A Novel Kinetic Analysis to Calculate Nucleotide Affinity of Proofreading DNA Polymerases

APPLICATION TO \( \delta 29 \) DNA POLYMERASE FIDELITY MUTANTS\(^a\)

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Amino acids Tyr\(^{254}\) and Tyr\(^{390}\) of \( \delta 29 \) DNA polymerase belong to one of the most conserved regions in eukaryotic-type DNA polymerases. In this paper we report a mutational study of these two residues to address their role in nucleotide selection. This study was carried out by means of a new kinetic analysis that takes advantage of the competition between DNA polymerization and 3' → 5' exonuclease activity to measure the \( K_m \) values for correct and incorrect nucleotides in steady-state conditions. This method is valid for any 3' → 5' exonuclease-containing DNA polymerase, without any restriction concerning DNA polymerase exonuclease rates of nucleotide incorporation.

The results showed that the discrimination factor achieved by \( \delta 29 \) DNA polymerase in the nucleotide binding step of DNA polymerization is 2.4 \( \times 10^3 \), that is, a wrong nucleotide is bound with a 2.4 \( \times 10^3 \)-fold lower affinity than the correct one. Mutants Y254F, Y390F, and Y390S showed discrimination values of 7.0 \( \times 10^2 \), >1.9 \( \times 10^2 \), and 2.9 \( \times 10^2 \), respectively. The reduced accuracy of nucleotide binding produced by mutations Y254F and Y390S lead us to propose that \( \delta 29 \) DNA polymerase residues Tyr\(^{254}\) and Tyr\(^{390}\), highly conserved in eukaryotic-type DNA polymerases, are involved in nucleotide binding selection, thus playing a crucial role in the fidelity of DNA replication. Comparison of the discrimination factors of mutants Y390S and Y390F strongly suggests that the phenyl ring of Tyr\(^{390}\) is directly involved in checking base-pairing correctness of the incoming nucleotide.

One of the most remarkable properties of DNA polymerases is their ability to replicate DNA with a very high fidelity (10^{-5}–10^{-8} errors/nucleotide) (Loeb and Kunkel, 1982; Echols and Goodman, 1991). This much is less than the error frequency of 2 \( \times 10^{-5} \), 6 \( \times 10^{-5} \), and 2 \( \times 10^{-5} \) errors per nucleotide polymerized, predicted on the basis of energy differences between correct and incorrect base pairs (Loeb and Kunkel, 1982). The accuracy of DNA polymerization is based upon template instruction, that is changed after each nucleotide incorporation event. Taking into account that DNA polymerases synthesize DNA at high velocity (100–1000 nucleotides/s) (Kornberg and Baker, 1992), their substrate specificity has to change very rapidly, while maintaining a strong discrimination against wrong nucleotides. The molecular mechanisms underlying this amazing behavior are poorly understood.

The considerable accuracy of DNA synthesis is achieved by the sequential operation of two fidelity mechanisms: (a) selection of the correct deoxynucleoside triphosphate in the polymerization reaction (base selection) and (b) exonucleolytic removal of an incorrectly inserted deoxynucleoside monophosphate from the end of the growing chain (editing). For base selection, the most likely discrimination principle is a strict demand for the precise geometry of the Watson-Crick base pair. This requirement might be manifested either in a more rapid dissociation from the enzyme active site of an incorrect nucleotide or in a considerably slower phosphodiester bond formation for mispaired bases, or most likely both. Additional mechanisms based on intermediate conformational changes have been described to discriminate against incorrect incorporation (Wong et al., 1991). Besides, the addition of the next correct nucleotide onto a mismatch occurs at a very slow rate, stalling the polymerase after misincorporation, and, therefore, allowing time for the exonucleolytic activity to attack the mispaired primer terminus (Donlin et al., 1991; Carroll et al., 1991; reviewed in Johnson (1993)).

In the absence of structural data, site-directed mutagenesis is a powerful tool to elucidate the contribution of individual amino acid residues of the DNA polymerization site to the fine nucleotide selection exhibited by most replication enzymes. However, very few studies have addressed properly the fidelity of mutant DNA polymerases. The high catalytic rate of nucleotide incorporation compromises the utilization of standard steady-state methods to describe the kinetic properties of DNA polymerization. Besides this, most DNA polymerases contain a 3' → 5' exonuclease activity that competes with DNA synthesis, this competition being unbalanced toward exonucleolytic degradation in the case of polymerization mutants, where nucleotide incorporation is impaired. Whereas this second point can be overcome by using exonuclease-deficient mutants, the study of very fast reactions in appropriate time windows seems to require rapid quench-flow techniques (Patel et al., 1991; Wong et al., 1991; Eger et al., 1991; Hsieh et al., 1993).

In this paper we report that meaningful biochemical information can be obtained from steady-state kinetics when the exonuclease activity is allowed to compete with DNA polymerization. We are using this approach to study the molecular mechanisms of DNA replication fidelity using \( \delta 29 \) DNA polymerase as model. This polymerase provides a simple and well-characterized model for studying template-directed DNA synthesis and identifying which amino acid side chains participate
Nucleotide Binding Affinity in DNA Replication Fidelity

in substrate selection and catalysis of phosphodiester bond formation. Extensive site-directed mutagenesis studies based on amino acid sequence comparison have allowed us to define the functional role for most of the residues belonging to highly conserved motifs in DNA polymerases (reviewed in Blanco and Salas (1995)). This large collection of 329 DNA polymerase mutants is now available to elucidate the contribution of individual amino acids of the active center to the mechanisms maintaining fidelity. Furthermore, a basic kinetic and fidelity description of both DNA replication and protein-primed initiation has been reported for 329 DNA polymerase (Esteban et al., 1993).

Recent structural and kinetic data indicate that polymerases achieve selectivity by mechanisms that differ in detail, although, from the available evidence, it is accepted that all polymerases follow general fidelity principles for base selection and editing (Echoes and Goodman, 1991). Therefore, the conclusions to be drawn from 329 DNA polymerase (a prototype enzyme for eukaryotic-type DNA polymerases) will shed some light into the basic mechanisms maintaining fidelity.

For the present study we have chosen two 329 DNA polymerase residues: Tyr254 and Tyr390. They belong to the highly conserved motifs DXXXLYP (region 1 according to Blasco et al. (1991), region A according to Delarue et al. (1990)) and XXXXNSLYG (region 2a according to Blasco et al. (1991), region B according to Delarue et al. (1990), respectively). The conservative mutations Y254F, Y390S, and Y390F were reported to show a defective dNTP binding affinity, closely related to a template-dependent hypersensitivity to nucleotide analogs, such as (2-(p-n-butylanilino)dATP and N2-(p-n-butylphenyl)dGTP (Blasco et al., 1992). Therefore, these DNA polymerase mutants appeared as ideal candidates for fidelity studies.

MATERIALS AND METHODS

Nucleotides and Proteins—Ultrapure unlabeled dNTPs were from Pharcopia Biotech Inc.; [32P]dATP (3000 Ci/mmol) was obtained from Amersham International Plc. T4 polynucleotide kinase was purchased from New England Biolabs. Wild-type 329 DNA polymerase and mutant derivatives Y254F, Y390F, and Y390S (Blasco et al., 1992) were purified as described by Lazaro et al. (1995).

DNA Templates—Complementary single-stranded oligonucleotides SP1 (15-mer) (5'-GATCACAGTGAGTAC) and SP1c (21-mer) (5'-32P-labeled), and mutant derivatives Y254F, Y390F, and Y390S (Blasco et al., 1992) were used to study the influence of 32P incorporation on DNA polymerization. The theoretical model described below).

Measurement of Incorrect Nucleotide Insertion Parameters—The reaction mixtures were as described above but, in this case, 20 µM dATP was included. This concentration was proven to be enough for the two first correct incorporations. For the incorrect nucleotide insertion on the third position of the template, different dCTP concentrations were assayed within a range of at least one order of magnitude. The dCTP concentration present in the reaction mixtures also allows the incorporation of the next correct nucleotide onto the mismatch. The ratio between the error-containing elongation products (misinsertion, 18-mer, and mismatch elongation, 19-mer) and the last correctly paired elongation product (17-mer) was calculated and plotted against the dCTP concentration, according to the theoretical method described in the text. The graphs were fitted to rectangular hyperbolas by nonlinear regression analysis using both the Newton and the steepest-descent methods. The kinetic parameters were obtained as described in the text.

RESULTS

Theoretical Model—Kinetics of single nucleotide incorporation catalyzed by DNA polymerases follow single or double exponential behavior (depending on experimental conditions), that usually reach equilibrium in the millisecond time scale (Papel et al., 1991). This rapid nucleotide insertion makes the steady-state studies difficult to interpret, because, in most of the cases, the rate-limiting step is DNA dissociation from the enzyme. Therefore, the information regarding the fast catalytic step is usually missed or mistaken.

We have studied the influence of 3' → 5' exonuclease activity on DNA polymerization kinetics. The mathematical treatment of pre-steady-state kinetics becomes considerably more complex when the exonuclease activity is included. However, we will show that, after the transient pre-steady-state, the final steady-state that is reached offers unambiguous information of some kinetic parameters governing DNA polymerization. This model represents a steady-state analysis of the competition between DNA polymerization and exonuclease activity (see below). Therefore, in order for this model to be applicable, two main conditions have to be fulfilled: DNA polymerase-DNA complex dissociation must not be rate-limiting, and solely the final steady-state phase of the time course has to be considered.

First, we will describe the validity of this approach to study the kinetic parameters of correct and incorrect nucleotide incorporation catalyzed by the wild-type 329 DNA polymerase. Then, we will apply this model to study the fidelity of Y254F, Y390S, and Y390F mutant derivatives of 329 DNA polymerase, previously described as deficient in nucleotide binding.
Correct Nucleotide Incorporation—We have considered the insertion of a correct nucleotide on a homopolymer tract as template, in the presence of the exonuclease activity. Two conditions should be imposed. First, the initial primer (D₀) has to be stable against exonucleolytic degradation, in order to keep constant the total amount of DNA template-primer in the reaction mixture. Second, the actual template has to be longer than the homopolymeric tract, to stop polymerization before reaching the end of the template. The reaction pathway that describes this system is shown in Scheme I.

In this scheme, E-Dᵢ stands for the binary complex formed by the i-mer primer DNA and DNA polymerase; kᵣ and kᵣ⁻ represent the catalytic rates of DNA polymerization and exonuclease reactions, respectively; kᵢ and kᵢ⁻ represent the association and dissociation rates, respectively, of nucleotide binding; n is the number of consecutive nucleotide incorporations allowed on the homopolymer tract.

Using steady-state kinetic analysis, the concentration of the different species can be derived as:

\[
Dᵢ = Dᵢ \cdot Nᵢ \cdot \left( \frac{kᵢ \cdot (KᵢM)^{n-i}}{kᵣ + (KᵢM)^{n-i}} \right)
\]

(Eq. 1)

\[
(Dᵢ \cdot N) = D₀ \cdot N \cdot \left( \frac{kᵢ \cdot (KᵢM)^{n-i}}{kᵣ + (KᵢM)^{n-i}} \right)
\]

(Eq. 2)

In these formulae, Dᵢ and (Dᵢ ⋅ N) represent the concentration of a certain DNA intermediate, i-bases long, and the complex i-mer DNA primer with the next nucleotide to be incorporated bound in its position, not yet catalyzed (see Scheme I); both D₀ and (Dᵢ ⋅ N) stand for the corresponding complexes with DNA polymerase; Dᵢ is the total amount of DNA polymerase/DNA complexes; N stands for dNTP concentration, and KᵢM = (kᵢ⁻ + kᵣ)/kᵣ.

These equations predict the steady-state distribution of the different elongated complexes as a function of kᵣ/kᵣ⁻, KᵢM, and nucleotide concentration. This theoretical distribution can be compared to experimental ones, generated at different dNTP concentrations, to fit the kinetic parameters kᵣ/kᵣ⁻ and KᵢM. A more straightforward approach can be followed by considering the ratio between the concentration of the two last elongated complexes (Dᵢ and Dᵢ⁻₁):

\[
\frac{Dᵢ}{Dᵢ⁻₁} = \frac{(KᵢM)^{n-i}}{(KᵢM)^{n-i-1}} \cdot \left( \frac{kᵢ}{kᵣ} \right)
\]

(Eq. 3)

The relative proportion between the two last DNA species appears as a function of nucleotide concentration, where kᵣ/kᵣ⁻ and KᵢM can be obtained from a nonlinear least square fit to the rectangular hyperbola.

For a practical approach to this model, we used as DNA substrate a 5’-labeled 15/21-mer primer-template oligonucleotide, which allows the consecutive incorporation of two correct dAMP residues; that is, the homopolymeric tract is restricted to two nucleotides (see Scheme II and Materials and Methods).

As described previously, the mathematical treatment presented here is valid only if the steady-state has been reached, and the DNA polymerase/DNA complex dissociation is not rate-limiting in this steady-state. These two points were addressed and confirmed experimentally. The incubation time required to reach the steady-state was determined by following the time course of the reaction. In this experiment, a very low concentration of dATP (20 nM) was added, so as to evaluate the time course at a very slow forward rate, the enzyme being far away from its maximal velocity. The diagnostic feature of the steady-state is the invariance of all the elongation product concentrations. In our case, due to the low dATP concentration, the only elongation product is D₂. As shown in Fig. 1, the intensity of the bands corresponding to D₀ and D₂ is invariant from the first time tested (1 min).

To evaluate whether dissociation or dissociation-reassociation are rate-limiting steps in the reaction, another experiment was carried out with increasing amounts of template-primer DNA and a fixed concentration of dATP DNA polymerase. As shown in Fig. 2, at the highest DNA concentrations, the relative proportions of elongated primers (D₂/D₀) is very low, indicating that dATP DNA polymerase is titrated out. However, the proportion between the longest elongation product and the shortest one (D₂/D₁) is independent from the DNA polymerase/DNA ratio. These results indicate that dATP DNA polymerase is not being dissociated from the template, and, therefore, complex dissociation does not influence the final steady-state.

Correct nucleotide insertion kinetics were carried out at different dATP concentrations, which bracketed the KᵢM value for this nucleotide, allowing the system to reach steady-state conditions. The ratio between the intensity of bands D₂ and D₁ was calculated and plotted against dATP concentration (see Fig. 3). Experimental data were fitted to Equation 3 by nonlinear regression analysis, and KᵢM and kᵣ/kᵣ⁻ were estimated as 0.5 μM and 2.6, respectively (see Table I).

Incorrect Nucleotide Insertion and Elongation—To study the incorporation of a wrong nucleotide, the same 5’-labeled 15/21-mer primer-template molecule was used. In this case, a sufficient concentration (20 μM) of dATP was included to allow the incorporation of the two first correct nucleotides. The misincor-
Nucleotide Binding Affinity in DNA Replication Fidelity

Fig. 1. Kinetics of nucleotide insertion catalyzed by φ29 DNA polymerase. Time course of nucleotide addition catalyzed by φ29 DNA polymerase on template-primer SP1/SP1c+6. •, relative proportion of the nonelongated primer to the total amount of DNA, obtained as the ratio $D_0/(D_0 + D_1)$, where $D_0$ corresponds to the primer position (15-mer), and $D_1$ stands for the addition of one nucleotide (16-mer). ○, relative proportion of the elongation product (16-mer) to the total amount of DNA, obtained as the ratio $D_2/(D_0 + D_1)$. □, quotient between the two DNA products, 16-mer and 15-mer, obtained as the ratio $D_2/D_0$. Upper chart, electrophoretic analysis of a primer extension reaction, where $D_0$ and $D_1$ indicate the primer position and the elongation product, respectively. The incubation times are indicated.

Fig. 2. Template-primer titration of φ29 DNA polymerase. Relative amount between the two last elongated products, calculated as the ratio $D_2/D_0$ (●), and relative proportion of elongated and non-elongated primers (○), calculated as $(D_1 + D_2)/D_0$. Both ratios are expressed relative to 1, taking the maximum value of the ratio as 1 (4.6 in the case of the ratio $(D_1 + D_2)/D_0$ and 0.8 in the case of the ratio $D_2/D_0$). Both ratios are plotted versus the concentration of template-primer. Upper chart, electrophoretic analysis of a primer extension reaction, where $D_0$ and $D_1$ indicate the primer position and the last elongated position, respectively. Template-primer concentrations are indicated.

Portion of C opposite A in the template was monitored at different dCTP concentrations. The dATP concentration present in the experiment allows the elongation of the mismatches using the next T in the template (see Scheme III).

In this model, $k_{cat}$ and $K_m$ are the catalytic rates for inserting an incorrect nucleotide and extending it with a correct one, respectively; $k_2$ and $k_{-2}$ stand for the association and dissociation rates, respectively, of dCTP binding; $k_3$ and $k_{-3}$ represent the association and dissociation rates, respectively, for binding the next correct nucleotide; $k_{exo}$, $k_{exo1}$, and $k_{exo2}$ are the catalytic rates of the exonuclease reaction on the properly paired primer terminus, on the mismatched one, and on the correctly elongated mismatch, respectively.

The steady-state analysis of this system leads to the following equations:

\[
D_2 = D_1 \cdot \frac{k_{cat} \cdot k_{exoi} \cdot k_{exoi2} \cdot N^2 \cdot K_m^{err} \cdot K_m^{ext}}{S} \quad (Eq. 4)
\]

\[
(D_2 - I) = D_1 \cdot \frac{k_{cat} \cdot k_{exoi} \cdot k_{exoi2} \cdot N^2 \cdot I \cdot K_m^{err}}{S} \quad (Eq. 5)
\]

\[
D_3 = D_1 \cdot \frac{k_{cat} \cdot k_{err} \cdot k_{exoi2} \cdot N^2 \cdot I \cdot K_m^{ext}}{S} \quad (Eq. 6)
\]

\[
(D_3 - N) = D_1 \cdot \frac{k_{cat} \cdot k_{err} \cdot k_{exoi2} \cdot N^3 \cdot I}{S} \quad (Eq. 7)
\]

\[
(D_4 = D_1 \cdot \frac{k_{cat} \cdot k_{err} \cdot k_{exoi2} \cdot N^3 \cdot I}{S} \quad (Eq. 8)
\]

where $D_2$, $(D_2 - I)$, $D_3$, $(D_3 - N)$, and $D_4$ are the different elongation products in the polymerization process (positions 17, 18, and 19, respectively; see oligonucleotide sequence in Scheme III). These DNA products are assumed to be always forming a complex with the enzyme. $D_0$ is the total amount of DNA polymerase/DNA complexes. $N$ and $I$ represent the correct and incorrect nucleotide concentrations, respectively. The different affinity constants are defined as follows:

\[
K_m^{err} = \frac{k_{-2} + k_{err}}{k_2} \quad (Eq. 9)
\]

\[
K_m^{ext} = \frac{k_3 + k_{ext}}{k_3} \quad (Eq. 10)
\]

$S$ stands for the following expression:

\[
S = k_{exo} \cdot k_{exo1} \cdot k_{exo2} \cdot K_m^{err} \cdot K_m^{ext} \cdot K_m^{m} + k_{exo} \cdot k_{exo1} \cdot k_{exo2} \cdot K_m^{err} \cdot K_m^{ext} \cdot K_m^{m} \cdot N + k_{exo} \cdot k_{exo1} \cdot k_{exo2} \cdot K_m^{err} \cdot K_m^{ext} \cdot K_m^{m} \cdot N
\]

\[
+ k_{exo} \cdot k_{exo1} \cdot k_{exo2} \cdot K_m^{err} \cdot K_m^{ext} \cdot N^2 + k_{exo} \cdot k_{exo1} \cdot k_{exo2} \cdot K_m^{err} \cdot K_m^{ext} \cdot N^3 \cdot I + k_{exo} \cdot k_{exo1} \cdot k_{exo2} \cdot K_m^{err} \cdot K_m^{ext} \cdot N^2 \cdot 1
\]


Km stands for the Michaelis constant of the two first correct nucleotide insertions, as defined for Equation 3. The ratio between the error-containing products (positions D3 plus D4) and the correct nucleotide insertion (position D2) gives us the following result:

\[
\frac{D_3 + (D_3 \cdot N) + D_4}{D_2 + (D_2 \cdot 1)} = \frac{k_{\text{eff}} \cdot (k_{\text{nat}} \cdot N + k_{\text{nat}} \cdot N + k_{\text{nat}} \cdot K_{m}^i)}{k_{\text{nat}} \cdot k_{\text{nat}} \cdot K_{m}^i} \cdot \frac{1}{K_{m}^i + 1}
\]

(Eq. 12)

Therefore, this ratio versus the concentration of the incorrect nucleotide (I) would give a rectangular hyperbola, from which the Km for the incorrect nucleotide (Km^i) can be obtained.

The incubation times required to reach the steady-state, as well as the absence of a DNA dissociation rate-limiting step, were assessed as described for the correct nucleotide (not shown).

The incorporation of the incorrect nucleotide was measured at different dCTP concentrations, and the ratio (D3 + D4)/D2 was calculated. The experimental data were fitted to Equation 12 by nonlinear regression analysis. The results are shown in Fig. 4 (see also Table I).

Fidelity Studies of φ29 DNA Polymerase Mutants Y254F, Y390S, and Y390F—φ29 DNA polymerase mutants Y254F, Y390F, and Y390S have been described as affected in dNTP binding (Blasco et al., 1992). Nevertheless, their deficiency in dNTP affinity could not be precisely assessed because Km could not be determined either for the wild type or for the mutant DNA polymerases. We have used the exonuclease-polymerization competition model, presented above, to study the steady-state kinetics of correct and incorrect nucleotide incorporation catalyzed by φ29 DNA polymerase mutants Y254F, Y390F, and Y390S.

As described for the wild-type enzyme, the absence of a rate-limiting DNA dissociation step and the incubation time required to reach steady-state conditions were evaluated experimentally for each mutant (not shown). In the case of mutants Y254F and Y390F, we observed that long incubation periods led to exonuclease degradation of most of the DNA primer substrate (not shown). This is probably due to their reduced DNA polymerization rate, that renders the standard dCTP concentration insufficient for protecting against their exonuclease activity. In order to avoid potential interferences with the incorporation of the correct nucleotide and taking into account that steady-state conditions were achieved after 10 s (not shown), we decided to use shorter incubation times (15 s) rather than to increase dCTP concentrations. It is worthy to note that, using this experimental approach, the incubation time is not relevant, as long as the steady-state has been reached.

The incorporation of correct (dATP) and incorrect (dCTP) nucleotides were evaluated at different dNTP concentrations, and the experimental data were analyzed as described for the wild-type enzyme. The results are shown in Fig. 5. Concerning the incorporation of the correct nucleotide, mutants Y254F, Y390F, and Y390S showed 10, 4.6, and 14-fold, respectively, reduction in dNTP affinity, compared to the wild-type enzyme. The discrimination factor achieved during nucleotide binding can be obtained from the different affinity for correct and incorrect nucleotides and represented by f_{\text{dis}} = K_m (incorrect)/K_m (correct). This factor was calculated for the wild-type and mutant φ29 DNA polymerases and is shown in Table I. Proteins Y254F and Y390S showed a reduced discrimination ability (7.0 × 10^2 and 2.9 × 10^2, respectively), whereas the discrimination value of Y390F (>1.9 × 10^3) was quite similar to the one of the wild-type enzyme (2.4 × 10^3). The quantification of these effects is also shown in Table I, as relative discrimination factors, that were calculated relative to the wild-type enzyme.

**DISCUSSION**

Since the first reports of in vitro studies concerning the fidelity of DNA polymerization (Trautner et al., 1962; Hall and
Lehman, 1968; Bruttig and Kornberg, 1972), a large number of DNA polymerases have been purified and their accuracies measured experimentally (reviewed in Echols and Goodman (1991), Kunkel (1992), Johnson (1993)). Based on these studies, the general strategies for achieving faithful DNA synthesis have been enlightened (reviewed in Johnson (1993)). Presently, many DNA polymerase studies are devoted to understand how the active center of these enzymes promotes such a highly selective reaction mechanism.

In this work, we have presented a new model for studying DNA polymerization kinetics in steady-state conditions, and we have applied it to get some insight into the molecular mechanisms of fidelity, operating at the active center of these enzymes. We have applied it to get some insight into the molecular mechanisms of fidelity, operating at the active center of these enzymes. We have applied it to get some insight into the molecular mechanisms of fidelity, operating at the active center of these enzymes.

The kinetic analysis proposed here takes advantage of the 3' → 5' exonuclease activity to compete DNA polymerization, leading to a true steady-state. Once this state has been reached, the relative amount of each DNA elongation product remains constant with time. This situation should not be taken as an equilibrium state, as there is a continuous (steady) interconversion between the different DNA products by means of deoxynucleoside triphosphate use and monophosphate production. Therefore, in order to apply this method to different systems, it has to be explicitly verified that the early transient stage of DNA product rearrangement is overcome. This requirement was specifically met with the wild-type and mutant φ29 DNA polymerases presented in this study.

Another key feature of this model, noticeable from Schemes I, II, and III, is the absence of dissociation rates ($k_{diss}$). As described in the text, all the DNA species are considered as DNA polymerase/DNA complexes, therefore, competent for nucleotide insertion or exonucleolytic degradation. The avoidance of dissociation and association steps between DNA polymerase and DNA allows a more straightforward interpretation of the experimental data in terms of the catalytically relevant rate constants. However, the experimental conditions have to be designed in order to fulfill the requirement for a DNA dissociation-independent reaction pathway. As demonstrated in the DNA titration experiment (this paper), φ29 DNA polymerase is able to remain stably bound to the template/primer, even when it has been stalled by the absence of the next correct nucleotide. In this experiment, the probability of inserting one or two nucleotides was measured at different DNA polymerase/DNA ratios. If the insertion of the second nucleotide is mediated by DNA polymerase dissociation and reassociation to the same DNA molecule, addition of a non-elongated DNA excess would lead to the accumulation of the DNA product corresponding to the insertion of only one nucleotide, because of the reduced probability of a DNA molecule to be encountered twice. On the other hand, if φ29 DNA polymerase dissociates after inserting the two correct nucleotides, the presence of an excess of non-elongated molecules would induce the accumulation of the DNA product corresponding to the insertion of two nucleotides. This is so because these elongated molecules no longer would be subjected to the pressure of the exonuclease activity. Taking into account that the ratio $D_2/D_1$ remained fairly constant over 100-fold increase in the concentration of the non-elongated DNA molecules, we can conclude that DNA polymerase/DNA complex dissociation is not relevant in the DNA polymerization pathway of φ29 DNA polymerase on these DNA molecules. This conclusion does not imply that the applicability of the model is restricted to very processive DNA polymerases. In the case of distributive DNA polymerases, the absence of a rate-limiting dissociation step can be accomplished simply by using high concentrations of the enzyme, to ensure that all the DNA species present in the reaction mixture are complexed with a DNA polymerase molecule.

Once verified these two conditions (reach of the steady-state and absence of a rate-limiting DNA dissociation step), the activity associated with many DNA polymerases makes it more difficult to correlate the experimental data with the kinetic parameters governing DNA polymerization. In this situation, an apparent $K_m$ is usually reported that simply reflects the nucleotide concentration at which half of the maximal rate of nucleotide incorporation is achieved. However, this parameter has no explicit relation with nucleotide binding affinity. In this paper, we propose a general method to calculate the true $K_m$ values for correct and incorrect nucleotides overcoming these limitations. As true $K_m$, we consider its original explicit definition: $K_m = (k_{-1} + k_{cat})^{-1}$.

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Once verified these two conditions (reach of the steady-state and absence of a rate-limiting DNA dissociation step), the activity associated with many DNA polymerases makes it more difficult to correlate the experimental data with the kinetic parameters governing DNA polymerization. In this situation, an apparent $K_m$ is usually reported that simply reflects the nucleotide concentration at which half of the maximal rate of nucleotide incorporation is achieved. However, this parameter has no explicit relation with nucleotide binding affinity. In this paper, we propose a general method to calculate the true $K_m$ values for correct and incorrect nucleotides overcoming these limitations. As true $K_m$, we consider its original explicit definition: $K_m = (k_{-1} + k_{cat})^{-1}$.

The kinetic analysis proposed here takes advantage of the 3' → 5' exonuclease activity to compete DNA polymerization, leading to a true steady-state. Once this state has been reached, the relative amount of each DNA elongation product remains constant with time. This situation should not be taken as an equilibrium state, as there is a continuous (steady) interconversion between the different DNA products by means of deoxynucleoside triphosphate use and monophosphate production. Therefore, in order to apply this method to different systems, it has to be explicitly verified that the early transient stage of DNA product rearrangement is overcome. This requirement was specifically met with the wild-type and mutant φ29 DNA polymerases presented in this study.

Another key feature of this model, noticeable from Schemes I, II, and III, is the absence of dissociation rates ($k_{diss}$). As described in the text, all the DNA species are considered as DNA polymerase/DNA complexes, therefore, competent for nucleotide insertion or exonucleolytic degradation. The avoidance of dissociation and association steps between DNA polymerase and DNA allows a more straightforward interpretation of the experimental data in terms of the catalytically relevant rate constants. However, the experimental conditions have to be designed in order to fulfill the requirement for a DNA dissociation-independent reaction pathway. As demonstrated in the DNA titration experiment (this paper), φ29 DNA polymerase is able to remain stably bound to the template/primer, even when it has been stalled by the absence of the next correct nucleotide. In this experiment, the probability of inserting one or two nucleotides was measured at different DNA polymerase/DNA ratios. If the insertion of the second nucleotide is mediated by DNA polymerase dissociation and reassociation to the same DNA molecule, addition of a non-elongated DNA excess would lead to the accumulation of the DNA product corresponding to the insertion of only one nucleotide, because of the reduced probability of a DNA molecule to be encountered twice. On the other hand, if φ29 DNA polymerase dissociates after inserting the two correct nucleotides, the presence of an excess of non-elongated molecules would induce the accumulation of the DNA product corresponding to the insertion of two nucleotides. This is so because these elongated molecules no longer would be subjected to the pressure of the exonuclease activity. Taking into account that the ratio $D_2/D_1$ remained fairly constant over 100-fold increase in the concentration of the non-elongated DNA molecules, we can conclude that DNA polymerase/DNA complex dissociation is not relevant in the DNA polymerization pathway of φ29 DNA polymerase on these DNA molecules. This conclusion does not imply that the applicability of the model is restricted to very processive DNA polymerases. In the case of distributive DNA polymerases, the absence of a rate-limiting dissociation step can be accomplished simply by using high concentrations of the enzyme, to ensure that all the DNA species present in the reaction mixture are complexed with a DNA polymerase molecule.
kinetic parameters of both correct and incorrect nucleotide incorporation, can be evaluated according to Equations 3 and 12. In the case of correct nucleotide, the maximal value of the ratio \( D_i/D_{i-1} \) (at saturating nucleotide concentration) is given by \( k_{\text{cat}}/K_{\text{m}(\text{ext})} \) (see Equation 3). In other words, the competition between DNA polymerization and 3′→5′ exonuclease becomes quantified explicitly in this analysis. On the other hand, the actual \( K_{\text{m}} \) value can be estimated from this equation, since it is not affected by the exonuclease rate. In the case of misincorporation, the value of the ratio defined in Equation 12, at saturating nucleotide concentrations, is given by a complex expression. This value depends on synthetic rates (misinsertion, \( k_{\text{err}} \), and mismatch elongation, \( k_{\text{ext}} \)) and exonuclease rates (error editing, \( k_{\text{ext}} \), and removal of the next correct nucleotide, \( k_{\text{exo2}} \)). But, interestingly, the affinity constant for the wrong nucleotide (\( K_{\text{m}(\text{err})} \)) can be calculated from Equation 12, without the interference of the other kinetic parameters. Potentially, other ratios can be calculated to get more information about the rate constants involved in the reaction pathway. For instance:

\[
\frac{D_{i+4}}{D_{i+3}} = \frac{k_{\text{cat}}}{k_{\text{exo2}}} \cdot \frac{N + K_{\text{m}(\text{ext})}}{N + K_{\text{m}(\text{err})}} \quad \text{(Eq. 13)}
\]

\[
\frac{D_{i+3}}{D_{i+2}} = \frac{k_{\text{cat}}}{k_{\text{exo}}} \cdot \frac{N + K_{\text{m}(\text{ext})}}{N + K_{\text{m}(\text{err})}} \frac{1}{1 + K_{\text{m}(\text{err})}} \quad \text{(Eq. 14)}
\]

By plotting \( D_{i+3}/D_{i+2} \) versus correct dNTP concentration, both the affinity for the next correct nucleotide and the ratio \( k_{\text{cat}}/k_{\text{exo2}} \) could be obtained from Equation 3. By plotting \( D_{i+3}/D_{i+2} \) versus incorrect nucleotide concentration, at subsaturating concentrations of the next correct nucleotide (\( N < K_{\text{m}(\text{err})} \)), the ratio \( k_{\text{cat}}/k_{\text{exo}} \) could be obtained from Equation 14. However, the proportion of the \( D_{i+3} \) elongation product in our system was always very low, precluding an accurate estimation of the parameters discussed above.

Another steady-state kinetic analysis for the study of 3′→5′ exonuclease-containing DNA polymerases has been recently described (Creighton and Goodman, 1995). The validity of their method relies on a dissociation step after each DNA polymerase/DNA encounter and a low probability of a DNA molecule to be bound twice during the reaction time. These conditions are fulfilled by using a large excess of DNA over DNA polymerase and by allowing the elongation of less than 20% of the total DNA molecules. This experimental strategy is just the opposite to the one described in this paper. However, there is an interesting conclusion obtained from both methods: the affinity constant, \( K_{\text{m}} \), is not affected by the exonuclease activity, when the ratio between the appropriate DNA elongation products is considered. Their analysis seems to allow the estimation of both \( V_{\text{max}} \) and \( K_{\text{m}} \) without the interference of the exonuclease activity. However, some limitations of their method, extensively discussed by the authors, compromise the use of high dNTP substrate conditions (which is most likely the case in an incorrect nucleotide insertion experiment). Therefore, their kinetic analysis is preferably restricted to determine incorporation efficiencies (\( V_{\text{max}}/K_{\text{m}} \)) rather than catalytic constants (\( k_{\text{cat}} \)).

The model presented in this paper has two major advantages. First, it has no restriction. Once verified the absence of a rate-limiting dissociation step, the validity of the analysis is independent of the value of the rate constants or the substrate concentrations. Second, this method can be applied to any reaction pathway in which DNA polymerization and exonucleolytic degradation are competing for the same DNA. After formulating and solving the corresponding steady-state equations, the distribution of the different DNA products will be predicted by the kinetic parameters governing the reaction pathway. Obviously, the more rate constants involved in the reaction, the more complex it will be to solve them separately. However, according to our experience, the affinity parameter (\( K_{\text{m}} \)) can always be obtained by plotting the ratio between appropriate DNA products versus dNTP concentration. Therefore, this method seems to be specially suitable for quickly determining true \( K_{\text{m}} \) values for correct or incorrect nucleotides in different DNA template contexts.

Using this approach, we have been able to calculate the true \( K_{\text{m}} \) values for both correct and incorrect nucleotides, in the case of the wild-type \( \phi 29 \) DNA polymerase. Using the synthetic DNA molecules described in this paper, dATP as the correct nucleotide and dCTP as the incorrect one, we have estimated a discrimination factor of \( 2.4 \times 10^2 \), based solely on nucleotide binding selection (\( K_{\text{m}}(\text{incorrect})/K_{\text{m}}(\text{correct}) \)). The global insertion fidelity of \( \phi 29 \) DNA polymerase, involving binding and catalysis, has been estimated between \( 10^3 \) and \( 10^5 \), depending on the mispair considered (Esteban et al., 1993). Although those values were calculated for a different template position, the comparison with the nucleotide binding discrimination factor reported here suggests that a significant contribution to the insertion discrimination ability of \( \phi 29 \) DNA polymerase comes from its nucleotide binding specificity. Discrimination factors based on the actual \( K_{\text{m}} \) for correct and incorrect nucleotides have been estimated for some other DNA polymerases. In the case of the Klenow fragment, T7 DNA polymerase, DNA polymerase \( \alpha \), and HIV-1 reverse transcriptase, the reported values were 1, that is, no discrimination (Eger et al., 1991), 450 (Wong et al., 1991), 3200 (Copeland et al., 1993), and 250 (Kati et al., 1992), respectively. Therefore, the nucleotide binding discrimination of \( \phi 29 \) DNA polymerase (2400) is one of the highest reported values.

Fidelity of \( \phi 29 \) DNA Polymerase Mutants—Using the kinetic analysis discussed above, we have been able to quantify the dNTP binding defect of mutants Y254F, Y390F, and Y390S of \( \phi 29 \) DNA polymerase. All these mutants were shown to have a reduced affinity for the correct nucleotide: 10-, 4.6-, and 14-fold, for mutants Y254F, Y390F, and Y390S, respectively.

Direct measurement of dNTP affinity of mutant DNA polymerases can not differentiate between direct effects, removal of a side chain that contacts the incoming dNTP, or indirect effects, via shaping the hydrophobic (or water-excluding) pocket that has to position the template-primer structure in the enzyme core. However, the considerable increase in \( K_{\text{m}} \) for incorrect nucleotide shown by mutants Y254F and Y390S lead us to propose that amino acid residues Tyr254 and Tyr390 are involved in direct contacts with the incoming dNTP.

Further support for this proposal came from the analysis of the nucleotide insertion fidelity of these \( \phi 29 \) DNA polymerase mutant derivatives. Mutants Y254F, Y390F, and Y390S showed a decreased affinity for the incorrect nucleotide, compared with the affinity of the wild-type protein. However, the relevant parameter for fidelity is the relation between the affinity for the correct and incorrect nucleotides. In the case of mutant Y390F, the discrimination value was \( 1.9 \times 10^3 \), showing a nucleotide selection ability quite similar to (or even higher than) that of the wild-type enzyme, whereas the values obtained for mutants Y254F and Y390S were \( 7.0 \times 10^3 \) and \( 2.9 \times 10^2 \), respectively, reducing the discrimination ability of \( \phi 29 \) DNA polymerase by factors of 3.5 and 8.3, respectively.

Taking into account the different phenotype of mutants Y390F and Y390S, it is tempting to speculate that the phenyl ring of Tyr390 is involved in hydrophobic interactions with the base moiety of the incoming nucleotide, playing a critical role in checking the correctness of base pairing. However, removal of the hydroxyl group of Tyr390 is not neutral for nucleotide binding. Mutation Y390F produced a 4-fold reduction in nucleotide
affinity for both correct and incorrect nucleotide. Therefore, this hydroxyl group seems to be important for general nucleotide binding, although it would not play a significant role in nucleotide selection.

The functional role of Tyr$^{254}$ in the nucleotide selection mechanism is more unclear, since removal of its hydroxyl group leads to a modest but significant reduction in the discrimination ability of the enzyme. One possibility is a direct role for the hydroxyl group in the interaction with the incoming nucleotide. Conversely, mutation Y$^{254}F$ could produce a local perturbation of the active center, reducing the stringency of base-pairing specificity. This mutant has also been shown to produce a drastic reduction in the fidelity of the protein-primed initiation reaction catalyzed by $\phi 29$ DNA polymerase$^{1}$ supporting the hypothesis that Tyr$^{254}$ is a key component in the nucleotide selection strategy.

Fidelity studies of DNA polymerase mutant derivatives have been reported recently (Carroll et al., 1991; Eger et al., 1991; Copeland et al., 1993; Dong et al., 1993a, 1993b; Donlin and Johnson, 1994). The results obtained with human DNA polymerase $\alpha$ are particularly relevant to our studies, because $\phi 29$ DNA polymerase belongs to the superfamily of $\alpha$-like DNA polymerases (Bernad et al., 1987; Wong et al., 1988; Blanco et al., 1991). Residue Tyr$^{865}$ of human DNA polymerase $\alpha$, the homolog to Tyr$^{254}$ in $\phi 29$ DNA polymerase, has been substituted by Phe or Ser, and the fidelity of the mutant derivatives has been addressed (Dong et al., 1993a). A 10-fold higher misinsertion frequency was observed for mutant Y$^{865}S$, whereas no fidelity deficiency was reported for mutant Y$^{865}F$. Based on these results, the authors proposed a role for the phenyl ring of Tyr$^{865}$ in nucleotide selection. However, it must be taken into account that, in the steady-state conditions of their analysis, the rate-limiting step for correct nucleotide incorporation is, very likely, DNA dissociation. In fact, the catalytic rate they reported for the wild-type enzyme is very low (1.4 s$^{-1}$), more comparable with a slow DNA dissociation step rather than with the fast catalytic rates calculated for other DNA polymerases (Kuchta et al., 1987; Patel et al., 1991; Kati et al., 1992). In addition, Dong et al. (1993b) also reported a 10-fold reduction in the affinity for the correct nucleotide of mutant Y$^{865}F$; however, this deficiency is surprisingly “compensated” with a 10-fold increase in its $V_{\text{max}}$ value, compared to the wild-type protein. One possible explanation for this striking behavior is based on a faster dissociation rate for mutant Y$^{865}F$, in agreement with its reduced processivity (as reported by the authors).

If DNA polymerase/DNA complex dissociation is rate-limiting in their steady-state analysis, this mutant would show both apparent $V_{\text{max}}$ and $K_m$ values higher than those of the wild-type enzyme, as they observed. The authors also presented a different assay that allows several consecutive incorporations per association event, with the DNA dissociation rate, therefore, being less critical. Using this assay, mutant Y$^{865}F$ showed a 6-fold reduction in the affinity for the correct nucleotide and a 3-fold reduction in catalytic rate. Taking into account that the affinity for the incorrect nucleotide was not affected by this mutation, these data would represent a reduction in its nucleotide binding discrimination ability of, at least, 6-fold. These results are in good agreement with the ones reported in this paper for mutant Y$^{254}F$ of $\phi 29$ DNA polymerase.

In summary, we have presented evidence showing that $\phi 29$ DNA polymerase residues Tyr$^{254}$ and Tyr$^{390}$ are involved in nucleotide binding selection, by means of the phenyl ring in the case of Tyr$^{390}$. Taking into account that these two amino acids are highly conserved in all the $\alpha$-like DNA polymerases, an evolutionary conservation of their functional role in DNA replication fidelity could be expected also. Further fidelity studies using different $\alpha$-like DNA polymerases would help to validate this hypothesis.

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APPENDIX

From Scheme I, the different DNA species can be obtained by derivation of the following differential equations.

First Nucleotide Incorporation Event

$$\frac{dD_i}{dt} = k_{\text{cat}} \cdot (D_0 \cdot N) + k_{\text{exo}} \cdot D_1 - k_1 \cdot D_0 \cdot N \quad (\text{Eq. A1})$$

$$\frac{d(D_0 \cdot N)}{dt} = k_1 \cdot D_0 \cdot N - (k_{1} + k_{\text{cat}}) \cdot (D_0 \cdot N) \quad (\text{Eq. A2})$$

$$\frac{dD_i}{dt} = k_{\text{cat}} \cdot (D_0 \cdot N) + k_{\text{exo}} \cdot D_2 - k_1 \cdot D_0 \cdot N \quad (\text{Eq. A3})$$

$$\frac{d(D_1 \cdot N)}{dt} = k_1 \cdot D_1 \cdot N - (k_{1} + k_{\text{cat}}) \cdot (D_1 \cdot N) \quad (\text{Eq. A4})$$

i-Nucleotide Incorporation Event

$$\frac{dD_{n-1}}{dt} = k_{\text{cat}} \cdot (D_{n-2} \cdot N) + k_{\text{exo}} \cdot D_n - k_1 \cdot D_{n-1} \cdot N \quad (\text{Eq. A5})$$

$$\frac{d(D_n \cdot N)}{dt} = k_1 \cdot D_{n-1} \cdot N - (k_{1} + k_{\text{cat}}) \cdot (D_n \cdot N) \quad (\text{Eq. A6})$$

Last Nucleotide Incorporation Event

$$\frac{dD_{n-1}}{dt} = k_{\text{cat}} \cdot (D_{n-2} \cdot N) + k_{\text{exo}} \cdot D_n - k_1 \cdot D_{n-1} \cdot N \quad (\text{Eq. A7})$$

$$\frac{d(D_n \cdot N)}{dt} = k_1 \cdot D_{n-1} \cdot N - (k_{1} + k_{\text{cat}}) \cdot (D_n \cdot N) \quad (\text{Eq. A8})$$

$$\frac{dD_n}{dt} = k_{\text{cat}} \cdot (D_{n-1} \cdot N) - k_{\text{exo}} \cdot D_n \quad (\text{Eq. A9})$$

Under steady-state conditions, the concentration of all the species present remain constant, and, therefore, all of the above equations are equal to 0. From Equations A5 and A6, since $K_m = (k_{\text{cat}} + k_{\text{exo}})$, the substitution leads to the following: $0 = k_1 \cdot D_{n-1} \cdot N - (k_{1} + D(n) \cdot N)$, then

$$D_n = (D_n \cdot N) \frac{K_m}{N} \quad (\text{Eq. A10})$$

This Equation A10 is valid for any value of $i$.

Now, all $D_i$ and $(D_i \cdot N)$ species can be expressed as a function of $D_n$ by solving the differential equations from bottom to top:

$$k_{\text{cat}} \cdot (D_{n-1} \cdot N) - k_{\text{exo}} \cdot D_n = 0 \quad (\text{Eq. A11})$$

$$(D_{n-1} \cdot N) = D_n \cdot \frac{k_{\text{exo}}}{k_{\text{cat}}} \quad (\text{Eq. A12})$$

and, equally

$$(D_{n-1} \cdot N) = D_n \cdot \frac{k_{\text{exo}}}{k_{\text{cat}}} \cdot \frac{K_m}{N} \quad (\text{Eq. A13})$$

$^1$J. Saturno, L. Blanco, M. Salas, and J. A. Esteban, unpublished results.
yield:

\[ D_{n-2} \cdot N + k_{-1} \cdot (D_{n-1} \cdot N) + k_{\text{exo}} \cdot D_n - k_{\text{exo}} \cdot D_{n-1} \]

\[ - k_1 \cdot D_{n-1} \cdot N = 0 \]  
(Eq. A14)

making the convenient substitutions:

\[ k_{\text{cat}} \cdot (D_{n-2} \cdot N) + k_{-1} \cdot D_n \cdot \frac{k_{\text{exo}}}{k_{\text{cat}}} + D_n - D_n \cdot \frac{k_{\text{exo}}}{k_{\text{cat}}} - \frac{k_m}{N} \]

\[ - k_1 \cdot D_{n-1} \cdot \frac{k_{\text{exo}}}{k_{\text{cat}}} - D_n \cdot \frac{k_m}{N} = 0 \]  
(Eq. A15)

\[ k_{\text{cat}} \cdot (D_{n-2} \cdot N) + k_{-1} \cdot D_n \cdot \frac{k_{\text{exo}}}{k_{\text{cat}}} + k_{\text{exo}} \cdot D_n - D_n \cdot \frac{k_{\text{exo}}}{k_{\text{cat}}} - \frac{k_m}{N} \]

\[ - k_1 \cdot D_{n-1} \cdot \frac{k_{\text{exo}}}{k_{\text{cat}}} - D_n \cdot \frac{k_m}{N} = 0 \]  
(Eq. A16)

\[ k_{\text{cat}} \cdot (D_{n-2} \cdot N) = D_n \cdot \frac{k_{\text{exo}}}{k_{\text{cat}}} \cdot \frac{k_m}{N} \]  
(Eq. A17)

These expressions can be summarized as follows:

\[ (D_{n-2} \cdot N) = D_n \cdot \frac{k_{\text{exo}}}{k_{\text{cat}}} \cdot \frac{k_m}{N} \]  
(Eq. A18)

\[ D_{n-2} = D_n \cdot \frac{k_{\text{exo}}}{k_{\text{cat}}} \cdot \frac{k_m}{N^2} \]  
(Eq. A19)

In general, we can conclude that:

\[ (D_i \cdot N) = D_n \cdot \left( \frac{k_{\text{exo}}}{k_{\text{cat}}} \right)^{n-i} \cdot \left( \frac{k_m}{N} \right)^{n-i-1} \]  
(Eq. A20)

\[ D_i = D_n \cdot \left( \frac{k_{\text{exo}}}{k_{\text{cat}}} \right)^{n-i} \cdot \left( \frac{k_m}{N} \right)^{n-i} \]  
(Eq. A21)

These are valid for any value of \( i \), from \( i = 0 \) to \( i = n - 1 \).

To calculate the value of \( D_n \) as a function of \( D_T \) (total DNA):

\[ D_T = \sum_{i=0}^{n-1} \left[ (D_i \cdot N) + D_h \cdot \frac{k_{\text{exo}}}{k_{\text{cat}}} \cdot \left( \frac{k_m}{N} \right)^{n-i-1} \right] + D_n = D_n \cdot \left( 1 + \left( \frac{k_m}{N} \right)^{n-1} \sum_{i=0}^{n-1} \left( \frac{k_{\text{exo}}}{k_{\text{cat}}} \right)^{n-i} \cdot \left( \frac{k_m}{N} \right)^{n-1-i} \right) \]

\[ + D_n \cdot \left( 1 + \left( \frac{k_m}{N} \right)^{n-i} \sum_{i=0}^{n-i-1} \left( \frac{k_{\text{exo}}}{k_{\text{cat}}} \right)^{n-i} \cdot \left( \frac{k_m}{N} \right)^{n-1-i} \right) + D_n = D_n \cdot \left( 1 + \left( \frac{k_m}{N} \right)^{n-1} \sum_{i=0}^{n-1} \left( \frac{k_{\text{exo}}}{k_{\text{cat}}} \right)^{n-i} \cdot \left( \frac{k_m}{N} \right)^{n-1-i} \right) \]

\[ + \left( 1 + \left( \frac{k_m}{N} \right)^{n-1} \sum_{i=0}^{n-1} \left( \frac{k_{\text{exo}}}{k_{\text{cat}}} \right)^{n-i} \cdot \left( \frac{k_m}{N} \right)^{n-1-i} \right) \]

then

\[ D_n = \frac{D_T}{1 + \left( \frac{k_m}{N} \right)^{n-1} \sum_{i=0}^{n-1} \left( \frac{k_{\text{exo}}}{k_{\text{cat}}} \right)^{n-i} \cdot \left( \frac{k_m}{N} \right)^{n-1-i}} \]

(Eq. A22)

This value can be substituted in Equations A20 and A21 to yield:

As can be seen, these equations are also valid for \( D_n \).
