

Communication

Enzyme-DNA Interactions Required for Efficient Nucleotide Incorporation and Discrimination in Human DNA Polymerase β*

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William A. Beardt, Wendy P. Osheroffs, Rajendra Prasad, Michael R. Sawaya†, Madhuri Jaju, Thomas G. Woold, Joseph Kraut†, Thomas A. Kunkel‡, and Samuel H. Wilson¶

From the Sealy Center for Molecular Science, University of Texas Medical Branch, Galveston, Texas 77555-1068, the Laboratory of Molecular Genetics, NIEHS, Research Triangle Park, North Carolina 27709, and the Department of Chemistry, University of California, San Diego, California 92039-0317

In the crystal structure of a substrate complex, the side chains of residues Asn279, Tyr271, and Arg283 of DNA polymerase β are within hydrogen bonding distance to the bases of the incoming deoxynucleoside 5'-triphosphate (dNTP), the terminal primer nucleotide, and the templating nucleotide, respectively (Pelletier, H., Sawaya, M. R., Kumar, A., Wilson, S. H., and Kraut, J. (1994) Science 264, 1891-1903). We have altered these side chains through individual site-directed mutagenesis. Each mutant protein was expressed in Escherichia coli and was soluble. The mutant enzymes were purified and characterized to probe their role in nucleotide discrimination and catalysis.

A reversion assay was developed on a short (5 nucleotide) gapped DNA substrate containing an opal codon to assess the effect of the amino acid substitutions on fidelity. Substitution of the tyrosine at position 271 with phenylalanine or histidine did not influence catalytic efficiency (kcat/ Km) or fidelity. The hydrogen bonding potential between the side chain of Asn279 and the incoming nucleotide was removed by replacing this residue with alanine or leucine. Although catalytic efficiency was reduced as much as 17-fold for these mutants, fidelity was not. In contrast, both catalytic efficiency and fidelity decreased dramatically for all mutants of Arg283 (Ala > Leu > Lys). The fidelity and catalytic efficiency of the alanine mutant of Arg283 decreased 160- and 5000-fold, respectively, relative to wild-type enzyme. Sequence analyses of the mutant DNA resulting from short gap-filling synthesis indicated that the types of base substitution errors produced by the wild-type and R283A mutant were similar and indicated misincorporations resulting in frequent T,dGTP and A,dGTP mispairing. With R283A, a dGMP was incorporated opposite a template thymidine as often as the correct nucleotide. The x-ray crystallographic structure of the alanine mutant of Arg283 verified the loss of the mutated side chain. Our results indicate that specific interactions between DNA polymerase β and the template base, but not hydrogen bonding to the incoming dNTP or terminal primer nucleotide, are required for both high catalytic efficiency and nucleotide discrimination.

Accurate DNA synthesis during replication and DNA repair is crucial in maintaining genomic integrity. Although DNA polymerases play a central role in these essential processes, the fundamental mechanism by which they select the correct deoxynucleoside 5'-triphosphate (dNTP) from a pool of structurally similar compounds and substrates to accomplish rapid and efficient polymerization is poorly understood. Vertebrate DNA polymerase β (β-pol) has been suggested to play a role in both DNA repair (1–5) and replication (6–8). The x-ray crystal structures of rat and human β-pol in complex with substrates have suggested a detailed model of the chemical mechanism for the nucleotidyl transfer reaction and also have suggested several protein/substrate interactions that may play a role in nucleotide discrimination (9–12). Additionally, these structures allow us to experimentally test model-derived predictions about the role(s) of individual amino acids.

DNA and RNA polymerases, for which the structure has been determined, have been described by analogy to the anatomical features of a hand as consisting of fingers, palm, and thumb subdomains (13). Conserved carboxylates, which bind catalytically essential divalent metal ions, are found in the palm subdomains of these polymerases. The dNTP binding site of β-pol is formed by the DNA template base, the 3'-terminal nucleotide of the primer strand, and the palm and thumb subdomains of the polymerase (10). Only three amino acid residues of the thumb subdomain have side chains that are within hydrogen bonding distance to the nucleotide bases within this binding pocket. These hydrogen bond donors are indiscernible in that they bond to the O2 of pyrimidines or the N3 of purines in the DNA minor groove (14). The structure of the β-pol ternary complex reveals a single hydrogen bond between the base of the incoming ddCTP and Asn775; Tyr271 and Arg283 are also within hydrogen bonding distance to the O2 and N3 atoms of the terminal primer and templating base, respectively (Fig. 1). To assess the role of these interactions in nucleotide selection and incorporation, we replaced Tyr271, Asn775, and Arg283 with alternate residues by site-directed mutagenesis to remove and/or alter each interaction.

EXPERIMENTAL PROCEDURES

Materials—Poly(dA),p(dT)10, and dNTPs were from Pharmacia Biotech Inc. [α-32P]dTTP (3000 Ci/mmol) was from DuPont NEN and T4 DNA ligase was purchased from New England Biolabs.

Mutagenesis of the Human β-Pol Gene—Oligonucleotide site-directed mutagenesis was performed using a procedure described previously (15). M13 phage containing the human β-pol target DNA was propagated using the bacterial host Cj 236 (dut- ung-) and phage DNA purified for use as template. Synthetic oligonucleotide primers containing the desired codon change were annealed to the template DNA and the

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† To whom correspondence should be addressed.

‡ Sealy Center for Molecular Science, University of Texas at Austin, 77555-1068, The University of Texas at Austin, Austin, Texas 78712.

§ Laboratory of Molecular Genetics, NIEHS, Research Triangle Park, North Carolina 27709.

¶ Department of Chemistry, University of California, San Diego, California 92039-0317.

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Whatman DE-81 filter disks and dried. Unincorporated NaCl, 10mM MgCl₂, 5% glycerol, 1mM ATP, 150ng (32fmol) of gapped DNA. The following mutations were introduced into the M13 primers extended with Sequenase Version 2.0 (U. S. Biochemical Corp.). The following mutations were introduced into the M13 primer (5.5 mg/ml in 20 mM MgSO₄) at room temperature. ddTTP was added in 40-fold molar excess to the enzyme-DNA complex. The template and primer sequences are 5'-CAAACTCACAT-3' and 5'-TGATGGTACG-3', respectively. Crystals of the complex were grown at room temperature from siting drops prepared by mixing 5mM of protein-DNA complex with 5mM of reservoir containing 13% polyethylene glycol 3350, 180mM NaCl, 50mM cacodylate, pH 6.5. Crystals appeared within a week. Macroseeding was employed to enlarge crystal size. Crystals belong to space group P2₁2₁2₁ (a = 100.6, b = 57.5, c = 47.9 Å) and are isomorphous with crystals obtained previously (11). The crystals grew to 0.6 × 0.3 × 0.2 mm and diffracted to 3.3 Å. Using a rotating anode x-ray generator equipped with Xuong-Hamlin multiwire area detectors (20), 43,435 observations of 8411 unique reflections were collected with 97% completeness to 3.3 Å.

RESULTS AND DISCUSSION

Expression constructs of human β-pol were prepared: Tyr²⁷¹ was replaced with either histidine or phenylalanine, Asn²⁷⁹ was replaced with either alanine or leucine, and Arg²⁸³ was replaced with either alanine, leucine, or lysine. Each altered human β-pol gene was expressed in E. coli and the recombinant enzymes were soluble in the crude cell extracts. Following purification, SDS-PAGE analysis indicated that the mutant β-pol polypeptides had the same apparent molecular weight as the wild-type enzyme and were greater than 99% homogeneous (data not shown).

To compare the catalytic efficiency of the wild-type and mutant enzymes, the steady-state kinetics on a simple T-P system, pol(dA)₃-(dT)₁₀ with ddTTP as the incoming nucleotide, were analyzed (Fig. 2). Whereas the catalytic activity of the Tyr²⁷¹ and Asn²⁷⁹ mutants were not significantly influenced (i.e. <10-fold), k₉₅ of the lysine, alanine, and leucine mutants of Arg²⁸³ were decreased greater than 20-, 150-, and 600-fold, respectively (Fig. 2A). In contrast, the K₉₅ for T-P was increased with the R283L mutant (17-fold), and the K₉₅ for ddTTP was increased to the greatest extent with the R283A mutant (29-fold) (Fig. 2A). Catalytic efficiency, as measured by the ratio of k₉₅ and K₉₅-ddTTP, was not influenced by the histidine substitution at Tyr²⁷¹ while the phenylalanine mutant displayed a modest...
(2-fold) decrease (Fig. 2B). Since the phenylalanine substitution had only a small effect on catalytic efficiency, substrate interactions with Tyr<sup>271</sup> appears to offer very little transition state stabilization. Elimination of the hydrogen bond between the incoming dNTP and the Asn<sup>279</sup> side chain with an alanine or leucine substitution decreased catalytic efficiency further, but again only modestly (~10-fold). In this case, catalytic efficiency was dependent solely on the apparent dNTP binding affinity<sup>2</sup> since <i>k<sub>cat</sub></i> of each mutant was similar to wild-type enzyme. The most dramatic decrease in catalytic efficiency was observed for the mutants of Arg<sup>283</sup> (Ala > Leu > Lys). A 5000-fold decrease in efficiency was observed for the alanine mutant, whereas catalytic efficiency of the lysine mutant, which could potentially hydroxyn bond to the template base, was decreased over 100-fold.

In vivo, β-pol is involved in short gap DNA repair (1–3, 5). DNA polymerase β is an ideal polymerase to examine “intrinsic” base substitution fidelity, because it lacks an associated 3’ – 5’ proofreading exonuclease. In vitro, pol-β fills these short gaps (<6 nucleotides) processively, whereas longer gaps are filled distributively (21). The fidelity of β-pol-dependent long gap DNA synthesis (i.e. >100 nucleotides) had previously been examined on undamaged (22, 23) and damaged DNA templates (24, 25). To determine the fidelity of wild-type β-pol on a physiologically relevant DNA substrate and to assess the effect of the amino acid substitutions on fidelity, a reversion assay was developed on a short (5 nucleotide) gapped DNA substrate containing an opal codon (Fig. 3A). This codon is within the non-essential lacZα gene of bacteriophage M13mp2. Polymerase errors that restore α-complementation activity yield a blue or light blue plaque phenotype. This assay can detect eight different base substitution errors.

The result of an in vitro gap filling synthesis by wild-type β-pol and the mutants described above on the reversion of the opal codon is shown in Fig. 3B. Wild-type β-pol produced one revertant per 370 filled gaps (reversion frequency of 2.7 × 10<sup>-4</sup>). Whereas deletion of the hydrogen bond donor at Tyr<sup>271</sup> did not alter the reversion frequency, alanine substitution at Asn<sup>279</sup> significantly reduced it signaling an apparent increase in fidelity. This apparent increase in fidelity could reflect a reduced misinversion rate or a reduced ability to extend mispairs, since both must occur to score a mutant. In contrast, alteration of the Arg<sup>283</sup> side chain, which interacts with the templating base, dramatically lowered fidelity, as demonstrated by the strong increases in reversion frequency (Fig. 3B).

Sequence analyses of the DNA of lacZα revertants resulting from short gap filling synthesis indicated that the types of base substitution errors produced by the wild-type and R283A mutant were similar (Table I). However, the frequency of each type of error was much greater for the R283A mutant. The base substitution errors observed in the polymerization products of both enzymes reflected misincorporations resulting in relatively frequent T-dGTP and A-dGTP mispairs. Seven of the eight mispairs detected by this reversion assay were observed in the products of wild-type enzyme and the strong mutator mutant R283A. For the mutant polymerase, a dGMP was incorporated opposite a template thymidine nearly 46% of the time, whereas the correct nucleotide was incorporated only 48% of the time. Additionally, sequence analysis often detected two misincorporations by both wild-type and R283A polymerases within the 5-nucleotide gap. These misincorporations were, in many instances, consecutive, and in one case, three consecutive misincorporations were observed. Consecutive misincorporations had not been observed previously in the forward mutation assay employing a long single-stranded template (22, 23). This suggests that a difference may exist between the fidelity of β-pol during short processive gap filling as compared

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**Table I**

<table>
<thead>
<tr>
<th>Template nucleotide</th>
<th>Mismatch (template dNTP)</th>
<th>Revertant nucleotide</th>
<th>Errors per detectable nucleotide&lt;sup&gt;a&lt;/sup&gt;</th>
<th>-Fold increase in errors relative to wild-type&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Wild-type β-pol revertants&lt;sup&gt;a&lt;/sup&gt;</td>
<td>R283A β-pol revertants&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>T</td>
<td>T-dGTP</td>
<td>C</td>
<td>2.9 × 10&lt;sup&gt;-3&lt;/sup&gt; (32)</td>
<td>460 × 10&lt;sup&gt;-3&lt;/sup&gt; (61)</td>
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<tr>
<td>T</td>
<td>T-dCTP</td>
<td>G</td>
<td>0.2 × 10&lt;sup&gt;-3&lt;/sup&gt; (2)</td>
<td>30 × 10&lt;sup&gt;-3&lt;/sup&gt; (4)</td>
</tr>
<tr>
<td>T</td>
<td>T-dTTP</td>
<td>A</td>
<td>≤0.1 × 10&lt;sup&gt;-3&lt;/sup&gt; (0)</td>
<td>30 × 10&lt;sup&gt;-3&lt;/sup&gt; (4)</td>
</tr>
<tr>
<td>G</td>
<td>G-dATTP</td>
<td>T</td>
<td>0.2 × 10&lt;sup&gt;-3&lt;/sup&gt; (2)</td>
<td>15 × 10&lt;sup&gt;-3&lt;/sup&gt; (2)</td>
</tr>
<tr>
<td>A</td>
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<td>C</td>
<td>0.2 × 10&lt;sup&gt;-3&lt;/sup&gt; (2)</td>
<td>≤7 × 10&lt;sup&gt;-3&lt;/sup&gt; (0)</td>
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<tr>
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<td>A-dCTP</td>
<td>G</td>
<td>0.3 × 10&lt;sup&gt;-3&lt;/sup&gt; (3)</td>
<td>60 × 10&lt;sup&gt;-3&lt;/sup&gt; (8)</td>
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<td>A</td>
<td>A-dTTP</td>
<td>A</td>
<td>0.6 × 10&lt;sup&gt;-3&lt;/sup&gt; (7)</td>
<td>110 × 10&lt;sup&gt;-3&lt;/sup&gt; (15)</td>
</tr>
<tr>
<td>A</td>
<td>A-dATP</td>
<td>T</td>
<td>0.1 × 10&lt;sup&gt;-3&lt;/sup&gt; (1)</td>
<td>30 × 10&lt;sup&gt;-3&lt;/sup&gt; (4)</td>
</tr>
</tbody>
</table>

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<sup>a</sup>The mutant fraction for wild-type and R283A β-pol DNA synthesis reactions was 27 × 10<sup>-4</sup> and 4400 × 10<sup>-4</sup>, respectively.

<sup>b</sup>Numbers in parentheses indicate number of base substitution errors observed.
The fingers and thumb subdomains are structurally diverse among the different classes of polymerases, and except for \( \beta \)-pol, the dNTP binding site is not clearly defined. Therefore, the functional role of each subdomain may be unique to each class of polymerase, and care must be taken in extrapolating the present results to the thumb subdomain of other DNA polymerases (27).

In summary, fidelity assays coupled with kinetic and structural evaluation of the alanine mutant of Arg283 indicate that this residue plays a central role in nucleotide discrimination by correctly positioning and stabilizing the templating base for efficient nucleotide incorporation. Although the guanidinium group of Arg283 is within hydrogen bonding distance to N3 of the template guanine, the hydrogen bond geometry is unfavorable. Therefore, correct van der Waal’s interactions may also be important at this site. This is consistent with the low catalytic efficiency and reduced fidelity exhibited by the lysine mutant of Arg283 which would be expected to preserve hydrogen bonding to the templating base. Our results support the hypothesis that discrimination and catalytic efficiency are modulated by polymerase interactions near the templating base and are sensitive to precise Watson-Crick base pairing by possibly “sensing” C1’ distances and bond angle geometry (28, 29). In contrast, alteration of direct interactions with the incoming dNTP decreased dNTP binding affinity but not fidelity. Thus, the coupling between catalytic efficiency and discrimination is residue-specific. Our results indicate that we can modulate discrimination and catalytic efficiency based upon ternary complex crystal structures, and site-directed mutagenesis will be a productive avenue for future analysis of polymerase structure-function relationships.

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REFERENCES