic uncompetitive inhibition (intercept effect) and linear competitive inhibition (slope effect) are detected, requiring at least 2 and possibly 3 binding sites for dGTP (Table III, Experiment 5, c and d). Simple linear competitive inhibition by the noncomplementary pyrimidine nucleotide dCTP is observed with respect to both free dTTP and its Mg^{2+} complex (Table III, Experiment 5, a and b).

In changing the template from a polydeoxyribonucleotide to a polyribonucleotide, the binding of dCTP at ancillary sites is no longer detected (Table III, Experiments 3, a and b; 5, a and b). However, there is less discrimination at the catalytic site, since dCTP can now compete with the substrate. This finding is in accord with the greater incorporation by Pol I of dTTP

TABLE III

Effects of noncomplementary deoxynucleotides on substrate kinetics of Escherichia coli DNA polymerase I using deoxynucleotide templates

Experiment No.	Template primer	Correct nucleotide substrate	Noncomplementary nucleotide	K_I or K_A[a]				Minimum No. of sites for noncomplementary nucleotides
				Intercept	Slope	Intercept	Slope	
					μM			
1								
a	Poly(dG)·oligo(dC)	$dCTP_f$	dTTP	$>1{,}200_a$	$282_i \pm 37$			1
b		Mg-dCTP		$>1{,}000_a$	$55_i \pm 7$ (hyperbolic)			1
c		$dCTP_f$	dATP	$223_i + 10$ (parabolic)	$173_i \pm 19$ (parabolic)			2
d		Mg-dCTP		$173_i \pm 9$ (parabolic)	$281_i \pm 44$ (parabolic)			2
2								
a	Poly(dT)·oligo(dA)	$dATP_f$	dCTP	$104_i \pm 16$ (parabolic)	$209_i \pm 50$ (parabolic)			2
b		Mg-dATP		$173_i \pm 32$ (parabolic)	$307_i \pm 70$ (parabolic)			2
c		$dATP_f$	dGTP	$40_i \pm 5$ (hyperbolic)	$232_i \pm 52$ (parabolic)			2
d		Mg-dATP		$328_i \pm 51$ (hyperbolic)	$379_i \pm 91$ (parabolic)			2
3								
a	Poly(dA)·oligo(dT)	$dTTP_f$	dCTP	$141_a \pm 10$	No effect	$406_i \pm 30$		2
b		Mg-dTTP		$178_a \pm 12$	No effect	$414_i \pm 29$		2
c		$dTTP_f$	dGTP	No effect	$72_i \pm 14$			1
d		Mg-dTTP		$360_i \pm 33$ (parabolic)	$110_i \pm 34$			2
4								
a	Poly(dC)·oligo(dG)	$dGTP_f$	dTTP	$158_a \pm 9$	No effect	$331_i \pm 19$		2
b		Mg-dGTP		$126_a \pm 7$	$570_a \pm 79$	$424_i \pm 24$		2
c		$dGTP_f$	dATP	$371_a \pm 24$ (parabolic)	No effect			2
d		Mg-dGTP		$480_a \pm 31$	No effect			1
5								
a	Poly(rA)·oligo(dT)	$dTTP_f$	dCTP	No effect	$440_i \pm 127$			1
b		Mg-dTTP		No effect	$380_i \pm 82$			1
c		$dTTP_f$	dGTP	$487_i \pm 47$ (parabolic)	$84_i \pm 22$			2
d		Mg-dTTP		$432_i \pm 42$ (parabolic)	$110_i \pm 28$			2

[a] *a*, activation; *i*, inhibition. The errors in K_I and K_A are the average of the standard errors of the weighted linear least square fits to the primary data (17).

with poly(rA) than poly(dA) as template. Smaller template-dependent differences were detected with dGTP as the noncomplementary nucleotide (Table III, Experiments 3c and d; and 5c and d).

DISCUSSION

In this investigation of the steady state kinetics of DNA synthesis, we have used homogeneous *Escherichia coli* DNA polymerase I, even though it is known to have several other catalytic properties such as 3′- and 5′-exonuclease activities (3). The times of incubation are such that less than 20% of the length of any template could have been copied. Control experiments indicate that exonuclease activities play no significant role in initial catalysis, *i.e.* the template and product are not hydrolyzed by the polymerase nor are deoxynucleoside triphosphates converted to monophosphates in the reaction mixture during the incubation period indicated[2] (21). Since all four template·primer complexes were studied, it has been possible to compare each of the deoxynucleoside triphosphates as a substrate using the complementary template and, in turn, as an inhibitor using the noncomplementary template.

The general rate equation for a 2-substrate reaction, in the

[2] G. Seal and L. A. Loeb, unpublished results.

TABLE IV

Calculated free energies of discrimination between complementary and noncomplementary nucleotides at active site

Experiment No.	Template · primer	Complementary nucleotide substrate	Noncomplementary nucleotide	K_i(noncomplementary nucleotide)/K_m(complementary nucleotide)	$-\Delta G$
					kcal/mol
1					
a	Poly(dG) · oligo(dC)	$dCTP_f$	dTTP	201	3.3
b		Mg-dCTP		>21	>1.9
c		$dCTP_f$	dATP	124	3.0
d		Mg-dCTP		20	1.8
2					
a	Poly(dT) · oligo(dA)	$dATP_f$	dCTP	56	2.5
b		Mg-dATP		8	1.3
c		$dATP_f$	dGTP	63	2.5
d		Mg-dATP		10	1.4
3					
a	Poly(dA) · oligo(dT)	$dTTP_f$	dCTP	>10,000	>5.6
b		Mg-dTTP		>344	>3.6
c		$dTTP_f$	dGTP	720	4.0
d		Mg-dTTP		38	2.2
4					
a	Poly(dC) · oligo(dG)	$dGTP_f$	dTTP	>2,564	>4.8
b		Mg-dGTP		>27	>2.0
c		$dGTP_f$	dATP	>2,564	>4.8
d		Mg-dGTP		>91	>2.8
5					
a	Poly(rA) · oligo(dT)	$dTTP_f$	dCTP	2,444	4.8
b		Mg-dTTP		76	2.7
c		$dTTP_f$	dGTP	467	3.8
d		Mg-dTTP		22	1.9

present case the template·primer complex and the deoxynucleotide, simplifies to an equation in terms of 1 substrate (the deoxynucleotide) when the other substrate (the template·primer complex) is saturating:

$$v = \frac{V}{1 + \frac{K_M}{[dNTP]}} \quad (1)$$

Although K_m values do not, in general, reflect true thermodynamic dissociation constants, they reflect the "effective affinity" of an enzyme for a substrate under steady state conditions, and therefore are directly relevant to the proper selection of the complementary substrate by the enzyme·template·primer complex under conditions of DNA synthesis.

The maximal rate of polymerization, V_{max}, depends upon the primary structure of the templates in the order poly(dC) > poly(dT) > poly(dA) ≫ poly(dG) (Table II), indicating that pyrimidine templates are copied faster than purine templates. Moreover, the sugar exerts an effect, since poly(rA) is copied 3 times faster than poly(dA) (Table II). This increase in V_{max} for poly(rA) as compared to poly(dA) may pertain only to certain homopolymers because natural RNAs are copied by Pol I, with turnover numbers 10^3-fold lower than natural DNAs (22). There is no apparent correlation of V_{max} with the secondary structure of the templates since the latter decreases under our experimental conditions in the order poly(dG) > poly(dC) = poly(dA) = poly(rA) > poly(dT) (23–25). Because of differences in the tertiary structures of the homopolymers, the maximal rate of copying a natural DNA of known composition cannot be predicted from data on homopolymers alone.

The kinetic data of Fig. 2*B* suggest more than 1 activating site for Mg^{2+} on DNA polymerase. Furthermore, the kinetic data of Table III indicate that as many as 2 binding sites in addition to the substrate site are required to account for the complex types of inhibition and activation observed with the noncomplementary nucleotides. These kinetic observations are in accord with previous nuclear magnetic resonance studies carried out in the absence of template which indicate that *E. coli* DNA polymerase has 5 ± 1 Mn^{2+} or Mg^{2+} binding sites, each of which can accommodate a nucleotide (5). In addition, the inhibition observed at Mg^{2+} concentrations above 1 mM (Fig. 1) is in accord with the large number of weak binding (~20) sites previously found for Mn^{2+} (5).

The kinetic experiments in this study involved polynucleotide templates containing only one nucleotide species. The results establish that noncomplementary nucleotides can bind to the enzyme at ancillary sites. However, this ancillary binding is template-dependent in that a change in the template alters not only the affinity of the enzyme for a given noncomplementary nucleotide but also the resulting kinetic effect. For example, with poly(dA) as template, dCTP causes activation and uncompetitive inhibition, while with poly(dT), the same nucleotide causes parabolic uncompetitive and competitive inhibition (Table III). These diverse effects argue against inhibition or activation resulting from complexation of Mg^{2+}. Moreover, calculated changes in the concentration of

TABLE V

Calculated free energies of discrimination for same nucleotide as substrate and as competitive inhibitor

Template	Substrate	K_m (complementary nucleotide)	K_I (noncomplementary nucleotide)	K_I/K_m	$-\Delta G$
		μM			*kcal/mol*
Poly(dA)	$dTTP_f$	0.10			
Poly(dG)			282	2,820	4.9
Poly(dC)			>1,000	>10,000	>5.6
Poly(dA)	Mg-dTTP	2.9			
Poly(dG)			>300	>103	>2.9
Poly(dC)			>300	>103	>2.9
Poly(dG)	$dCTP_f$	1.4			
Poly(dA)			>1,000	>714	>4.0
Poly(dT)			209	149	3.1
Poly(dG)	Mg-dCTP	14.0			
Poly(dA)			>1,000	>71	>2.6
Poly(dT)			307	22	1.9
Poly(dC)	$dGTP_f$	0.39			
Poly(dA)			72	185	3.2
Poly(dT)			232	595	3.9
Poly(dC)	Mg-dGTP	11.00			
Poly(dA)			110	10	1.4
Poly(dT)			379	35	2.2
Poly(dT)	$dATP_f$	3.7			
Poly(dG)			173	47	2.4
Poly(dC)			>1,000	>270	3.4
Poly(dT)	Mg-dATP	38.0			
Poly(dG)			281	7.4	1.2
Poly(dC)			>1,000	>26	>2.0

free Mg^{2+} are insufficient to explain the observed effects. The binding of free nucleotide to the free template, prior to interaction with enzyme, is an unlikely cause of the observed inhibition or activation, since interactions between mononucleotides and polynucleotides are too weak (27) to account for the low K_I and K_A values of the noncomplementary nucleotides. Inhibition due to displacement of the bound template by the noncomplementary nucleotide is unlikely because of the high affinity of the enzyme for the template (28) and the high concentrations of template·primer complex used in the present study (400 nM). It could be argued that the ancillary nucleotide binding sites, detected in this study with homopolymer templates, could function as substrate binding sites with heteropolymers and natural DNA templates. This interpretation would be in accord with our previous proposal of prealignment of nucleotides on the enzyme in preparation for polymerization (5). Evidence for multiple substrate sites on DNA

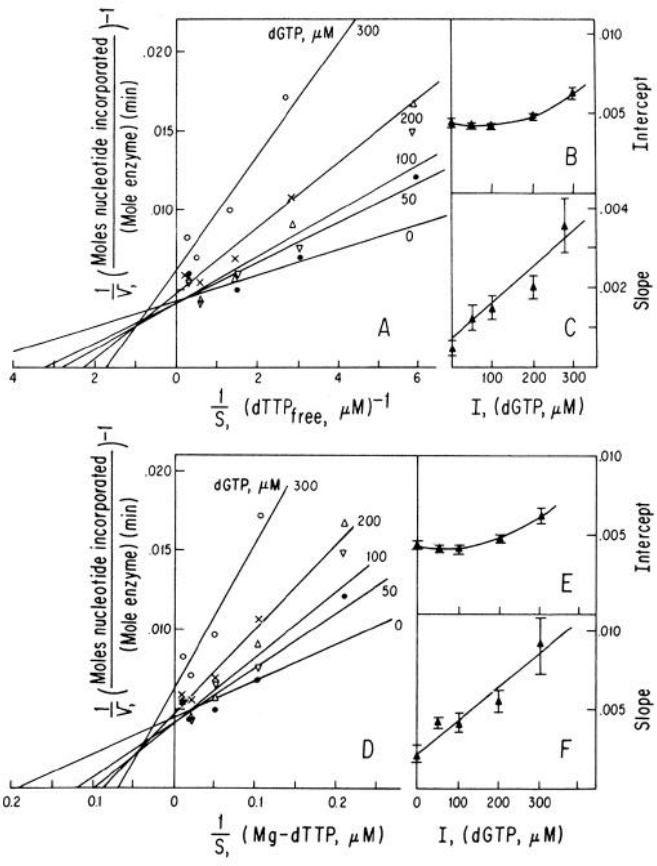

FIG. 5. Kinetic analysis of the effects of an incorrect deoxynucleotide triphosphate on Pol I using poly(rA)·oligo(dT) as the template·primer complex. *A*, a double reciprocal plot of the rate of incorporation of [^{3}H]dTTP into poly(dT) as a function of the concentration of free dTTP at the indicated concentrations of the incorrect nucleotide, dGTP. *B* and *C*, secondary plots of the data illustrated in *A*. *D*, a double reciprocal plot similar to that of *A* but incorporation is measured as a function of the concentration of Mg-dTTP at the indicated concentrations of dGTP. *E* and *F*, secondary plots of the data illustrated in *D*. The concentrations of reactants are as follows: 2 mM $MgCl_2$; 400 nM poly(rA); 200 nM oligo(dT); 7.2 nM enzyme; 5, 10, 50, and 100 μM dTTP substrate; and 0, 50, 100, 200, and 300 μM dCTP inhibitor. Incubation was for 2 min at 37°. Further details are described under "Methods." The data are analyzed as described for Fig. 3.

polymerase has also been found in kinetic studies using avian myeloblastosis virus DNA polymerase which incorporates a high percentage of noncomplementary deoxynucleotides during polynucleotide synthesis (9). This finding, together with the lack of competition between the substrate nucleotide and the noncomplementary nucleotide, indicates more than 1 functional nucleotide binding site on the viral polymerase (26).

Differences in the kinetic effects of complementary and noncomplementary nucleotides yield clues on the mechanism of fidelity of DNA synthesis (Tables IV and V). Assuming that the ratio of the K_I (slope) of the noncomplementary nucleotide to the K_m of the complementary nucleotide reflects the relative functional affinity of the active site for the noncomplementary and complementary substrate, one can estimate the free energy of discrimination against the incorrect nucleotide at the active site from the relationship: $\Delta G = -RT \ln K_I/K_m$. In seven of the 20 cases studied (Table III, Experiments 1b; 3a and b; 4a to d) no linear or parabolic competitive inhibition was observed; hence, no binding of the noncomplementary nucleotide at the substrate site was detected. From the experimental errors, lower limits to $-\Delta G$ of 1.9 to 5.6 kcal/mol have been calculated (Table IV). In those cases in which linear or parabolic competition is observed (Table III), a precise value for the free energy of discrimination can be obtained (Table IV). Values of $-\Delta G$ ranging from 1.3 to 4.8 kcal/mol can be calculated. These values are equivalent to 1 to 2 additional hydrogen bonds which are required for the binding of the correct nucleotide as compared to the incorrect nucleotide by the Watson-Crick base-pairing scheme (27). These values are insufficient to explain the high fidelity of template copying by Pol I (see below).

A second clue to the mechanism of fidelity can be obtained by comparing the K_m of a nucleotide, when it is a substrate, with its K_I, when it is a competitive inhibitor; *i.e.* the nucleotide is kept constant and the template is changed. This comparison is made in Table V. In the seven cases in which no linear or parabolic competitive inhibition was detected, lower limits to $-\Delta G$ of 2.0 to 5.6 kcal have been estimated. Where absolute numerical values for the K_I (slope) can be assigned, $-\Delta G$ may be as great as 4.9 kcal/mol, again suggesting 2 hydrogen bonds in the complementary system. The error frequency of Pol I with homopolymer templates has been estimated to be 1:100,000 (28, 29) which would require a $-\Delta G$ of 7.0 kcal/mol (30). Hence, only the lower limit values for the free energy of discrimination calculated in Tables IV and V may be sufficient to explain the accuracy of DNA synthesis by Pol I. In most cases, where linear or parabolic competitive inhibition is detected, the absolute numerical values calculated for ΔG cannot explain the highly accurate DNA synthesis observed with this enzyme. Hence, the criterion of overlapping binding sites for correct and incorrect nucleotide, which is sufficient to produce competitive inhibition, does not assure the incorporation of the incorrect nucleotide. The observed accuracy of Pol I could be achieved by an "error-correcting" 3'-exonuclease activity, as pointed out by Brutlag and Kornberg (31) and theoretically considered by Hopfield (32). Alternatively, the required free energy for discrimination can be derived from selective orientation of the nucleotides, *i.e.* by an entropy change (33). The existence of eukaryotic DNA polymerases which copy templates with high fidelity yet lack any associated exonuclease argue for the latter concept (2). Moreover, our most recent nuclear magnetic resonance studies (33) indicate that when Mn-dTTP binds to the enzyme in the absence of template, the base-sugar conformation of this substrate is adjusted (or selected) to be that of a nucleotide unit in double helical DNA B. Such a restricted structure of the enzyme-bound substrate would, in the presence of a template, provide an orientational or entropic force to increase complementary nucleotide binding as well as to decrease noncomplementary nucleotide interactions.

In conclusion, the kinetic data in this paper indicate the presence of multiple sites for deoxynucleotides on the enzyme which influence the rate of polymerization. These sites may be ancillary active sites or regulatory sites. These findings are consistent with our previous suggestion of prealignment of substrates on the enzyme. The data also indicate that the presence of the template facilitates rejection of the noncomplementary nucleotide, a fact which contributes significantly to the fidelity of DNA polymerase. In addition, the template may also raise the affinity of the enzyme for the complementary nucleotide (3). To clarify these points, it was necessary to use homopolymers as templates.

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