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Mechanism of the idling-turnover reaction of the large (Klenow) fragment of *Escherichia coli* DNA polymerase I

(exonuclease/pyrophosphorolysis)

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ABSTRACT **The mechanism of the idling-turnover reaction catalyzed by the large (Klenow) fragment of *Escherichia coli* DNA polymerase I has been investigated. The reaction cycle involved is one of excision/incorporation, in which the 3′ deoxynucleotide residue of the primer DNA strand is partitioned into its 5′-mono- and 5′-triphosphate derivatives, respectively. Mechanistic studies suggest the 5′-monophosphate product is formed in the first step by simple 3′ → 5′ exonucleolytic cleavage. Rapid polymerization follows with the concomitant release of inorganic pyrophosphate. In the second step, the 5′-triphosphate product is generated by a pyrophosphorolysis reaction, which, despite the low concentration of pyrophosphate that has accumulated, occurs at a rate that is comparable with that of the parallel 3′ → 5′ hydrolysis reaction.**

The multifunctional DNA polymerase I of *Escherichia coli* has served as the widely studied model for describing, at the molecular level, certain enzymatic processes involved in the replication of DNA (1). In addition to its polymerase activity, the enzyme also catalyzes DNA degradation by distinct 5′ → 3′ and 3′ → 5′ exonuclease activities, as well as by net pyrophosphorolysis. Extensive kinetic (2, 3) and stereochemical (4, 5) studies of the various activities have elucidated the important underlying features of the phosphodiester bond-forming and bond-breaking reactions. In addition, the availability of a 3-Å resolution x-ray structure of the large proteolytic (Klenow) fragment of DNA polymerase I (6) has generated considerable interest in the area of structure–function assignment (7).

The conversion of a fraction of the available deoxynucleoside 5′-triphosphate (dNTP) pool into a corresponding monophosphate pool has provided evidence in support of alternating polymerase and 3′ → 5′ exonuclease expression during the course of DNA synthesis (8, 9). In view of the convincing evidence implicating the 3′ → 5′ exonuclease activity in ensuring fidelity (10–13), the extent of the dNTP → dNMP conversion may thus reflect the degree of proofreading accompanying replication by DNA polymerases possessing such an exonuclease activity (14). In the absence of the following complementary dNTP that is required for normal polymerization, the turnover process is exaggerated since the enzyme is constrained to "idle" at the primer terminus until depletion of the available dNTP pool is complete (15). Comparative kinetic studies of the turnover reaction with the enzyme held in such an idling mode have allowed the evaluation of base misinsertion frequencies, thus demonstrating an important application of the idling-turnover process (16).

In this paper, we report our results on the idling-turnover reaction catalyzed by the Klenow fragment (KF), which bear on the general problem of describing a unified mechanism of the interrelated activities of this enzyme.

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MATERIALS AND METHODS

The Klenow fragment was purified from *E. coli* CJ155 according to Joyce and Grindley (17). The *E. coli* strain was kindly provided by C. Joyce. Polyacrylamide gel electrophoresis of the purified enzyme indicated at least 95% homogeneity. *Eco*RI restriction endonuclease was generously furnished by P. Modrich. *Bam*HI restriction endonuclease was from New England Biolabs. Myosin subfragment 1 ATPase was kindly provided by K. A. Johnson. Plasmid pBR322 was isolated from transformed *E. coli* HB101 by a standard procedure (18).

[^{3}H]dATP (20 Ci/mmol; 1 Ci = 37 GBq) was from ICN. [^{3}H]dATP (73 Ci/mmol) was from Amersham. [α-^{32}P]dATP and [α-^{32}P]dGTP (>3200 Ci/mmol) were from New England Nuclear. Carrier-free [^{32}P]P_i was from ICN. [γ-^{32}P]dATP was prepared by the method of Johnson and Walseth (19).

Scintiverse II liquid scintillation fluid was from Fischer. DE-81 filters were from Whatman. DEAE-Sephadex A-25 was from Pharmacia. TLC was performed on polyethylene-cellulose plates with fluorescent indicator (Baker), developing in 0.3 M potassium phosphate (pH 7.0). Anion-exchange HPLC was performed on a Pharmacia fast protein liquid chromatography (FPLC) system equipped with a 1-ml Pharmacia Mono Q column, eluting with a 15-ml linear gradient of 0.05–0.3 M NaCl in 20 mM Tris·HCl (pH 7.8) at a flow rate of 1.0 ml/min. Retention times for dAMP, dADP, and dATP were 4.8, 8.6, and 11.9 min, respectively. Column elutions were monitored spectrophotometrically at 260 nm and were collected with a Pharmacia Frac-100 fraction collector.

Restriction Digestion of pBR322. Linearization of pBR322 with either *Eco*RI or *Bam*HI was carried out by a standard procedure (18).

3′-End Labeling. A mixture containing 220–300 nM 3′ ends, 14 nM Klenow fragment (KF), 5 mM $MgCl_2$, 2 mM dithiothreitol and the appropriate dNTP at 2 μM {[α-^{32}P]dATP ($\approx$4.5 $\times$ 10^5 cpm/pmol) or unlabeled dATP for *Eco*RI-digested pBR322; [α-^{32}P]dGTP ($\approx$5.5 $\times$ 10^5 cpm/pmol) for *Bam*HI-digested pBR322} in 50 mM Tris·HCl (pH 7.4) was incubated at 21°C for 2–5 min. Control experiments in which end-labeling was monitored by the DE-81 filter assay (2) had shown quantitative end labeling to be complete under the above conditions. The reaction was quenched by the addition of 2 vol of a PhOH/$CHCl_3$ mixture (1:1, vol/vol), followed by a brief Vortex mixing. The end-labeled DNA was recovered by ethanol precipitation and was redissolved in 0.3 ml of 10 mM Tris·HCl (pH 8.0) at a concentration of 0.15–0.3 μg of DNA/μl. The remaining mononucleotides were removed by centrifuge desalting as follows (20): The sample was loaded onto a 2.5-ml syringe column of Sephadex G-25 equilibrated with 50 mM Tris·HCl (pH 8.0), which had been spun to dryness (100 $\times$ *g*, 3 min) after two 0.3-ml washes with 10 mM Tris·HCl (pH 8.0) just prior to sample loading. The DNA was

Abbreviation: KF, Klenow fragment of *E. coli* DNA polymerase I.

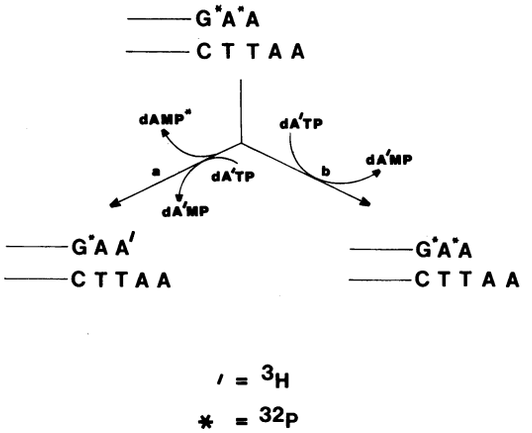
ethanol precipitated from the solution recovered after centrifugation (100 × *g*, 3 min). This procedure reproducibly gave 70–80% recovery of DNA and >99.99% mononucleotide removal. The DNA was stored at 1.2–1.5 μg of DNA/μl in 10 mM Tris·HCl (pH 8.0)/1 mM EDTA.

Idling Reaction Assay. Unless otherwise indicated, enzyme-initiated reaction mixtures (total vol, 50 μl) contained 140 nM 3′-labeled DNA ends, 50 nM KF, 6.6 mM $MgCl_2$, 2 mM dithiothreitol and dNTPs, as specified in the text and table and/or figure legends, in 50 mM Tris·HCl (pH 7.4). Samples were withdrawn at given times and quenched with EDTA (2 μl, pH 7.5) to a final concentration of 40 mM. To the quenched aliquots (5 μl) was added 5 μl of an appropriate UV-visualization marker solution (dAMP, dADP, and dATP each at 3 mM for the 3′-[^{32}P](dA)$_4$-labeled *Eco*RI-digested pBR322 reactions, and dGMP and dGTP each at 3 mM for the 3′-[^{32}P](dG)$_2$-labeled *Bam*HI-digested pBR322 reactions). For the TLC assay, a 5-μl portion of the quenched, marker-containing solution was spotted at the origin prior to developing the plate. Following drying and visualization by UV and autoradiography, the DNA (which remains at the origin), mono-, di-, and triphosphate spots were accordingly cut out, placed in scintillation vials and treated with 1 ml of 1 M HCl for 10 min. The radioactivity was quantitated after the addition of 10 ml of Scintiverse II. Control experiments showed good agreement (± 10%) between the above TLC assay and the standard DE-81 filter assay for the quantitation of [^{32}P]DNA.

For the HPLC assay, the quenched marker-containing solutions (10 μl) were diluted with 20 mM Tris·HCl (pH 7.8) to 60 μl, of which 30 μl was assayed by fast protein liquid chromatography. Peak volumes (0.3–0.6 ml for each dAMP, dADP, dATP) were diluted to 1 ml with 20 mM Tris·HCl (pH 7.8), and the resulting samples were counted in 10 ml of Scintiverse II.

RESULTS

The time course of the KF-catalyzed idling reaction on the 3′-[^{32}P](dA)$_4$-labeled *Eco*RI-digested pBR322, in the presence of a 10 μM pool of [^{3}H]dATP, is illustrated in Fig. 1. Approximately one-half of the total ^{32}P initially present was lost over the 90-min period during which the reaction was monitored. Concomitant with this process was the linear appearance of the idling product [^{3}H]dAMP that is formed at the expense of [^{3}H]dATP. The results of this experiment thus suggest an excision/incorporation mode of action of the enzyme under idling conditions (Scheme 1, pathway *a*) rather than the alternative misincorporation/excision mode (pathway *b*).



SCHEME 1.

The fate of the 3′-[^{32}P]-end label was traced by a TLC assay in a series of idling experiments on 3′-end-labeled *Eco*RI-digested pBR322 conducted in the presence of an unlabeled

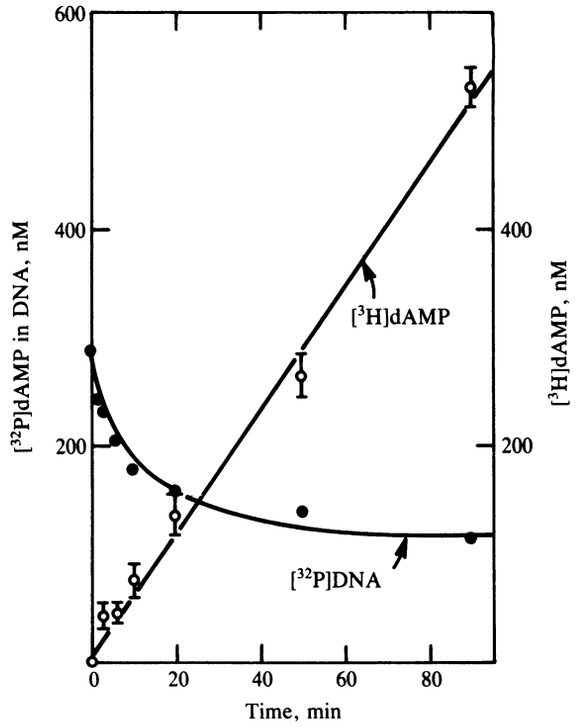


FIG. 1. Double-label idling-turnover time course of KF on 3′-[^{32}P](dA)$_4$-labeled *Eco*RI-digested pBR322 with [^{3}H]dATP. A 50-μl reaction mixture containing 140 nM 3′ ends, 50 nM KF, 6.6 mM $MgCl_2$, 2 mM dithiothreitol and 10 μM [^{3}H]dATP (5640 cpm/pmol) in 50 mM Tris·HCl (pH 7.4) was incubated at 21°C. Aliquots (3 μl) were withdrawn at various times and analyzed for [^{3}H]dAMP formation (○) by the TLC assay, and for ^{32}P loss from 3′-end labeled *Eco*RI-digested pBR322 (●) by the DE-81 filter assay.

dATP pool of varying initial concentration. The results of one such experiment at 1 μM dATP are illustrated in Fig. 2. Surprisingly, the total ^{32}P lost from the polymer is accounted for in the form of two distinct products. Besides yielding the expected hydrolysis product [^{32}P]dAMP, the idling reaction also releases a product that comigrates with an authentic dATP marker. Identification of the second product as [^{32}P]dATP was confirmed as follows: (*i*) An independent HPLC analysis of the product mixture showed that the two ^{32}P-labeled mononucleotide products coelute with authentic dAMP and dATP markers, thus corroborating the TLC results. (*ii*) ATPase treatment of the product mixture. Selected time points of the idling reaction were subjected to an incubation with a large excess of myosin subfragment 1 ATPase for a period of time predetermined to be sufficiently long to quantitatively hydrolyze the remaining pool of dATP to dADP. The products of the ATPase treatment were analyzed by TLC against ATPase-free controls, to identify the changes, if any, in chromatographic behavior of the ^{32}P-labeled species present. While the 3′-end-labeled *Eco*RI-digested pBR322 and [^{32}P]dAMP migrations were unaffected, the radioactivity initially eluting with the dATP marker quantitatively shifted to the dADP position following ATPase treatment. These results confirm the identification of the second ^{32}P-labeled idling-turnover product as [^{32}P]dATP.

Similar idling-turnover experiments conducted at 10 μM and 1 mM dATP pool concentrations yielded qualitatively similar results, with ^{32}P distributing into both [^{32}P]dAMP and [^{32}P]dATP. In all cases, the triphosphate/monophosphate product ratio was not constant during the course of the reaction but was found to steadily increase to a limiting value. Thereafter, depletion of the available triphosphate pool resulted in a gradual decrease of the product ratio to a final value of zero. Neither the initial rate of label loss nor the

limiting ratio of the two products formed was observed to significantly depend upon the initial dATP pool concentration over a 1 μM to 1 mM range (Table 1).

The appearance of a ^{32}P-labeled triphosphate product is subject to specific conditions. (*i*) In the presence of a 10 μM pool of the noncomplementary triphosphate, dGTP, the "idling" reaction on 3'-[^{32}P](dA)$_4$-labeled *Eco*RI-digested pBR322 forms [^{32}P]dAMP exclusively.* (*ii*) The replacement of dATP by ATP, which, although complementary, is not a polymerization substrate under the reaction conditions employed, similarly results in the exclusive formation of [^{32}P]dAMP from 3'-end-labeled *Eco*RI-digested pBR322. (*iii*) In the absence of an added triphosphate pool, simple 3' → 5' exonucleolytic degradation prevails yielding, as expected, 5'-^{32}P-labeled monophosphate as the exclusive product (1). The results of such a control experiment further validate the TLC assay. (*iv*) The generality of the [^{32}P]dNTP-forming reaction under idling-turnover conditions was confirmed by repeating the experiment at a different 3'-terminal sequence. For this purpose, the labeled duplex 3'-[^{32}P](dG)$_2$-labeled *Bam*HI-digested pBR322 was employed. As expected, the idling-turnover reaction on this substrate in the presence of a requisite pool of dGTP yielded both [^{32}P]dGMP and [^{32}P]dGTP as products. (*v*) Finally, the triphosphate formation observed during idling-turnover was completely suppressed by the inclusion of inorganic pyrophosphatase (1–10 unit/ml) in the reaction mixture. Under such conditions, the idling reaction on 3'-end-labeled *Eco*RI-digested pBR322 in the presence of 10 μM dATP resulted in the loss of *ca.* one-half of the total ^{32}P label from the DNA as [^{32}P]dAMP over a 90 min period.

The observation of [^{32}P]dNTP formation during idling-turnover demands the involvement of a nucleophilic displacement reaction by PP_i on the terminal phosphodiester linkage of the primer strand. To trace the source of PP_i involved in such a reaction, an idling-turnover experiment was conducted on 3'-(dA)$_4$-labeled *Eco*RI-digested pBR322 in the presence of a 1 μM pool of [γ-^{32}P]dATP. If the PP_i is derived from the triphosphate pool, a certain amount of [β-^{32}P]dATP would be formed by random attack of the pyrophosphatase-trappable [^{32}P]PP_i on the 3' terminus of the DNA substrate. TLC analysis of the reaction mixture following ATPase treatment showed a time-dependent appearance of [^{32}P]dADP, confirming that positional isotope exchange of the dATP pool does indeed occur during idling-turnover.

In an attempt to define a mechanism to account for the dual

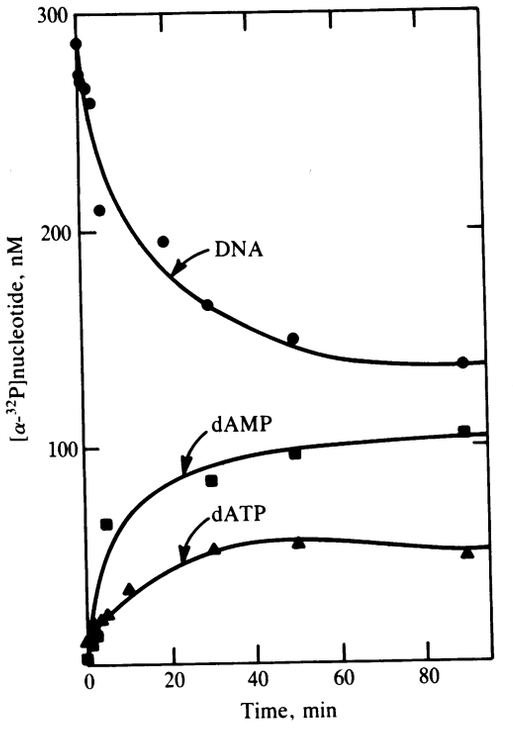
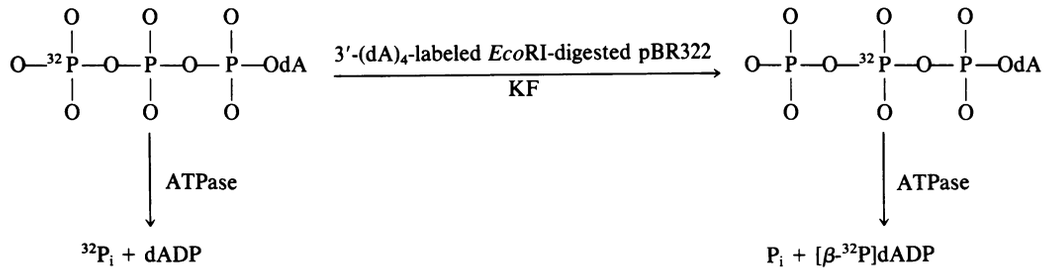


FIG. 2. Analysis of the ^{32}P-labeled products of the idling-turnover reaction of KF on 3' [^{32}P](dA)$_4$-labeled *Eco*RI-digested pBR322 with dATP. A 30-μl reaction mixture containing 140 nM 3' ends, 50 nM KF, 6.6 mM $MgCl_2$, 2 mM dithiothreitol, and 1 μM dATP in 50 mM Tris·HCl (pH 7.4) was incubated at 21°C. Aliquots (3 μl) were withdrawn at various times and analyzed by the TLC assay. The loss of label from 3'-end-labeled *Eco*RI-digested pBR322 (●) is accompanied by the formation of [^{32}P]dAMP (■) and [^{32}P]dATP (▲).

product formation observed during idling-turnover, the effect of increasing the enzyme concentration (10–200 nM) on the course of the reaction on 3'-[^{32}P](dA)$_4$-labeled *Eco*RI-digested pBR322 in the presence of 10 μM dATP was investigated. The initial dATP/dAMP product ratio, as measured during the first 5% of the reaction, was found to decrease with increasing enzyme concentration. Double reciprocal analysis yielded a limiting initial product ratio of 0.12 ± 0.05 at a saturating concentration of enzyme, for DNA concentrations over the range of 50–140 nM 3' ends.

Finally, a description of the mechanism of the triphosphate-forming reaction required the independent measurement of rates of KF-catalyzed pyrophosphorolysis under conditions similar to those of the idling-turnover reaction. We initially sought to develop a coupled assay in which the [^{32}P]dNTP product of the pyrophosphorolysis reaction is quantitatively trapped with ATPase, and as such, is prevented from participating in the reverse polymerization reaction. Unfortunately, technical problems associated with this assay precluded its use.† An alternative method of "trapping" the labeled dNTP is



*On the basis of the misinsertion kinetic data of Fersht *et al.* (16), the extrapolated dGTP → dGMP turnover rate at an initial concentration of 10 μM dGTP is much slower than the rate of 3' → 5' exonuclease activity. Negligible protection of the 3' terminus against exonucleolytic degradation is thus expected, in accordance with the earlier observations of Brutlag and Kornberg (10).

†The relatively low V_{max} of the ATPase trap necessitates a large molar excess of ATPase over Klenow fragment (>1000-fold) for efficient trapping. This in turn sets a lower limit on the concentration of PP_i in the assay, since ATPase is apparently capable of binding PP_i.

Table 1. Distribution of the ^{32}P-labeled mononucleotide products of the idling reaction.

DNA substrate	Unlabeled nucleotide	Conc., μM	^{32}P-labeled nucleotide formed*, pmol	
			[^{32}P]dNMP	[^{32}P]dNTP
3'[^{32}P](dA)$_4$-labeled *Eco*RI-digested pBR322	dATP	1	0.28 ± 0.03	0.13 ± 0.01
		10	0.23 ± 0.04	0.22 ± 0.04
		1000	0.23 ± 0.02	0.16 ± 0.02
	dGTP	10	0.43 ± 0.02	<0.01
	ATP	1000	0.37 ± 0.02	<0.005
	—†	—	0.33 ± 0.02	<0.005
3'[^{32}P](dG)$_2$-labeled *Bam*HI-digested pBR322	dGTP	10	0.16 ± 0.02	0.18 ± 0.02
	—†	—	0.15 ± 0.01	<0.01

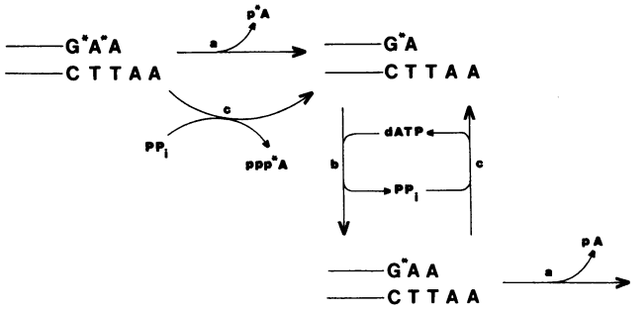
^{32}P-labeled products were quantitated by the TLC assay. Results represent mean ± SEM.
*At 50 min, from DNA initially containing 0.86 pmol (3'[^{32}P](dA)$_4$-labeled *Eco*RI-digested pBR322 case) or 0.43 pmol (3'[^{32}P](dG)$_2$-labeled *Bam*HI-digested pBR322 case) of ^{32}P-labeled nucleotide.
†Exonuclease control.

by dilution into an unlabeled triphosphate pool. Using this approach, the effect of added PP_i on the initial rate of formation of [^{32}P]dATP from 3'-[^{32}P](dA)$_4$-labeled *Eco*RI-digested pBR322 was investigated. The results obtained indicated an approximately linear increase in rate over the 1–5 μM PP_i range.

DISCUSSION

During the course of an idling-turnover reaction catalyzed by KF, the 3'-terminal deoxynucleotide residue of the DNA substrate is partitioned into two products. The dual product formation is strictly contingent upon the presence of a complementary dNTP pool in the idling reaction mixture (Table 1). The ratio of the 5'-mono- to the 5'-triphosphate product shows little dependence upon the initial concentration of the dNTP pool over a 1 μM to 1 mM range. The dNMP product is readily accounted for by 3' → 5' exonucleolytic cleavage of the terminal residue. More perplexing, however, is the appearance of a dNTP product that necessarily results from a pyrophosphorolytic-type cleavage in which PP_i attacks a 3'-terminal phosphodiester linkage. Since each polymerization event catalyzed by KF releases an equivalent of PP_i, the dNTP pool may provide the source of PP_i necessary for this type of cleavage. Indeed, the time-dependent [γ-^{32}P]dATP → [β-^{32}P]dATP positional isotope exchange observed in a 3'-(dA)$_4$-labeled *Eco*RI-digested pBR322-dependent idling-turnover experiment provides qualitative evidence consistent with this notion. Moreover, the triphosphate-forming pathway of the idling-turnover reaction can be totally suppressed by the inclusion of an inorganic pyrophosphatase trap in the reaction mixture, suggesting that the pyrophosphorolytic cleavage reaction is effected by PP_i that was free in solution.

The simplest possible mechanism to account for the dual product formation observed in the idling reaction is illustrated in Scheme 2.



SCHEME 2.

The initial step is 3' → 5' exonucleolytic cleavage of a fraction of the DNA substrate to produce the monophosphate product. This reaction converts the DNA into a form capable of reacting specifically with dATP in a rapid polymerization step, with the concomitant release of PP_i (pathway b). As long as the available dATP pool concentration is sufficiently high to suppress the competing exonuclease activity, this intermediate DNA substrate retains its second labeled dA residue. At this stage, the released PP_i is free to react with the DNA in a pyrophosphorolysis step to yield a triphosphate product. Since the idling reactions were typically conducted at substoichiometric enzyme/DNA levels, a certain fraction of the double-labeled DNA substrate remains available to participate in the pyrophosphorolysis reaction. The labeled triphosphate product of this reaction is diluted into the dATP pool already present and thus appears to accumulate.

The mechanism proposed in Scheme 2 was tested by first investigating the response of the labeled dNTP/dNMP product ratio to various concentrations of KF. As discussed above, the product ratio increases over the course of the reaction as a result of the time-dependent increase in the concentration of PP_i, which in turn accelerates the dNTP-forming reaction. To minimize this complicating feature, which biases the ratio in favor of the dNTP product, only initial product ratios were considered. The model proposed in Scheme 2 ideally predicts a limiting product ratio of zero at saturating enzyme with loss of the entire 3'-terminal label as [^{32}P]dNMP in the first turnover.‡ A limiting ratio of 0.12 ± 0.05 was, however, observed. One possible explanation for the discrepancy between the observed and predicted ratios is the presence of contaminating PP_i in the reaction mixture. Since the unlabeled triphosphate pool provides the only reasonable source of contaminating PP_i, one would predict a sharp rise in the dNTP/dNMP product ratio at higher initial triphosphate pool concentrations. However, the data presented in Table 1 rule out this hypothesis. Alternatively, the discrepancy may be unavoidable because, even at the earliest measurable times, the product ratio is still biased in favor of the triphosphate product for the reasons outlined above. As such, we believe that despite this discrepancy, the observed KF concentration-dependence data are fully consistent with the proposed mechanism.

As proposed above, triphosphate formation during idling-turnover results from a conventional pyrophosphorolysis reaction on the base-paired primer terminus of the DNA substrate. To test this hypothesis, an independent measurement of the rate of pyrophosphorolysis under similar condi-

‡Pyrophosphorolysis could certainly occur as indicated in subsequent turnovers, but would be radiochemically silent.

tions (i.e., at low PP_i levels and on the same template-primer substrate) was required. For the reasons outlined above, pyrophosphorolysis rates were measured in the presence of an unlabeled triphosphate pool. It is important to note that equating a rate thus measured to the actual rate of pyrophosphorolysis, assumes that the triphosphate pool is not involved in the formation of an "activated" enzyme·DNA complex, which may be particularly reactive toward nucleophilic attack by PP_i. The kinetics of triphosphate formation during idling-turnover are complicated by the constantly changing level of PP_i during the course of the reaction, and initial rate measurements are, therefore, only approximate. Comparison of these approximations with the rate data obtained in the presence of 1–5 μM supplemented PP_i indicates that the triphosphate-forming reaction during idling-turnover corresponds to a pyrophosphorolysis reaction involving 0.2–0.5 μM PP_i. In all cases, the PP_i concentration thus extrapolated was less than the maximum theoretical amount that could be generated by complete turnover of the available triphosphate pool (1 μM to 1 mM; Table 1). On the basis of these results, the triphosphate formation observed during idling-turnover may indeed be ascribed to a pyrophosphorolysis reaction effected by PP_i generated *in situ* via pathways *a* and *b* of Scheme 2.

In conclusion, the idling-turnover reaction catalyzed by KF has been found to proceed via an alternating excision/incorporation cycle over the dNTP concentration range studied (1 μM to 1 mM). There is no evidence whatsoever for reaction via a misincorporation/excision pathway under these experimental conditions. This result may, therefore, raise some doubt concerning the mismatch specificity when determining misinsertion frequencies by the turnover assay (14). Perhaps the most significant observation emerging from this study is the unusually low concentration of PP_i that is sufficient to effect the triphosphate formation during idling-turnover. The early kinetic studies of Deutscher and Kornberg (21) on the PP_i exchange and pyrophosphorolysis reactions catalyzed by DNA polymerase I yielded K_M values in the 0.5–0.7 mM range. Consequently, later diverse studies that ranged from elucidating the stereochemistry of the PP_i exchange reaction (3) to probing the effect of PP_i exchange on the fidelity of DNA replication (8, 22), were all conducted at PP_i levels that are at least 10^3- to 10^4-fold higher than those applicable to the present study. The constraint of the idling-turnover reaction (i.e., the absence of the following complementary dNTP) has allowed us to demonstrate that at the extremely low PP_i concentration of 0.1 μM, the rates of pyrophosphorolytic and $3' \rightarrow 5'$ exonucleolytic degradation of duplex DNA by the KF of DNA polymerase I are equivalent.

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