O-helix Mutant T664P of Thermus aquaticus DNA Polymerase I

ALTERED CATALYTIC PROPERTIES FOR INCORPORATION OF INCORRECT NUCLEOTIDES BUT NOT CORRECT NUCLEOTIDES*

Previous studies indicate that the O-helix of Thermus aquaticus (Taq) DNA polymerase I (pol I) plays an important role in the replication fidelity of the enzyme. This study examines the role of Thr-664, which lies in the middle of the O-helix of Taq pol I. A mutant of Taq Pol I with a proline substitution of Thr-664 (T664P) exhibits much lower replication fidelity than the wild type enzyme in a forward mutation assay. T664P produces base substitution, single-base deletion, and single-base insertion errors at 20-, 5, and 50-fold higher rates than wild type, respectively. In specific activity and steady-state kinetic experiments, T664P was catalytically robust for insertion of correct nucleotides. In contrast, it incorporated incorrect nucleotides 6.1- to 10-fold more efficiently than wild type at a template dC. Mismatched primer termini were extended by T664P 4.2- to 9.5-fold more efficiently than wild type. These data imply that the O-helix with a proline at position 664 functions like wild type Taq pol I for correct nucleotide incorporations, but bends and enlarges the catalytic pocket of the enzyme and increases the rate of nucleotide misincorporation.

Chromosomal DNA is subject to damage from naturally occurring in vivo processes such as hydrolysis, oxidation, and methylation (1). Cells have several DNA repair pathways that act as mutation avoidance systems and prevent the potentially deleterious effects of DNA damage. In addition to these systems, DNA polymerases discriminate between correct and incorrect nucleotides during DNA synthesis, thus providing accurate DNA synthesis and enhancing genomic stability (2).

During DNA synthesis by a polymerase, both the efficiency and the fidelity catalysis depends on the same phosphoryl transfer reaction and involves functionally and physically overlapping regions of the polymerase active site. The characteristics of mutant DNA polymerases are often useful for understanding the mechanisms that determine DNA replication fidelity. Among the mutants, we are interested in mutants with reduced fidelity but normal levels of catalytic activity. These types of mutations might be advantageous to a cell under conditions when low replication fidelity is desirable (i.e. to bypass DNA lesions) and could have played a role during evolution of error-prone DNA polymerases.

Nucleotide selection fidelity could be altered by mutations that modify the interaction between polymerase and substrate/template-primer DNA (2). For example, ribonucleotides are excluded from the DNA polymerase active site by structural/steric interference involving the 2'-OH group of the ribose sugar (3–5). Mutations in Glu-615 in Taq pol I, and the corresponding amino acid in Klenow or HIV reverse transcriptase, remove the steric clash with the 2'-OH group. Mutations in Ile-614 also reduce steric hindrance for incoming ribonucleotides and allow ribonucleotide misincorporation. In other mutant polymerases, an increase or decrease in the rate of base substitution or frameshift mutation is observed. Taq pol I F667L is a transversion antimutator in which the stacking force against bulky purine-purine base pairs may be reduced (6). This mutant and most other polymerases with altered replication fidelity show altered catalytic efficiency; Taq pol I F667L demonstrates a 23-fold lower A→T transversion frequency and ~30- to 100-fold lower catalytic efficiency than wild type Taq pol I.

This work reports the properties of Taq pol I mutants that have substitutions at Thr-664 in the O-helix. Taq pol I T664P is a mutator polymerase whose catalytic properties are near wild type for insertion of correct nucleotides. It is proposed that Taq pol I T664P may be a mutator because of increased plasticity in a wall of the catalytic pocket. Evidence supporting this hypothesis is discussed.

EXPERIMENTAL PROCEDURES

Materials—Wild type and mutants in Taq pol I were purified as described previously (7). Activity was measured in assays containing 200 μg/ml activated calf thymus DNA, 100 μM each dATP, dGTP, dCTP, and dTTP and 0.2 μCi of 3H]dATP in 50 mM Tris-HCl (pH 8.0), 2 mM MgCl₂; incubation was for 5 min at 72 °C. Incorporation of radioactivity into an acid-insoluble product was determined according to Yoshida et al. (8). One unit represents incorporation of 10 nmol dNMP in 1 h, corresponding to 0.1 unit as defined by PerkinElmer Life Sciences (Foster City, CA). Oligonucleotides were synthesized and purified by Amersham Pharmacia Biotech (Buckinghamshire, UK).

Forward Mutation Assay—Replication error was quantitated by the M13mp2 forward mutation assay. A gapped M13mp2 substrate was constructed in which the single-stranded gap region contained the lacZa-complementation target sequence (9). Gap-filling reactions were monitored by electrophoresis of the reaction products in 0.8% agarose gels. The reaction mixture contained 100 units of wild type or mutant Taq pol I, 400 ng of gapped M13mp2, 50 mM Tris-HCl (pH 8.0), 7 mM MgCl₂, 50 mM KCl, and 200 μM each dNTP (6). After incubation at 72 °C for 5 min, double-stranded phage DNA was transfected, the cultures were plated and incubated, and plaques were scored. Except for the experiment with T664P, mutant plaques were identified by their color. In some cases, plaques were isolated, replated and re-evaluated. DNA sequence was determined with an ABI PRISM™ Dye Primer Cycle Sequencing Core kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

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In earlier experiments, the number of background mutant plaques was as high as 3.5 $\times$ 10$^2$ (7), making it difficult to estimate the increase or decrease in mutation frequencies for polymerases. However, the background decreased to 0.87 $\times$ 10$^2$ (8), when a different preparation of gapped DNA substrate was used. In addition, the magnesium concentration was raised to 7 mM to force misincorporation (10). These changes in assay conditions made it possible to easily measure the mutation frequency of wild type polymerases and provided a sufficient number of mutant plaques for analysis. Deoxynucleotide concentrations were also increased to 200 $\mu$m to ensure efficient incorporation by polymerases with high $K_v$ values.

**Polymerase-DNA Equilibrium Binding Constants**—The 14-mer DNA primer (5'-CGCGCCGAATTCCCT) was 32P-labeled at the 5'-end by incubating with T4 polynucleotide kinase and ($\gamma$-32P)ATP and annealed to a 2-fold molar excess of the 46-mer DNA template, 5'-CGCGCGAAGCTTGCTGCAAGATATCGTACGGGAAATTCGCGCCG. The labeled template-primer was mixed with unlabeled DNA in a 1:1 ratio and used at a final concentration of 50 nM in each reaction. Reaction mixtures contained 50 mM Tris-HCl (pH 8.0), 10 mM MgCl$_2$, 50 mM KCl, 20 $\mu$m each dNTP, and 20 nM wild-type protein or 3.5 nM T664P in a final volume of 20 $\mu$L. In some experiments, either dCTP or dGTP was omitted from the mixture. Primer extension was carried out at 45 °C for 0, 2, 5, 10, 20, and 60 min.

**Single Nucleotide Incorporation Kinetics**—Assays measuring single nucleotide incorporation were essentially the same as described previously (11). A 14-mer template-primer was mixed with unlabeled template-primer in a 1:10 ratio and used at a final concentration of 50 nM in each 20-$\mu$L reaction containing 50 mM Tris-HCl (pH 8.0), 10 mM MgCl$_2$, 50 mM KCl, and specified concentration of dNTP and enzyme as described below. To measure incorporation of dC, dA, and dTTP, respectively. Reactions were performed at 45 °C for 5 min.

**Replication Fidelity**—Replication fidelity was measured using a gapped M13mp2 DNA with the lacZ and Thr-664 (Thr and Pro) are in green and red, respectively. The arrow indicates the hypothetical C-terminal domain on the O-helix. The coordinate sets were obtained from the Protein Data Bank (22). The drawing was made using the program package Insight II (Molecular Simulations Inc, San Diego, CA).

**RESULTS**

In the crystal structure models of Thermus aquaticus (Taq) DNA polymerase I (pol I) that are demonstrated by Li et al. (14), we found that substitution of Thr-664 with proline (T664P) removes at least two hydrogen bonds in the closed complex structure (Fig. 1). In wild type Taq pol I, the hydroxyl group of Thr-664 hydrogen bonds with the O$^\beta$ position of the guanine base pairing with the incoming ddCTP (Fig. 1A), and a similar interaction occurs between the Thr-664 hydroxyl group and the oxygen or amino group for other bases. However, this interaction does not form in the open or binary complex (15). Thr-664 also forms a hydrogen bond between the amino group and carboxyl group on an α-helical turn ahead (Fig. 1B). These hydrogen bonds do not form in the T664P mutant. To study the functions of these interactions, we measured replication fidelity and kinetic constants of wild type and Thr-664 mutant polymerases.

**Forward Mutation Assay**—Replication fidelity was measured as the frequency of mutations during a single round of DNA synthesis in vitro using a gapped M13mp2 DNA with the reporter lacZa gene as template for gap-filling DNA synthesis. These assays were carried out using a modification of the previously published procedure (see Ref. 7 and "Experimental Procedures"). Under the modified conditions wild type Taq pol I generates more mutant plaques, thereby the mutation frequencies of high and low fidelity mutants are more easily measured and compared with the wild type enzyme (6).

In experiments with wild type and Thr-664 mutants of Taq...
Pol I, the mutant frequency of wild type was $8.1 \times 10^{-2}$; however, the frequency of $16.5 \times 10^{-2}$ is used in this work, which is the average of several determinations (see Table I of Ref. 6 in detail). The mutant frequency of T664S and T664I is similar to wild type (Table I). The mutant frequency of T664N and T664R is slightly higher than wild type, at 53.8 and 63.8 $\times 10^{-3}$, respectively. The mutant frequency of T664R was similar to that reported previously (7), although relative value to wild type was lower because of the 6.6-fold higher mutant frequency for wild type.

In most cases, mutant plaques are scored by counting pale blue and white plaques on plates that have a much larger number of dark blue plaques. In cases where plaque colors were not clearly identified, phages are isolated, mixed with wild type phages and plated again in a fresh plate. However, it was very difficult to score the plaques formed by Taq Pol I T664P by this method, because the number of mutant plaques was unusually high, and because the mutant plaques had a large range of coloration. It was roughly estimated that at least 20% of the plaques were mutant, but the phenotype of the remaining plaques was very uncertain. Thus, we considered the remaining plaques as also carrying considerable populations of mutations and determined the DNA sequence for all the plaques on the plate to determine the mutant frequency for T664P. By this method, the mutant frequency of T664P was $529 \times 10^{-3}$ in the forward mutation assay (Table I).

**Mutational Specificity of T664P**—The DNA sequence was determined for DNA from 189 plaques produced during a forward mutation assay with T664P. There were 47 clones with one mutation and 53 clones with two or more mutations. Base substitution and frameshift frequencies were $4.2 \times 10^{-3}$ (one error per 240 nucleotides) and $3.0 \times 10^{-4}$ (one error per 3300 nucleotides), respectively. These error frequencies are 20- and 9.1-fold higher than wild type for base substitution and frameshift errors, respectively (Table II).

The types of base substitutions and frameshifts made by T664P are indicated in Table II. Base substitution frequencies were in the range of 6.7- (G→C) to 150-fold (G→A) higher than wild type. T664P produces T→C transitions (26%) and A→T (17%) transversions at a high rate, and these rates are 25- and 11-fold higher in the mutant than in wild type. In addition, T→A transversions (12%) and G→A transitions (11%) are made by T664P at a 55- and 150-fold higher rate than wild type. These data show that T664P discriminates poorly between the correct and incorrect nucleotide and thus is a base substitution mutator. Taq pol I T664P is also a frameshift mutator; frequencies of single base insertions and deletions were 50- and 5.2-fold higher than wild type, respectively (Table II).

The mutational spectrum of wild type and T664P was compared for the mutational target region used in the forward mutation assay. The base substitutions made by T664P map relatively evenly and randomly throughout the sequenced region; in contrast, the base substitutions made by wild type Taq pol I are in discrete hot spots (Fig. 2). Thus, base substitutions occurred in the mutational spectrum with little or no apparent sequence context effect and are randomly distributed throughout the target region. This character is quantitatively demonstrated by counting number of base appearance at one base upstream the substitution sites. For twenty-two G→A transi-

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**TABLE I**

| Taq     | Plaques scored | Mutant frequency |
|---------|----------------|------------------|
|         | Total          | Wild type        |
|         | $\times 10^{-3}$| $\times 10^{-3}$ |
| WT      | 27564          | 16.5$^a$         |
| T664S   | 2284           | 28.5             |
| T664N   | 1317           | 53.8             |
| T664 I  | 4153           | 15.0             |
| T664 P  | 189            | 529              |
| T664 R  | 2315           | 63.8             |

$^a$ Mutant frequency of wild type and mutant Taq pol I enzymes (except mutant T664P) was determined by inferring plaque genotype from plaque color. Background frequency for this method ($0.87 \times 10^{-3}$) was subtracted from each data point. For assays with Taq pol I T664A, plaques were sequenced to determine their genotype.

**TABLE II**

| Error type | WT$^b$ | T664P | Fold (T664P/WT) |
|------------|--------|-------|-----------------|
|            | N      | %     | $\times 10^{-5}$| N      | %     | $\times 10^{-5}$ |
| All errors |        |       |                 |        |       |                 |
| Base substitutions |        |       |                 |        |       |                 |
| Transitions |        |       |                 |        |       |                 |
| T→C        | 14     | 19    | 4.8             | 50     | 26    | 120             |
| C→T        | 6      | 8.0   | 2.1             | 14     | 7.2   | 32              |
| A→G        | 1      | 1.3   | 0.34            | 7      | 3.6   | 16              |
| G→A        | 3      | 1.3   | 0.34            | 22     | 11    | 51              |
| Transversions |       |       |                 |        |       |                 |
| T→A        | 3      | 4.0   | 1.0             | 24     | 12    | 55              |
| T→G        | 1      | 1.3   | 0.34            | 5      | 2.6   | 12              |
| A→T        | 21     | 28    | 7.2             | 33     | 17    | 76              |
| A→C        | 0      | 0     | 0               | 2      | 1.0   | 4.6             |
| G→T        | 13     | 17    | 4.5             | 15     | 7.7   | 35              |
| G→C        | 1      | 1.3   | 0.34            | 1      | 0.5   | 2.3             |
| C→A        | 0      | 0     | 0               | 7      | 3.6   | 16              |
| C→G        | 0      | 0     | 0               | 2      | 1.0   | 4.6             |
| Frameshifts |        |       |                 |        |       |                 |
| Deletes    | 13     | 17    | 3.1             | 7      | 3.6   | 16              |
| ≥2 base deletions | 0     | 0     | 0               | 5      | 2.6   | 12              |

$^b$ Data from Ref. 6.

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*a* Error frequency was determined as described previously by Tindall and Kunkel (16). The mutational target region included coordinates −70 to +145. This region includes 107 targets for base substitution and 154 targets for frameshift mutation (9).

* Error frequency was determined by sequencing 229 nucleotides in 189 clones. The number of mutations was divided by the number of nucleotides sequenced.
tions, 3A, 7C, 6G, and 6T were found at the N of 5'-NG, respectively. For twenty-four T→A substitutions, 6A, 4C, 6G, and 8T were found at 5'-NT, respectively (Table II). Sequence context also influences mutation frequency differently for wild type and mutant forms of Taq pol I. For example, ~26% of base substitutions made by T664P are T→C transitions. In the wild type mutational spectrum, nine of 14 T→C transitions occur at the sequence 5'-CT. However, in the mutational spectrum of T664P, T→C transitions are more frequent with A, G, or T at the 5'-NT (i.e. A:C:G:T = 12:4:16:18).

**Primer Extension**—Experiments were also performed with wild type and T664P using a primer extension assay in the absence of dCTP or dGTP. The template-primer, appropriate nucleotides, and wild type or mutant enzyme were incubated for up to 60 min. As shown in Fig. 3A, wild type Taq pol I rapidly incorporates the first nucleotide, dGMP, at template C, but does not efficiently incorporate the next nucleotide because correct dCTP is lacking. Hence, large amounts of the 15-mer reaction product accumulate. However, by misincorporation, product at the 16-mer position gradually increases with incubation time. Evidently, the 16-mer product is not efficiently extended, presumably due to the mismatched nucleotide opposite template G at the primer terminus. Base discrimination at both the incorporation and extension steps also occurs in the absence of dGTP (Fig. 3B, lanes 2–6). Most of the primer remains as a 14-mer, much less is extended to a 15-mer, and further extension is inefficient, presumably because of the mismatched nucleotide opposite template C at the primer terminus. At the time point of 60 min, wild type Taq pol I extends 7.0% of primers past the first template dG, and 8.7% of primers past the first template dC, when dCTP or dGTP is omitted from the reaction, respectively. In contrast, Taq pol I T664P extends 64% of the primer past the first template dG in the reaction omitting dCTP, and 68% of the primer past the first template dC in the reaction omitting dGTP. In addition to this high misinsertion and extension rate, many reaction products elongate past 4 target residues (lane 12). Under the minus dCTP condition, where the first dGTP was normally incorporated by polymerase, a comparable proportion of primers is extended by Taq pol I T664P at all time points. These data indicate that T664P has a greater capacity than wild type to elongate in the minus dCTP condition because of its increased ability to utilize non-complementary bases and mispaired primer termini, rather than because of a difference in the amount of activity present in the reaction with mutant or wild type enzyme.

**Kinetic Analyses**—Under steady-state conditions, reaction products were further quantitated, and the kinetic constants were determined (Table IIIa). Despite the structural changes in T664P (Fig. 1), its catalytic properties were similar to wild type. The insertion efficiency (Vmax/Km) for dCTP by Taq pol I T664P (0.12) was very similar to wild type (0.2), and was several-fold better than wild type for dGTP, dATP, and dCTP (Table IIIa). The Kf values for C:G matched and mismatched primer termini were also very similar for wild type and T664P mutant Taq pol I. This is consistent with the observations that the specific activity of T664P was 29% of the wild type value (19,000 versus 66,000 units/mg protein), and that T664P com-
implemented the growth defect of *Escherichia coli* strain recA718 polA12 (16).

Wild type and T664P mutant *Taq* pol I polymerase have comparable *Kₘ* values for misincorporation of dAMP, dTMP, and dCMP. In contrast, *Vₘₐₓ* values were higher for the T664P mutant; 31-fold (0.68 versus 2.2 × 10⁻² min⁻¹), 35-fold (0.3 versus 8.5 × 10⁻⁴ min⁻¹), and 12-fold (6.3 × 10⁻³ versus 5.2 × 10⁻⁴ min⁻¹) higher than wild type *Taq* pol I for dAMP, dTMP, and dCMP misincorporation opposite dC, respectively. As a result, misincorporation efficiencies by T664P were 6.1 (dAMP) and 10 (dTTP and dCMP)-fold higher than wild type. In the primer extension reaction using primers that have mismatched nucleotides at the 3’-termini, wild type and T664P also have similar *Kₘ* values for dGMP incorporation with a C-G (template:primer) and a C-A terminus, but T664P has a 6.9- and 3.7-fold lower *Kₘ* value for C-T and C-C, respectively (Table IIIb). *Vₘₐₓ* values were also different for wild type and T664P *Taq* pol I; C:A (9.2 × 10⁻⁴ and 0.12 min⁻¹), C:T (1.2 × 10⁻² and 6.6 × 10⁻⁴ min⁻¹), and C:C (1.2 × 10⁻⁴ and 1.3 × 10⁻³ min⁻¹). For *Taq* pol I T664P, mismatch extension efficiencies of C:A, C:T, and C:C mispairs were 4.2 (1.3 × 10⁻⁴ versus 5.5 × 10⁻⁴), 8.7 (1.0 × 10⁻⁵ versus 8.7 × 10⁻⁶), and 9.5 (1.0 × 10⁻⁶ versus 9.5 × 10⁻⁶)-fold higher than for wild type *Taq* pol I.

**DISCUSSION**

*Taq* pol I produces frequent T–C transitions and deletions at runs of single nucleotides (17). When performed at a high enzyme concentration, *Taq* pol I is more error-prone; the enzyme extends A:A and G:A mismatched primer termini, and the frequency of A→T transversions and G→T transversions increases. In addition, −1 frameshift errors occur at pyrimidine:purine dinucleotides involving loss of the downstream purine (6). This study reports a forward mutation analysis of T664P, an O-helix mutant of *Taq* pol I that demonstrates significant reduction in replication fidelity. This mutant synthesizes DNA with an increased frequency of all base substitution and frameshift errors (i.e. 150-fold increase in G→A transitions). These data indicate that *Taq* pol I T664P is impaired in its ability to discriminate between correct and incorrect incoming nucleotides during gap-filling DNA synthesis in vitro.

*Taq* pol I T664P displays reduced replication fidelity (Table II) and an altered mutational spectrum (Fig. 2), but its kinetic properties (*Vₘₐₓ* and *Kₘ*) for correct deoxynucleotide incorporation, as well as *Kₐ* values for matched and mismatched primer termini, are essentially the same as or better than wild type *Taq* pol I (Table II). This observation is consistent with the non-essential function of Thr-664 for catalytic efficiency of the enzyme; the amino acid corresponding to Thr-664 is not conserved among the pol I class of DNA polymerases. These data suggest that the catalytic pocket structure of T664P assumes a conformation nearly identical to wild type when encountering a correct nucleotide during DNA synthesis.

Two processes contribute to the ability of a polymerase to discriminate correct from incorrect nucleotides during DNA synthesis; discrimination occurs at the insertion step (selection of incoming nucleotide during catalysis) and at the extension step (discrimination between matched and mismatched primer termini). Single nucleotide incorporation kinetics for T664P indicate that misinsertion efficiency for incorrect incorporation of dATP, dTTP, and dCTP are 6-, 10-, and 10-fold higher than wild type, respectively (Table IIIa). Furthermore, mismatch extension efficiency for C-A, C-T, and C-C mispairs are 4.2-, 8.7-, and 9.5-fold higher than wild type, respectively (Table IIIb). These data show that T664P is impaired in discrimination at the insertion and the extension step. It is possible that kinetic values would differ if determined using a different template than the one arbitrarily chosen for the experiment presented here. However, this possibility is less likely for T664P because the data set is internally consistent for the forward mutation assay and the kinetic studies; 15-, >47-, and >14-fold increase is observed in the forward mutation assay in C–T, C–A and C–G base substitution rates, respectively (Table II). To understand the mechanism underlying the altered repli-
cation fidelity of T664P, other mutant polymerases that have amino acid substitutions at Thr-664 were also purified and characterized. If the interaction between Thr-664 and the template base plays a major role in determining replication fidelity (Fig. 1A), it is likely that the effects of substitution of Thr-664 would depend on the side chain length and polarity of the substituting amino acid. Substitution with the polar amino acid serine does not significantly reduce replication fidelity of Taq pol I in the forward mutation assay; substitution of Thr-664 with isoleucine (a non-polar side chain) caused no change in the wild type mutant frequency (Table II). These data indicate that the Thr664 may influence base selection by Taq pol I, but it seems to play a relatively inessential and dispensable role.

Proline is a unique amino acid because its side chain is covalently bound to the backbone nitrogen atom forming a ring structure. Proline is underrepresented in protein α-helices, but a substantial number of prolines are found in the helical regions of proteins (18). When proline substitutes for Thr-664 in the O-helix of Taq pol I, one of the nine intrahelical hydrogen bonds in the wild type structure fails to form (Fig. 1B); this may result in a steric hindrance within the α-helix conformation and may produce a kink (18–20). Our data indicate that substitution of proline in the O-helix at position 664 does not alter catalytic activity for insertion of correct nucleotides by Taq pol I (Table IIIa), despite the hypothetical structural hindrance observed in model structures (19). One interpretation of this result is that the helix may be sufficiently stable with eight hydrogen bonds in the O-helix backbone for incorporation of the correct nucleotide, instead of the nine hydrogen bonds that form in the wild type enzyme.

The results presented here indicate that T664P is catalytically altered during incorporation of incorrect nucleotides or when extending mismatched primer termini (Tables IIIa and IIIb). These data suggest that the O-helix bends and enlarges the catalytic pocket at the position of the hydrogen bond that does not form because of the T664P substitution (Fig. 1). In wild type Taq pol I, improper base pairs may dislocate the C-terminal residues Phe-667 and Tyr-671 that form a “wall” in the hydrophobic catalytic pocket (14). This stress is transferred to the N-terminal amino acids Arg-659 and Lys-663 that interact with the substrate phosphates, unless the O-helix assumes a bent structure. Thus proline is substituted near the middle of the O-helix, it may promote a transition between the near-intact (correct base pair) and bent structure (incorrect base pair) that acts as an elastic hinge.

The low replication fidelity mutant A661E of Taq pol I has also recently been characterized (6). The results of that study suggest that an interaction between the O-helix and the primer might stabilize the closed form of Taq pol I, allowing more time for chemistry and increasing the probability of phosphoryl transfer involving incorrectly paired dNTPs. It is also possible that amino acid substitution in the O-helix might create slightly more space in the catalytic pocket to accommodate incorrect base pairs. However, the mechanisms causing low replication fidelity may differ between Taq pol I T664P and A661E.

The amino acid 664 is not conserved in 9 Pol I class DNA polymerases and 47 Pol α class DNA polymerases (21). Nevertheless, proline is not found at any of the corresponding residues or in regions that correspond to the O-helix in this group of related proteins. Our data also suggest that proline substitution, if it ever happened during evolution of the replicative DNA polymerases, might have been excluded to ensure a sufficient level of replication fidelity.

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