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

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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 deoxy nucleoside 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 assess the role of the amino acid substitutions on fidelity. A reversion assay was developed on a short (5 nucleotide) probe their role in nucleotide discrimination and catalytic efficiency (tyrosine.Substitution of the tyrosine at position 271 with phenylalanine did 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 (Fig. 1). To assess the role of these interactions in nucleotide selection and incorporation, we replaced Tyr271, Asn279, and Arg283 with alternate residues by site-directed mutagenesis to remove and/or alter each interaction.

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 deoxy nucleoside 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 deoxynucleotide 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 indiscriminate 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 Asn279; 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, Asn279, and Arg283 with alternate residues by site-directed mutagenesis to remove and/or alter each interaction.

EXPERIMENTAL PROCEDURES

Materials—Poly(dA), d(dT)10, and dNTPs were from Pharmacia Biotech Inc. [α32P]dATP (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 Cj236 (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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Mutagenesis of Pol-β dNTP Binding Pocket to Probe Fidelity

**Fig. 1. Ribbon drawing of the dNTP binding pocket of rat DNA polymerase β.** The side chains of residues Asn729, Tyr271, and Arg833 of DNA polymerase β are within hydrogen bonding distance (dashed lines) to the bases of the incoming dideoxynucleoside triphosphate (ddCTP), the terminal primer nucleotide, and the templating nucleotide, respectively (10). These residues can hydrogen bond indiscriminately to the O2 of pyrimidines or the N3 of purines in the DNA minor groove. They are part of two α-helices, M and N, which are interrupted by a cistepide bond between residues Gly322-$$^{\text{Ser}}$$-324. The observed distances from the side chain hydrogen bond donor and O2 or N3 are 2.7, 3.0, and 3.2 Å for Tyr271, Asn729, and Arg833, respectively (10). Also indicated are active site carboxylate side chains (Asp190, Asp192, Asp250) which coordinate two Mg$$^{2+}$$ ions in the palm subdomain. This figure was made with MOLSCRIPT (30).

**Fig. 2. Steady-state kinetic parameters for wild-type and mutant β-pol.** Assays were performed as described under “Experimental Procedures.” The substrate concentrations were varied from at least 0.3 to 3 × $K_{\text{m}}$ under saturating concentrations of the other (i.e. > 4 × $K_{\text{m}}$). Initial velocities were fitted to the Michaelis equation by nonlinear least squares methods. The results represent the mean and S.E. of at least two independent determinations. A, the $k_{\text{cat}}$, $K_{\text{m}}$, and $K_{\text{dDTTP}}$ values for the mutant enzymes relative to wild-type are presented in the left, center, and right panels, respectively. The corresponding values for $k_{\text{cat}}$, $K_{\text{m,TP}}$, and $K_{\text{dDTTP}}$ with wild-type enzyme are 0.8 ± 0.1 s$$^{-1}$, 120 ± 30 nm, and 6.6 ± 1.3 μM, respectively. B, the catalytic efficiency ($k_{\text{cat}}/K_{\text{dDTTP}}$) relative to wild-type enzyme is presented in order of decreasing efficiency. The corresponding value for $k_{\text{cat}}/K_{\text{dDTTP}}$ with wild-type enzyme is 8.8 ± 1.6 × 10$$^{4}$$ M$$^{-1}$ s$$^{-1}$.

Primers extended with Sequenase Version 2.0 (U. S. Biochemical Corp.). The following mutations were introduced into the M13 β-pol vector, 5′ to 3′: Y271F (TAT to TTT), Y271H (TAT to CAC), N279A (AAT to CTG), R283A (AGG to GCG), R283K (AGG to GCG), R283L; 250:1 for R283A. for wild-type, N279A, N279L; 100:1 for Y271H, Y271F; 200:1 for R283K, R283L; 250:1 for R283A.

**Expression Constructs**—Wild-type and mutant enzymes were purified as described previously (16). All enzyme preparations were assayed for containing 3′ → 5′ exo/nuclease activity on a mismatched primer and had at least 10-fold lower exo/nuclease activity relative to Klenow fragment (17).

β-Pol Polymerization Assays—Enzyme activities were determined using a standard reaction mixture (50 μl) containing 5 mM Tris-HCl, pH 7.4 (22°C), 5 mM MnCl2, and 100 mM KCl. Other reaction conditions are described in the figure legends. Reactions were initiated by addition of enzyme, incubated at 22°C, and stopped by the addition of 20 μl of 0.5 M EDTA, pH 8. Quenched reaction mixtures were spotted onto Whatman DE-81 filter disks and dried. Unincorporated [α-32P]dCTP was removed, and filters were counted as described (18).

Short Gap Fidelity Assay—A gapped DNA substrate was constructed in which the single-stranded gap contains the 5′-3′ exonuclease activity on a mismatched primer and had at least 10-fold lower exonuclease activity relative to T7 DNA polymerase (17).

FIG. 1. Photograph showing a close-up view of the dNTP binding pocket of rat DNA polymerase β. The dNTP binding pocket is delineated by the side chains of residues Asn729, Tyr271, and Arg833 of DNA polymerase β. The dNTP binding pocket is shown in red, the terminal primer nucleotide in blue, and the templating nucleotide in green. The figure was made with MOLSCRIPT (30).
(2-fold) decrease (Fig. 2B). Since the phenylalanine substitution had only a small effect on catalytic efficiency, substrate interactions with Tyr871 appears to offer very little transition state stabilization. Elimination of the hydrogen bond between the incoming dNTP and the Asn779 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, since Kcat of each mutant was similar to wild-type enzyme. The most dramatic decrease in catalytic efficiency was observed for the mutants of Arg283 (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 hydrogen 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, β-pol 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 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 × 10⁻⁴). Whereas deletion of the hydrogen bond donor at Tyr871 did not alter the reversion frequency, alanine substitution at Asn779 significantly reduced it signifying an apparent increase in fidelity. This apparent increase in fidelity could reflect a reduced misinsertion rate or a reduced ability to extend mispairs, since both must occur to score a mutant. In contrast, alteration of the Arg283 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 mutants 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 1). 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

![Image](https://example.com/image.png)

**Fig. 3. Short gap fidelity assay.** A, experimental outline for the short gap fidelity assay as described under “Experimental Procedures.” B, mutation frequencies for the products synthesized by the wild-type and mutant β-pol polymerases. The background reversion frequency for the assay was ≤0.001%. Frequencies are shown as the mean and standard deviation of at least two independent determinations.

| Template nucleotide | Mismatch (template/dNTP) | Revertant nucleotide | Errors per detectable nucleotide a | Fold increase in errors relative to wild-type |
|---------------------|--------------------------|----------------------|-----------------------------------|---------------------------------------------|
| T                   | T-dGTP                   | C                    | 2.9 × 10⁻³ (32)                   | 460 × 10⁻³ (61) 160                        |
| T                   | T-dCTP                   | G                    | 0.2 × 10⁻³ (2)                    | 30 × 10⁻³ (4) 150                          |
| T                   | T-dTTP                   | A                    | ≤0.1 × 10⁻³ (0)                   | 30 × 10⁻³ (4) ≥100                          |
| G                   | G-dATP                   | T                    | 0.2 × 10⁻³ (2)                    | 15 × 10⁻³ (2) 75                            |
| G                   | G-dGTP                   | C                    | 0.2 × 10⁻³ (2)                    | ≤7 × 10⁻³ (0) ≤35                           |
| A                   | A-dCTP                   | G                    | 0.3 × 10⁻³ (3)                    | 60 × 10⁻³ (8) 200                           |
| A                   | A-dGTP                   | C                    | 0.6 × 10⁻³ (7)                    | 110 × 10⁻³ (15) 180                         |
| A                   | A-dATP                   | T                    | 0.1 × 10⁻³ (1)                    | 30 × 10⁻³ (4) 300                           |

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

b 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 β-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

1. Wiebauer, K., and Jiricny, J. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 5842–5845
2. Diano, G., and Lindahl, T. (1994)Curr. Biol. 4, 1069–1076
3. Horton, J. K., Srivastava, D. K., Zmutzka, B. Z., and Wilson, S. H. (1995)Nucleic Acids Res. 23, 3810–3815
4. Singhal, R. K., Prasad, R., and Wilson, S. H. (1995)J. Biol. Chem. 270, 549–557
5. Sobol, R. W., Horton, J. K., Kühn, R., Gu, H., Singhal, R. K., Prasad, R., Rajewsky, K., and Wilson, S. H. (1996)Nature 379, 183–186
6. Lino, S. (1991)Cell 66, 183–187
7. Jenkins, T. M., Saxena, J. K., Kumar, A., Wilson, S. H., and Ackerman, E. J. (1992)Science 258, 475–478
8. Sweesy, J. B., and Loeb, L. A. (1992)J. Biol. Chem. 267, 1407–1410
9. Sawaya, M. R., Pelletier, H., Kumar, A., Wilson, S. H., and Kraut, J. (1994)Science 264, 1930–1935
10. Pelletier, H., Sawaya, M. R., Wolfe, W., Wilson, S. H., and Kraut, J. (1994)Biochemistry, in press
11. Pelletier, H., Sawaya, M. R., Wolfe, W., Wilson, S. H., and Kraut, J. (1996)Biochemistry, in press
12. Helms, W., Sawaya, M. R., Wolfe, W., Wilson, S. H., and Kraut, J. (1996)Biochemistry, in press
13. Kohlstadt, L. A., Wang, J., Friedman, J. M., Rice, P. A., and Steitz, T. A. (1992)Science 256, 1783–1790
14. Seeman, N. C., Rosenberg, J. M., and Rich, A. (1976)Proc. Natl. Acad. Sci. U.S.A. 73, 804–808
15. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987)Methods Enzymol. 154, 367–382
16. Beard, W. A., and Wilson, S. H. (1995)Methods Enzymol. 262, 98–107
17. Roberts, J. D., Bebenek, K., and Kunkel, T. A. (1988)Science 242, 1171–1173
18. Beard, W. A., and Wilson, S. H. (1993)Biochemistry 32, 9745–9753
19. Bebenek, K., and Kunkel, T. A. (1995)Methods Enzymol. 262, 217–232
20. Hamlin, R. (1985)Methods Enzymol. 114, 416–452
21. Singhal, R. K., and Wilson, S. H. (1993)J. Biol. Chem. 268, 15906–15911
22. Helms, W., and Wilson, S. H. (1996)J. Biol. Chem. 271, 5767–5756
23. Kunkel, T. A., and Soni, A. (1988)J. Biol. Chem. 263, 14784–14789
24. Feig, D. J., and Loeb, L. A. (1993)Biochemistry 32, 4466–4473
25. Feig, D. J., and Loeb, L. A. (1994)J. Mol. Biol. 235, 33–41
26. Prasad, R., Beard, W. A., and Wilson, S. H. (1994)J. Biol. Chem. 269, 18096–18101
27. Jocic, C. M., and Steitz, T. A. (1995)J. Bacteriol. 177, 6321–6329
28. Kendall, O. (1988) in Structure and Function, DNA and Its Drug Complexes (Sarma, R. H., and Sarma, M. H., eds) Vol. 2, pp. 1–25, Adenine Press, Guilford, NY
29. Edwards, R., and Goodman, M. F. (1991)Annu. Rev. Biochem. 60, 477–511
30. Kraulis, P. J. (1991)J. Appl. Crystallogr. 24, 946–950

Fig. 4. Structural comparison of wild-type and alanine mutant of Arg283 of human β-pol complexed with DNA and ddTTP. The structure of the ternary substrate complex of the wild-type and R283A mutant were determined as described under “Experimental Procedures.” A Fobs - Fcalc difference Fourier map reveals a negative peak enveloping the Arg283 side chain consistent with mutation to alanine. No other significant changes were detected in the structure. The DNA, helices M and N (Arg283), and active site carboxylates (Asp190, Asp213, Asp216) of the wild-type enzyme are superimposed on the difference map. Although the thymidine moiety of ddTTP is observed, the 5′-triphosphate is disordered. In contrast to the rat β-pol ternary complex where the thumb subdomain is in a closed conformation (Fig. 1)(10), the human enzyme (wild-type and mutant) crystallized with the thumb in the open conformation. Therefore, Ala283 is moved over 12 Å away from where it is observed in the closed ternary complex with distributive DNA synthesis on large gaps. Processive short gap filling synthesis is modulated by the binding of the amino-terminal 8-kDa domain to the downstream 5′-phosphate group in gapped DNA (26).

To understand the structural basis for the lower catalytic efficiency and fidelity of the alanine mutant of Arg283, we determined the x-ray crystal structure of this mutant in complex with substrates (Fig. 4). In contrast to the rat β-pol ternary complex, where the thumb subdomain is in a closed conformation (10), the human mutant β-pol ternary complex crystallized in a different crystal packing form with the thumb in the open conformation (space group P212121). Hence, the difference map. Although the thymidine moiety of ddTTP is observed, the 5′-triphosphate is disordered. In contrast to the rat β-pol ternary complex, the N3 position of the templating base and site-directed mutagenesis will be a productive avenue for future analysis of polymerase structure-function relationships.