Efficiency of Correct Nucleotide Insertion Governs DNA Polymerase Fidelity*

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DNA polymerase fidelity or specificity expresses the ability of a polymerase to select a correct nucleoside triphosphate (dNTP) from a pool of structurally similar molecules. Fidelity is quantified from the ratio of specificity constants (catalytic efficiencies) for alternate substrates (i.e. correct and incorrect dNTPs). An analysis of the efficiency of dNTP (correct and incorrect) insertion for a low fidelity mutant of DNA polymerase β (R283A) and exonuclease-deficient DNA polymerases from five families derived from a variety of biological sources reveals that a strong correlation exists between the ability to synthesize DNA and the probability that the polymerase will make a mistake (i.e. base substitution error). Unexpectedly, this analysis indicates that the difference between low and high fidelity DNA polymerases is related to the efficiency of correct, but not incorrect, nucleotide insertion. In contrast to the loss of fidelity observed with the catalytically inefficient R283A mutant, the fidelity of another inefficient mutant (G274P) is not altered. Thus, although all natural low fidelity DNA polymerases are inefficient, not every inefficient DNA polymerase has low fidelity. Low fidelity polymerases appear to be an evolutionary solution to how to replicate damaged DNA or DNA repair intermediates without burdening the genome with excessive polymerase-initiated errors.

The equilibrium between genome stability and instability is tightly regulated since mutations are central to aging, disease, and evolution. Thus, cellular strategies that modulate this equilibrium are of general and immense interest. The structure of DNA was proposed nearly 50 years ago and provided the first clue to how “genetic material” could be replicated faithfully (1). It is now recognized that DNA polymerases play pivotal roles in both genome replication and maintenance (i.e. DNA repair). Polymerases copy the parental (template) strand to generate a new or repaired complementary daughter strand, and accurate DNA synthesis during replication and repair is essential in maintaining genomic integrity. Although DNA polymerases play a central role in these essential processes, the fundamental mechanism(s) by which they select the correct deoxynucleoside 5’-triphosphate (dNTP) from a pool of structurally similar molecules to accomplish efficient and faithful polymerization is poorly understood. The intrinsic base substitution error frequency for DNA replication and repair polymerases is generally between 10⁻³ and 10⁻⁶ (2). These frequencies represent one error per thousand or million nucleotides synthesized, respectively. These levels of discrimination are far greater than predicted by free energy differences between matched and mismatched DNA termini (predicted error frequency of ~0.4; one error per 3 nucleotides synthesized), indicating that DNA polymerases can enhance fidelity by a large factor (3). However, even this remarkable specificity is inadequate to faithfully replicate a genome of more than 10⁹ nucleotides. Thus, replicative DNA polymerases often have an intrinsic proofreading exonuclease to remove misinserted nucleotides, and cells possess a postreplication DNA mismatch repair pathway that can correct misinserted nucleotides that escape proofreading.

DNA polymerase (pol) fidelity, specificity, or discrimination represent relative kinetic terms used to describe the propensity of a polymerase to produce a base substitution error. Polymerase specificity may be quantified in vitro by measuring the insertion kinetics of a single nucleotide (correct or incorrect) opposite a defined templating base. The absolute rate or probability that a pol inserts a correct or incorrect nucleotide follows Michaelis-Menten kinetics. A steady-state kinetic approach defines substrate specificity as catalytic efficiency, k_cat/K_m,dNTP, for formation of a specific base pair. Substrate specificity determined by a pre-steady-state kinetic approach is k_cat/K_m, where K_m is the equilibrium dissociation constant for the incoming dNTP and k_cat is the kinetic step that limits insertion of the first nucleotide. This step may be either chemistry (i.e. nucleotidyl transfer) and/or conformational transitions of the ternary polymerase/substrate complex. Although these two kinetic approaches may measure different steps along the catalytic pathway, they measure the same specificity constant (i.e. k_cat/K_m = k_cat/K_m) (4). DNA polymerase specificity is typically quantified by comparing the ratio of catalytic efficiencies for correct and incorrect nucleotide insertion and typically expressed as relative misinsertion efficiency, f_ins = (k_cat/K_m,correct)/(k_cat/K_m,incorrect), or fidelity (1/f_ins) (5, 6).

Since fidelity represents a ratio of catalytic efficiencies for correct and incorrect nucleotide insertion, differences in fidelity can be due to changes in one or both specificity constants. We had noted previously that a mutant of pol β had low fidelity and catalytic efficiency for correct insertion (7). By examining the catalytic efficiencies for correct and incorrect nucleotide insertion for wild-type and mutant forms of pol β and comparing them with those reported for other polymerases exhibiting divergent fidelities, we find that the fidelity of natural DNA polymerases is determined primarily by the efficiency for correct nucleotide insertion.

EXPERIMENTAL PROCEDURES

Protein Purification—Wild-type human pol β and mutant derivatives (R283A and G274P) were expressed and purified as described previ
Enzyme concentrations were determined by Coomassie dye binding using purified pol β as the standard. The concentration of purified pol β was determined by total amino acid analysis.

**DNA Preparation**—A 34-mer oligonucleotide DNA substrate containing a single nucleotide gap was prepared by annealing 3 gel-purified oligonucleotides (Oligos Etc., Wilsonville, OR) to create a single-nucleotide gap at position 16. The sequence of the gapped DNA substrate was: primer, 5'-CTGAGTTGATCCG-G-3'; downstream oligonucleotide, 5'-GTACGGATCCCCGGGTAC-3'; and template, 3'-AGGGCGGATCCATCGGGGCGAT-5'. The resultant single-nucleotide gapped DNA has a templating G in the gap. The oligonucleotides were quantified, resuspended in a buffer, and annealed in a sharp bend between two α-helices. Gly-274 interacts with the sugar of the incoming nucleotide. The van der Waals surface of Gly-274 and the template, 3'-CGGGCGGATCCATCGGGGCGAT-5'.

**RESULTS AND DISCUSSION**

**Exhibiting Low Fidelity**—Ratio or random mutagenesis of the pol active site generally results in mutant enzymes that have moderately reduced or improved specificity. However, alanine substitution for an arginine residue of pol β (R283A, Fig. 1), suggested to stabilize the templating base, results in a dramatic loss of catalytic efficiency for correct insertion and fidelity, implying that catalytic efficiency for correct nucleotide insertion and discrimination were coupled (7). Since those measurements were performed on dissimilar DNA substrates, we reexamined the catalytic efficiency for correct insertion and fidelity on the same single-nucleotide gapped DNA substrate, a substrate prepared by pol β in base excision repair (8). A steady-state kinetic analysis of the R283A mutant of human pol β indicates that there is a 33,100-fold loss in catalytic efficiency for insertion of dCTP opposite a templating guanine within a single-nucleotide gapped DNA substrate relative to the wild-type enzyme. In contrast, the mutant enzyme inserts dTTP opposite guanine 15-fold less efficiently than wild-type enzyme. Since fidelity or relative misinsertion efficiency are relative ratios of catalytic efficiencies, the mutant enzyme has a 2,400-fold lower ability to discriminate against dTTP insertion relative to dCTP (Fig. 2A).

**Relative misinsertion efficiency for formation of the dG-dTTP mispair as a function of catalytic efficiency for nucleotide insertion.** The reported catalytic efficiencies for dCTP or dTTP insertion opposite guanine were analyzed for exonuclease-deficient pols that span five pol families (Table I): α, A-family; β, B-family; γ, RT-family; δ, X-family; ε, Y-family. The DNA polymerases included are T7 pol (T7), Klenow fragment (Kf), pol ξ (ξ), RB69 pol (RB69), pol X (X), rat and human pol β (rβ and hβ, respectively) on non-gapped or gapped (g) DNA, R283A mutant of pol β (R283A), pol η (η), pol ε (ε), and pol κ (κ). For some of these polymerases, several reported catalytic efficiencies are given. These represent alternate determinations from independent laboratories. The dotted line (f_{ins} = 0.6) represents the calculated free energy difference determined for matched and mismatched terminal base pairs (∆ΔG ~0.3 kcal/mol at 37 °C) (3). As shown in A, the efficiencies for correct nucleotide insertion (θ_{ins}/K_{act}) span 5 orders of magnitude for the DNA polymerases surveyed with a corresponding decrease in the relative misinsertion efficiencies (f_{ins}). As shown in B, in contrast, the efficiencies for dTTP insertion occurred over a much narrower range for most polymerases and do not correlate with fidelity in a systematic way.
This loss in discrimination is entirely due to the loss of the ability to insert the correct nucleotide, dCTP, since dTTP insertion efficiency is reduced in the mutant enzyme.

**Low Fidelity DNA Polymerases Are Inefficient**—To determine whether the loss of catalytic efficiency for the correct nucleotide generally leads to a loss in discrimination, as observed with the R283A mutant of pol β, we analyzed the reported insertion kinetics for 12 exonuclease-deficient DNA polymerases from a variety of biological sources that span five pol families (Table I). Replicative and repair DNA polymerases have generally been reported to insert the correct nucleotide with a catalytic efficiency of about 1–10 μM⁻¹ s⁻¹ with corresponding relative misinsertion efficiencies of 10⁻³–10⁻⁶ for a G-T (template base-incoming nucleotide) base pair (Fig. 2A). Since DNA polymerases can typically misinsert three common nucleotides opposite a templating guanine, albeit with low efficiency, the data points for these mispairs are spread vertically (Fig. 3).

More recently, a new family of DNA polymerases (i.e. Y-family, which includes pols η, ι, and κ) has been described (9). These polymerases lack an intrinsic proofreading exonuclease, exhibit low processivity, replicate DNA with low fidelity, and are believed to assist replication complexes stalled at DNA lesions (for a recent review, see Ref. 10). A survey of the catalytic efficiencies for nucleotide insertion by members of this family indicates that they generally insert dCTP opposite a templating guanine with low efficiency (<0.2 μM⁻¹ s⁻¹). DNA polymerase η inserts dCTP with an efficiency of about 10⁻¹ to 10⁻² μM⁻¹ s⁻¹ (11, 12), whereas the efficiency for pol ι for this base pair is about 10⁻³ μM⁻¹ s⁻¹ (13, 15). Thus, the efficiencies for insertion of dCTP opposite guanine can vary 5 orders of magnitude depending on the identity of the pol (Fig. 2A). As observed for the R283A mutant of pol β, discrimination is coupled to the catalytic efficiency for correct insertion, but not for incorrect insertion (e.g. dTTP insertion opposite guanine; Fig. 2B). The loss in the ability to insert the correct nucleotide results in the loss of discrimination (i.e. higher f_mis).

The trend in this analysis suggests that if the catalytic efficiency for correct insertion was reduced further, insertion of
Inefficient Mutant of Pol β Exhibiting High Fidelity—As a first approximation, the correlation between correct nucleotide insertion efficiency and fidelity suggests that if the catalytic efficiency of a mutant Pol were greatly diminished, then it may also exhibit a correspondingly low fidelity (e.g. R283A mutant of Pol β). Traditionally, site-directed mutagenesis of the Pol active site (e.g. metal-coordinating carboxy...
lates) of the A, B, X, and RT families results in a 100–1000-fold loss in catalytic efficiency (22–25). Due to this considerable loss of catalytic efficiency, fidelity was not assessed with these “inactive” mutant enzymes. During a steady-state kinetic-screen of site-directed mutants of pol β, a G274P mutant exhibited a 102-fold decrease in catalytic efficiency relative to wild-type enzyme. A rare cis-peptide bond is observed between Gly-274 and Ser-275 that creates a sharp turn between two α-helices that contribute significant interactions with the nascent base pair. Gly-274 interacts with the sugar of the incoming nucleotide (Fig. 1). Proline substitution for Gly-274 would be expected to sterically clash with the incoming nucleotide and alter helix interactions with the nascent base pair. The enormous loss of catalytic efficiency is consistent with this proposal. To discount the possibility that the mutant enzyme did not fold properly, resulting in a large fraction of inactive protein, we performed a single-turnover analysis (pol Δ DNA substrate) to directly address the rate of insertion into a single-nucleotide gapped DNA substrate with a templating guanine. Under these assay conditions, the rate of insertion was nearly identical to that determined when substrate concentration is in excess (i.e. steady-state assay), indicating that the poor efficiency was intrinsic to the mutant enzyme (data not shown). To address whether there was a corresponding decrease in fidelity, we attempted to measure dTTP insertion efficiency opposite the templating guanine. However, misinsertion efficiency was very weak, precluding an accurate determination of the templating guanine. However, misinsertion efficiency was intrinsic to the mutant enzyme (data not shown). To determine when substrate concentration is in excess (i.e. steady-state assay), indicating that the poor efficiency was intrinsic to the mutant enzyme (data not shown).

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