An Induced-Fit Kinetic Mechanism for DNA Replication Fidelity: Direct Measurement by Single-Turnover Kinetics†

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ABSTRACT: An exonuclease-deficient mutant of T7 DNA polymerase was constructed and utilized in a series of kinetic studies on misincorporation and next correct dNTP incorporation. By using a synthetic oligonucleotide template–primer system for which the kinetic pathway for correct incorporation has been solved [Patel, S. S., Wong, I., & Johnson, K. A. (1991) *Biochemistry* (first of three papers in this issue)], the kinetic parameters for the incorporation of the incorrect triphosphates dATP, dCTP, and dGTP were determined, giving, respectively, k_{cat}/K_m values of 91, 23, and 4.3 M^{-1} s^{-1} and a discrimination in the polymerization step of 10^5–10^6. The rates of misincorporation in all cases were linearly dependent on substrate concentration up to 4 mM, beyond which severe inhibition was observed. Competition of correct incorporation versus dCTP revealed an estimated K_i of ~6–8 mM, suggesting a corresponding k_{cat} of 0.14 s^{-1}. Moderate elemental effects of 19-, 17-, and 34-fold reduction in rates were measured by substituting the α-thiotriphosphate analogues for dATP, dCTP, and dGTP, respectively, indicating that the chemistry step is partially rate-limiting. The absence of a burst of incorporation during the first turnover places the rate-limiting step at a triphosphate binding induced conformational change before chemistry. In contrast, the incorporation of the next correct triphosphate, dCTP, on a mismatched DNA substrate was saturable with a K_m of 87 μM for dCTP, 4-fold higher than the K_d for the correct incorporation on duplex DNA, and a k_{cat} of 0.025 s^{-1}. A larger elemental effect of 60, however, suggests a rate-limiting chemistry step. The rate of pyrophosphorolysis on a mismatched 3′-end is undetectable, indicating that pyrophosphorolysis does not play a proofreading role in replication. These results show convincingly that the T7 DNA polymerase discriminates against the incorrect triphosphate by an induced-fit conformational change and that, following misincorporation, the enzyme then selects against the resultant mismatched DNA by a slow, rate-limiting chemistry step, thereby allowing sufficient time for the release of the mismatched DNA from the polymerase active site to be followed by exonucleolytic error correction.

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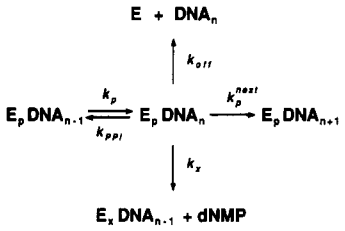
Models to explain the high fidelity of DNA replication have long been proposed in the literature [reviewed in Loeb and Kunkel (1982)]. For example, several models have invoked a role for pyrophosphorolysis in fidelity by mechanisms sometimes referred to as "kinetic proofreading" (Brutlag & Kornberg, 1972; Deutscher & Kornberg, 1969; Hopfield, 1976; Ninio, 1975). It is, however, worth noting that the original models have been proposed as purely mathematical constructs, and the only evidence in support of a role for pyrophosphorolysis is the observation that polymerization is less accurate at high pyrophosphate concentrations (Kunkel et al., 1986). Although induced-fit mechanisms have been suggested, it has also been argued that induced-fit models cannot account for increased selectivity (Fersht, 1974, 1985; Page, 1986). Thus, because of a void of mechanistic information, almost all of these models take considerable liberties with invoking specific but unsubstantiated intermediates in the kinetic pathway of polymerization. Furthermore, most of the published mechanistic studies available on misincorporation rely solely on steady-state kinetic data, which, as we will show, are at best difficult to interpret and at worst extremely misleading. The only transient kinetic study on the mechanism of DNA polymerization relied upon the use of the Klenow fragment of the DNA repair enzyme, Pol I, and some surprising results were obtained, the most notable of which is that the polymerase bound correct and incorrect dNTPs with nearly equal affinities (Kuchta et al., 1987, 1988).

Scheme I



In this report, we propose a mechanism for DNA replication fidelity as a part of our kinetic study of the T7 DNA polymerase (Patel et al., 1991; Donlin et al., 1991). The T7 system lends itself especially well to our approach to fidelity because its kinetic scheme has been completely solved (Patel et al., 1991; Donlin et al., 1991). Consequently, our results can be interpreted by
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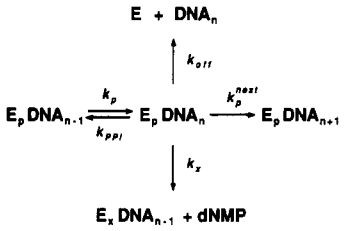
Scheme I



In this report, we propose a mechanism for DNA replication fidelity as a part of our kinetic study of the T7 DNA polymerase (Patel et al., 1991; Donlin et al., 1991). The T7 system lends itself especially well to our approach to fidelity because its kinetic scheme has been completely solved (Patel et al., 1991; Donlin et al., 1991). Consequently, our results can be interpreted by a direct comparison of the differences between correct and incorrect polymerization. In general, T7 DNA polymerase constitutes a nearly ideal model system for any DNA replication studies because it functions in vivo as a true replication enzyme with a minimal number of components.

The problem of replication fidelity is summarized in Scheme I. Conceptually, we divide the issue of fidelity into two parts. First, we are concerned with the mechanism of making an error; this primarily involves solving the kinetics of misincorporation. Second, we are interested in the kinetic conse-

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Table I: Oligonucleotides

25/36-mer	5'-GCCTCGCAGCCGTCCAACCAACTCA CGGAGCGTCGGCAGGTTGGTTGAGTAGGTCTTGTTT-5'
25A/36-mer	5'-GCCTCGCAGCCGTCCAACCAACTCA^{A} CGGAGCGTCGGCAGGTTGGTTGAGTAGGTCTTGTTT-5'

contribution—the kinetic partitioning—between the four possible exit pathways from the central "enzyme–error" complex: (1) pyrophosphorolysis, which represents the microscopic reversal of polymerization, (2) incorporation of the next correct dNTP, (3) dissociation of the mismatched DNA from the enzyme into free solution, and (4) direct transfer of the mismatched DNA into the exonuclease site for repair.

Paradoxically, the chief disadvantage of the T7 system lies in its high replication fidelity. Wild-type enzyme does not form detectable levels of stable misincorporations in vitro. This, as we will show, is due to the highly efficient 3′–5′ proofreading exonuclease, which is particularly good at excising mismatches. For this reason, we have constructed an exonuclease-deficient mutant enzyme (D5A,E7A) (Patel et al., 1991) on the basis of sequence homology studies (Reha-Krantz, 1988a,b; Leavitt & Ito, 1989; Bernad et al., 1989). Using this mutant polymerase, we are able to study the kinetic mechanism and consequences of misincorporation. On the basis of these studies, we propose here that, in a normal cycle of polymerization involving correct incorporation, a rate-limiting conformational change step in an induced-fit mechanism bears the primary burden of substrate selectivity. The issues of (1) V_{max} versus K_m discrimination in substrate selectivity, (2) the role of pyrophosphorolysis in error correction, and (3) the kinetic partitioning mechanism for exonucleolytic error repair will all be discussed in the context of this induced-fit model.

Experimental Procedures

Materials

Bacterial Strains, Plasmids, and Phage. *Escherichia coli* A179 (Hfr-C)(λ)*trxA*::*kan*) and plasmids of pGP5-3 and pGP1-3 were obtained from S. Tabor and C. C. Richardson (Harvard Medical School; Tabor & Richardson, 1987). The plasmid containing the exo$^-$ T7 gene 5, pGA1-14, was constructed in this laboratory as described in the preceding paper (Patel et al., 1991).

Proteins and Enzymes. *E. coli* thioredoxin was purified as described in the preceding paper. Klenow fragment (KF) was kindly provided by R. Kuchta and C. Catalano (The Pennsylvania State University). T4 polynucleotide kinase was purchased from New England Biolabs.

Nucleoside Triphosphates. dNTPs were purchased from Pharmacia Molecular Biologicals at >98% purity. ATP was purchased from Sigma. [α-^{32}P]dTTP, [α-^{32}P]dCTP, [α-^{32}P]dATP, and [γ-^{32}P]ATP were purchased from New England Nuclear.

Synthetic Oligonucleotides. Synthetic oligonucleotides (see Table I) used were synthesized on either an Applied Biosystems 380A DNA synthesizer or a Milligen/Biosearch 7500 DNA synthesizer and purified by electrophoresis through a denaturing gel (20% acrylamide, 1.5% bisacrylamide, and 8M urea in Tris–borate buffer). The major DNA band was visualized by UV shadowing and excised. DNA was electroeluted from the gel slice in an Elutrap apparatus purchased from Schleicher & Schuell. Triethylammonium bicarbonate (TEAB; 2 M, pH 7.5) was added to a final concentration of 0.5 M, and the eluate was applied to an Alltech Maxi-Clean 7.5, purified DNA was eluted in 50% ethanol. Concentrations of purified oligonucleotides were determined by UV absorbance at 260 nm in 8 M urea the following extinction calculated coefficients: 20-mer, ϵ = 202 450; 25-mer, ϵ = 249 040; 25A-mer, ϵ = 261 040; 36-mer, ϵ = 377 000 cm^2/μmol.

Duplex Oligonucleotides. Duplex oligonucleotides were annealed at room temperature in TE buffer containing 100 mM NaCl. They were then purified by electrophoresis through a nondenaturing gel (20% acrylamide and 1.5% bisacrylamide in Tris–borate buffer). The major DNA band was visualized, excised, and electroeluted as above.

Methods

25A/36-mer. Template–primer containing a 3′-terminal A-A mismatch was enzymatically synthesized by using exo$^-$ T7 DNA polymerase. The 25/36-mer (1 μM) was incubated with enzyme (500 nM) and dATP (2 mM) for 5 min at room temperature. Reaction was quenched by the addition of EDTA to 50 mM, followed by two extractions with buffer-saturated phenol–chloroform (1:1). Unincorporated dATP and EDTA as well as residual phenol–chloroform were removed by centrifugation through Bio-Spin 30 centrifuge desalting columns.

3′-^{32}P-Labeled 25A/36-mer. The 25/36-mer (1 μM) was incubated with enzyme (500 nM) and [α-^{32}P]dATP (3000 Ci/mmol at a final concentration of 2–3 μM) for 45 min at room temperature. Cold dATP (2 mM) and an additional aliquot of enzyme (250 nM) were added, and incubation was continued for an additional 5 min. Workup of labeled DNA was as described above.

Reaction Buffer, 5′ ^{32}P Labeling, Reconstitution of T7 DNA Polymerase, Rapid-Quench Experiments, Product Analysis by Denaturing Acrylamide Gels, and PEI-Cellulose TLC. These protocols were performed exactly as described in detail in the first paper in this series (Patel et al., 1991).

Results

Determination of K_m and k_{cat} for Incorporation of the Incorrect dNTPs. We began our studies by attempting to determine the steady-state kinetic parameters, K_m and k_{cat}, for incorrect dNTP incorporation. The DNA substrate used was a 5′-labeled 25/36-mer, and the time courses of incorporation were monitored by analysis of the products on denaturing sequencing gels. The misincorporation of dATP and dGTP (versus A in the template) led to elongation of the 25-mer by one base, while the misincorporation of dCTP led to a series of four bands of sizes 26–29 bases. This resulted from the fact that, after the initial misincorporation to generate the 26-mer, dCTP was the correct base for the next two additions (27- and 28-mers); at higher concentrations of dCTP and at longer time points, a second misincorporation on the 28-mer generated the 29-mer. All four bands were excised, counted, and summed to yield the total products, thus defining the kinetics of the first misincorporation.

The rates of misincorporation for the three incorrect dNTPs were determined over a range of dNTP concentrations from 5 μM up to 10 mM. The results in the millimolar range are shown in Figure 1A. Misincorporation rates were found to be linearly dependent on dNTP concentrations up to 4 mM. Beyond 4 mM, severe inhibition was observed, presumably due to inhibition of DNA binding. Regression analysis gave linear best fits to the data with the slopes defining the apparent second-order rate constant, k_{cat}/K_m. The k_{cat}/K_m values for dATP, dCTP, and dGTP were 91, 23, and 4.3 M^{-1} s^{-1}, respectively. Although there was no indication of curvature in

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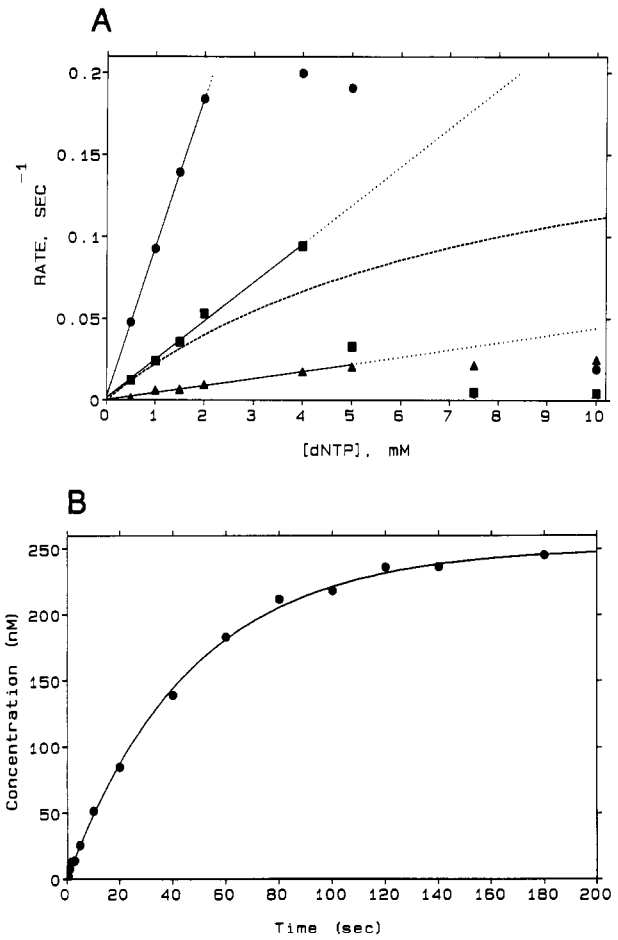

FIGURE 1: Misincorporations. Panel A shows a plot of rates of misincorporation onto 25/36-mer as a function of dNTP concentration. The slopes of the solid lines yield the values of k_{cat}/K_m: (▲) dGTP, k_{cat}/K_m = 4.2 M^{-1} s^{-1}; (■) dCTP, k_{cat}/K_m = 23 M^{-1} s^{-1}; (●) dATP, k_{cat}/K_m = 91 M^{-1} s^{-1}. The concentration dependence continues to be linear until 4 mM, beyond which the rate of misincorporation is severely inhibited. The dashed line shows an attempted hyperbolic fit to estimated lower limits for K_m of 8 mM and a k_{cat} of 0.2 s^{-1} for the dCTP data with a resultant k_{cat}/K_m of 25 M^{-1} s^{-1}. Reactions were all carried out under steady-state conditions with 1 μM 25/36-mer and 5 nM enzyme. Reactions were quenched by the addition of EDTA to 125 mM, and the products were analyzed by denaturing sequencing gels. Quantitation of products was by excision and liquid scintillation counting of gel bands. Panel B shows the time course of a single-turnover dCTP misincorporation at 250 nM enzyme, 250 nM DNA, and 1 mM dCTP. The best fit of the data to a single exponential yields a rate of 0.021 s^{-1}, indicating that the rate during the first turnover is the same as the steady-state rate.

estimate the K_m on the basis of data describing the inhibition of correct incorporation by the incorrect dCTP (see below). The dashed line in Figure 1A represents the calculated hyperbola for a K_m of 8 mM at a k_{cat} of 0.2 s^{-1} (k_{cat}/K_m = 25 M^{-1} s^{-1}), setting a lower limit on the magnitudes of K_m and k_{cat}.

While these experiments were carried out under steady-state conditions in which DNA was in 200-fold excess over enzyme, an experiment performed under pre-steady-state conditions, with a 1:1 ratio of DNA to enzyme, indicated that the rate during the first turnover was the same as that for subsequent turnovers (Figure 1B). Even given the subsaturating concentration of dNTP used in the single-turnover experiments, biphasic kinetics would have been expected if the steady-state rate measured some rate-limiting step after chemistry, given a detection limit of 5%. Consequently, within this limit, we conclude that the steady-state rates reported in Figure 1 reflect

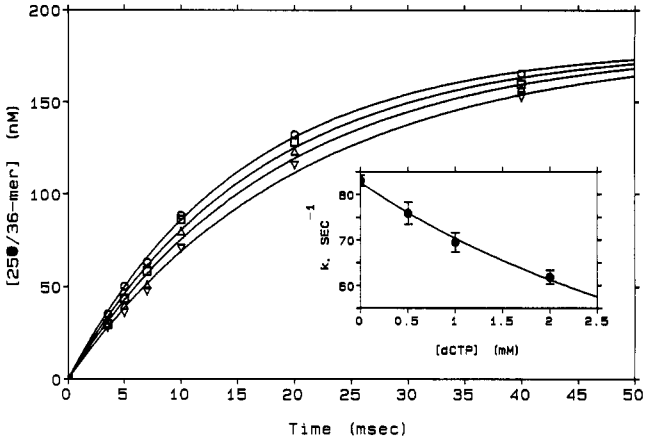

FIGURE 2: Inhibition of correct incorporation by incorrect dNTP. Time course of correct incorporation at 10 μM dTTP in the presence of 0 (○), 0.5 (□), 1.0 (△), and 2.0 mM (▽) of the incorrect dCTP. Solid curves are best fits of the data to single exponentials with rates of 83, 76, 69, and 62 s^{-1}, respectively. Inset shows rate of correct incorporation as a function of dCTP concentration fitted to a hyperbola to yield an extrapolated K_i for dCTP of 4 ± 2 mM. Enzyme and DNA concentrations were 250 nM each. The degree of inhibition observed is so small that the error on the K_i is correspondingly large. Taken together with the estimated lower limit of 8 mM observed in the experiment described in Figure 1, we would estimate a reasonable range for the K_m of dCTP to be around 6–8 mM. See text for further descriptions on the practical constraints in designing these experiments.

(DNA) dissociation, as is the case for correct incorporation.

In a separate attempt to extract discrete values of k_{cat} and K_m for misincorporation, a competition experiment was performed to determine a K_i for inhibition of correct incorporation (dTTP) by an incorrect dNTP (dCTP). DNA (5′-labeled 25/36-mer at 250 nM) was preincubated with 250 nM exo$^-$ enzyme and then was reacted with 10 μM Mg·dTTP (correct dNTP) to measure the rate of the burst of correct incorporation in the presence of 0, 0.5, 1, and 2 mM dCTP (Figure 2). From the concentration dependence of the effect of the incorrect dNTP on the rate of correct incorporation, a K_i for the incorrect dNTP can be very roughly estimated at 4 ± 2 mM. However, the overall degree of inhibition observed was slight, and therefore, the value of K_i thus derived was heavily extrapolated. Taken together with the limit of 8 mM estimated in Figure 1A (see above description), we estimate an approximate range for K_m of 6–8 mM to be within reasonable limits of experimental errors. Unfortunately, the severe inhibition observed at higher dNTP concentrations precluded a better measurement of the K_m for misincorporation.

Elemental Effect for Misincorporation. In order to estimate the extent to which the chemistry step is rate-limiting, we compared the rates of misincorporations of dATP, dCTP, and dGTP with their α-thio analogues dATP(αS), dCTP(αS), and dGTP(αS). A full elemental effect, resulting in a 100-fold reduction of rate when the thio analogues are substituted for the oxy-dNTPs, would indicate a completely rate-limiting chemistry step. For the incorporation of the correct nucleoside triphosphate, dTTP, a small elemental effect of 3 has been observed (Patel et al., 1991), indicating that the chemistry step is not rate-limiting during a normal cycle of polymerization.

In these experiments, 5′-labeled 25/36-mer was used at a 5-fold molar excess over enzyme. Data were quantitated by excision and liquid scintillation counting of bands from a denaturing sequencing gel. The time courses of incorporation of the oxy-dNTPs and the α-thio-dNTPs are shown in Figure 3. Substitution by the thio analogues resulted in reduction in incorporation rates by factors of 19, 17, and 37 for dATP,

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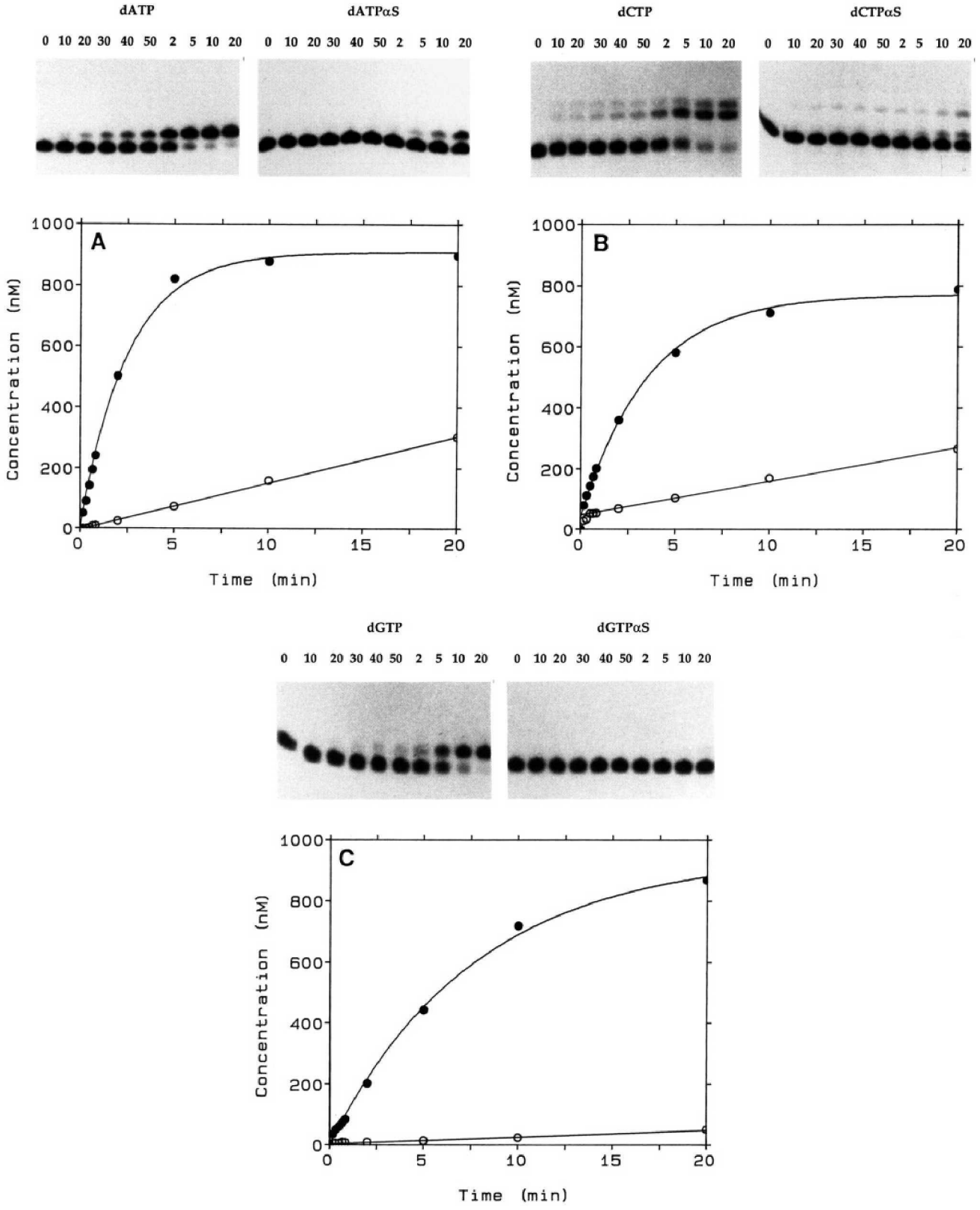

FIGURE 3: Elemental effects on misincorporations. Time course of misincorporation of dATP, dCTP, and dGTP (●) and their α-thio analogues dATP(αS), dCTP(αS), and dGTP(αS) (○). Panel A shows dATP and dATP(αS) misincorporations at 2.4×10^{-2} and 1.3×10^{-3} s^{-1}, respectively. Panel B shows dCTP and dCTP(αS) misincorporations at 1.6×10^{-2} and 9.3×10^{-4} s^{-1}, respectively. As noted in the text, because dCTP is the correct triphosphate for the next two incorporations, a ladder of products from 26 to 29 bases was observed. The difference in banding patterns between the dCTP and dCTP(αS) lanes is illustrative of the difference in elemental effects on dCTP misincorporation and next correct incorporation. With dCTP, the incorporation of the correct dCTPs after the misincorporation is faster than misincorporation and therefore no net accumulation of the 25C-mer is observed. However, because the elemental effect on misincorporation of dCTP is smaller than that for the next correct incorporation, the rate of addition of the next dCTP(αS) in the correctly base-paired positions is now actually slower than the rate of misincorporation, resulting in the accumulation of the 25C-mer. Panel C shows dGTP and dGTP(αS) misincorporations at 7.1

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