Low Fidelity Mutants in the O-Helix of Thermus aquaticus DNA Polymerase I*

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We screened 67 mutants in the O-helix of Thermus aquaticus (Tag) DNA polymerase I (pol I) for altered fidelity of DNA synthesis. These mutants were obtained (Suzuki, M., Baskin, D., Hood, L., and Loeb, L. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9670–9675) by substituting an oligonucleotide containing random sequences for codons 659–671, and selecting for complementation of a growth defect in Escherichia coli caused by temperature-sensitive host pol I. Thirteen mutants decreased fidelity in a screen that employed primer extension reactions lacking one of four complementary deoxyribonucleoside triphosphates (dNTPs). Three mutants were purified and exhibited 29–68% of wild-type specific activity. Homogeneous polymerases A661E, A661P, and T664R extended primers further than the wild-type, synthesizing past template nucleotides for which the complementary dNTP was absent. The data indicate that both misinsertion of incorrect nucleotides and extension of mispaired primer termini were increased. In a lacZα forward mutation assay, A661E and T664R yielded mutation frequencies at least 7- and 25-fold greater, respectively, than that of the wild-type polymerase. These findings emphasize the importance of the O-helix in substrate recognition and are compatible with a role for pyrophosphate release in enhancing fidelity of DNA synthesis.

Thermus aquaticus (Tag) DNA polymerase I (pol I) is used extensively in the amplification of DNA by the polymerase chain reaction. Based on its primary sequence, crystal structure, and catalytic mechanism, Taq pol I is classified in the same family as Escherichia coli pol I (1–3). The amino acid sequences of Taq pol I and E. coli pol I are 38% homologous, both enzymes have 5′-3′ exonuclease domains (4, 5), and the crystal structures of the polymerase domains are nearly identical (5, 6). The structure of the vestibial 3′-5′ exonuclease domain of Taq pol I is very different, however; unlike E. coli pol I, purified Taq pol I exhibits no proofreading activity (7).

The O-helix in E. coli pol I is constituted of 15 amino acids which form part of the DNA binding cleft (8). Crystallographic, biochemical, and mutagenesis studies indicate that four conserved residues in the O-helix which face toward the cleft interact with either the incoming dNTP or the template-primer. Considerable evidence indicates that in E. coli pol I, Arg-754 and Lys-758 interact with the phosphate groups in the incoming dNTP, that Phe-762 interacts with the deoxyribose moiety in the dNTP, and that Tyr-766 binds to the template-primer (9–11). The nearly identical structures of the polymerase domains of Taq pol I and E. coli pol I (6, 7) suggest that the corresponding amino acids in Taq pol I have analogous functions. By using random sequence mutagenesis together with genetic complementation, we recently observed that the O-helix of Taq pol I contains four amino acids which are immutable (or nearly immutable) and are apparently essential for catalysis in vivo (12). These residues, Arg-659, Lys-663, Phe-667, and Tyr-671, correspond to the four above mentioned residues in E. coli pol I. Importantly, Phe-667 in Taq pol I and the corresponding Phe-762 in E. coli pol I are critical for substrate discrimination (13). Substitution of Tyr for Phe results in increased incorporation of deoxyribonucleoside triphosphates. Analysis of the effects of this and other mutations in the O-helix on the incorporation of non-complementary nucleotides may be important to understanding substrate recognition by DNA polymerases.

The fidelity of DNA polymerases is central to the accurate transmission of genetic information. This fidelity is conferred primarily by the accuracy of polymerization, with an additional contribution from proofreading for enzymes which possess an editing 3′-5′ exonuclease (14). In the case of Taq pol I, which lacks proofreading activity, the error rate during a single round of replication has been estimated as one misincorporation for every 9000 nucleotides polymerized (15). This estimate is in approximate agreement with studies on the product of polymerase chain reactions obtained after multiple rounds of DNA synthesis in some (16) but not all studies (17). The frequency of misincorporation by Taq pol I is greater than that of the Klenow fragment of E. coli pol I, even when assayed in 1 mM dNTP (conditions that substantially reduce the Klenow-associated 3′-5′ exonuclease activity). This comparison implies that despite the similarity in structure of the two enzymes, there are subtle differences that affect fidelity. Interestingly, the fidelity of Taq pol I is markedly influenced by reaction conditions; both base substitution and frameshift error rates of less than 1 × 10⁻⁵ are observed at pH 5–6 (18).

The fidelity of polymerization depends on the ability of the polymerase site to discriminate correctly from incorrect substrates, i.e. complementary from non-complementary nucleoside triphosphates (19) and matched from mismatched primer termini (20). A molecular understanding of polymerization fidelity will require identification of the amino acids which participate in substrate discrimination and elucidation of the role(s) played by each. As recently pointed out, lack of mutants with altered accuracy, particularly for DNA polymerases whose three-dimensional structure is known, is a limiting factor in...
obtaining a molecular description of fidelity (9). Some accuracy mutants have been obtained by site-directed mutagenesis (9). However, this approach does not readily permit examination of large numbers of amino acid replacements and frequently yields mutants with markedly reduced catalytic efficiency.

In the present work, we illustrate a different approach to obtaining fidelity mutants of Taq pol I. We have previously used random sequence mutagenesis together with genetic complementation to create a collection of catalytically active mutants with amino acid replacements in the O-helix (12), and we have now screened some of these mutants for reduced fidelity to examine the effects of the mutations on accuracy. The importance of the O-helix in interactions with incoming dNTPs and with the template-primer suggests that amino acid substitutions within this segment that alter fidelity are likely to do so by altering these interactions. Moreover, because our mutant selection depends on complementation of a temperature-sensitive phenotype in vivo, Taq pol I derivatives with near wild type activity are obtained (21), and the effects of mutation on fidelity are therefore observed in the absence of major impairments of catalysis.

**EXPERIMENTAL PROCEDURES**

**Polymerase Sources, Strains, and Oligonucleotides—**Wild-type Taq pol I and its mutant derivatives were selected by genetic complementation as previously reported (12). E. coli DH5α [F' , 68 DIN lacZΔM15, ΔlacZYA-argF/U169, deoR, recA1, endA1, phaA, hsdR17(r b )], supE44, thi-1, gyrA96, relA1] was used to express Taq pol I protein. E. coli MC1061 [hsdR, merC, araD, (139.A araABC) leuD, Δ 7676lacX74, galU, galK, rpsL, thi-1] was used for transfection in the forward mutation assay, and transfectants were plated on the indicator strain E. coli CSH50 [Δpro-Blac-F' + rdsD, thi-1, ara-proAB, lac-24, ΔM15]. Oligonucleotides were synthesized and purified by Operon Technologies Inc. (Alameda, CA).

**Preparation of Extracts—**A single colony of E. coli DH5α carrying wild-type or mutant Taq pol I was inoculated into 40 ml of 2xYT (16 g/liter tryptone, 10 g/liter yeast extract, 5 g/liter NaCl, pH 7.3) containing 30 mg/liter chloramphenicol. After incubation at 37 °C overnight with vigorous shaking, an equal amount of fresh medium with 0.5 mM isopropyl-β-D-thiogalactoside was added, and incubation was continued for 4 h. Cells were harvested, washed once with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and suspended in 100 μl of buffer A (50 mM Tris-HCl, pH 8.0, 2 mM MgCl₂, 50 μM KCl, 20 μM each dNTP, 0.2 μM of [³²P]dATP, and 200 μg/ml activated calf thymus DNA). Incorporation of radioactivity into an acid-insoluble product was measured according to Battula and Loeb (23). One unit represents incorporation of 10 nmol of dNTP in 1 h, corresponding to 0.1 unit as defined by Perkin-Elmer.

**Primer Extension Assay**—The 14-mer primer 5'-GGCGGGCAT- TCCC was ³²P-labeled at the 5' end by incubation with [³²P]dATP and T4 polynucleotide kinase and annealed to an equimolar amount of the template 46-mer 5'-GGCGGAAGGTTCGGACAAAGTTGGCTGCGGCGC. Crude extracts containing 0.3–1 unit of mutant or wild-type Taq pol I were incubated at 45 °C for 60 min in 50 mM Tris-HCl (pH 8.0), 2 mM MgCl₂, 50 mM KCl, 20 μM each dATP, dGTP, dCTP, and TTP, and 1.4 ng of the annealed template-primer. A set of four additional reactions, each lacking a different dNTP, was carried out for each polymerase. Purified enzyme (1 unit) was incubated for the times indicated under the same conditions as for crude extracts. After electrophoresis in a 14% polyacrylamide gel containing 8 M urea, reaction products were analyzed by autoradiography. Extension was quantified by using an NIH imaging program.

**Forward Mutation Assay and DNA Sequence Analysis**—The non-coding strand of the lacZa gene contained in 200 ng of gapped M13mp2 DNA (24) was copied by using 5 units of wild-type or mutant Taq pol I in a reaction mixture containing 50 mM Tris-HCl (pH 8.0), 2 mM MgCl₂, 50 μM each dNTP, and 50 mM KCl. After incubation at 72 °C for 5 min, the DNA was transfected and the plaques were scored (15). The nucleotide sequence of mutants was determined by using a Thermo Sequenase™ cycle sequencing kit (Amersham LIFE SCIENCE, Cleveland, OH).

**RESULTS**

**Screening for Fidelity Mutants**—We have previously generated a large collection of functional mutants of Taq pol I (12). Extracts of E. coli expressing these mutated Taq pol I were used to screen for mutations that alter fidelity. The mutants were created by substituting random nucleotides for the 13 codons specifying amino acids 659–671 in the O-helix of plasmid-borne Taq pol I. Functional mutations were then selected based on their ability to complement the growth defect of an E. coli strain carrying a temperature-sensitive host pol I (12). Sequence analysis of the selected, functional mutants revealed that 4 of the 13 mutagenized amino acids, Arg-659, Lys-663, Phe-667, and Tyr-671, tolerated no substitutions or only a few conservative substitutions, in accord with biochemical and crystallographic evidence that Arg-659 and Lys-663 participate in the formation of a metal-dNTP binding pocket (10, 11). The nine remaining residues were tolerant of several or many replacements. To explore the contribution of individual amino acids in the O-helix to replicational fidelity, we screened functional mutants in a primer elongation assay to identify substitutions that affect ability to utilize non-complementary dNTPs.

We screened 67 of the 75 active mutants previously sequenced, including all 38 with single amino acid substitutions (described in Fig. 3A of Ref. 12). Plasmids encoding the mutant polymerases were cloned, purified, and grown in E. coli, and host cells were analyzed for expression of Taq pol I by measuring the activity of crude extracts (see “Experimental Procedures”). E. coli DNA polymerases and nucleases were inactivated by heating at 72 °C for 20 min. The ability of heat-treated extracts to elongate primers in the absence of a complete complement of four dNTPs was then determined using a set of five reactions. One reaction contained all four complementary triphosphates while each of the others lacked a different dNTP ("minus conditions"). Elongation in the minus reactions is lim-
ited by the rate of misincorporation at template positions complementary to the missing dNTP.

Fig. 1 illustrates representative results obtained for wild-type Taq pol I and for mutants with elongation patterns that differed from wild-type. In the presence of all four dNTPs, every extract examined extended more than 90% of the hybridized primer to a product of length similar to that of the template. In the minus reactions, wild-type Taq pol I (lanes 2–6) extended 48–60% of the primer up to, but not opposite, the first template position complementary to the missing dNTP. The remaining primer was terminated opposite the missing dNTP, presumably by incorporation of a single non-complementary nucleotide, or was terminated further downstream, presumably by extension of the mispaired primer terminus. A variety of elongation patterns was observed for the 67 mutants. Thirteen mutants extended more of the primer and/or synthesized a greater proportion of longer products than the wild-type enzyme in three or four of the minus reactions (e.g. mutant 2 (lanes 12–16) which formed full-length products in reactions lacking dGTP (lane 15) or TTP (lane 16)). This increased extension presumably reflects increased incorporation and/or extension of non-complementary nucleotides. Other mutants (e.g. mutant 5, lanes 27–31) extended less of the primer or synthesized shorter products than the wild-type enzyme. In several cases, different amino acid substitutions at the same position either increased or decreased extension in comparable minus reactions.

Fig. 2 contains a compilation of amino acid replacements in the 13 mutants that displayed increased extension in at least three of the minus reactions. With the exception of Gly-668, one or more substitutions that putatively reduce the accuracy of DNA synthesis were observed for each of the 9 non-conserved amino acids. Eleven mutants harbored substitutions at either Ala-661 or Thr-664, including several single mutants. This initial screen with crude extracts suggested that a large number of changes are permitted in the O-helix that do not reduce

FIG. 1. Screening for altered fidelity of Taq pol I mutants in a primer extension assay. Heat-treated extracts of E. coli expressing wild-type Taq pol I or one of its mutant derivatives were used to catalyze primer elongation in the absence of a full set of complementary dNTPs. For each pol I, 32P-labeled primer was extended in a control reaction with all four dNTP and in a set of four additional reactions in which one of the dNTPs was omitted, as indicated in the figure. Elongation patterns for seven extracts are shown to exemplify results of the screen, including wild-type Taq pol I (lanes 2–6) and a catalytically non-functional derivative (lanes 32–36) in which the O-helix was replaced with another sequence (12). M1, A661E, I665T; M2, A661P, N666I; M3, T664N, L670I; M4, F667Y; M5, R660K. The non-functional mutant was displayed in a separate portion of the gel and spliced with the others. Open and filled triangles represent the positions of the primer and the fully extended product, respectively. Positions of nucleotides complementary to the template are shown at the right.

FIG. 2. Low fidelity mutants of Taq pol I identified in the primer extension screen. Amino acid replacements are listed for mutants that elongated primers to a greater extent than wild-type Taq pol I in at least three of the “minus” reactions in the primer extension screen (see Fig. 1). Amino acid substitutions at positions Arg-659 through Tyr-671 are shown below the wild-type (WT) sequence. Mutant designations at the left are the same as described previously (12). Note that Arg-659, Lys-663, Phe-667, and Tyr-671, for which no replacements are listed, were previously found to be immutable or nearly immutable (12).
the ability of Taq pol I to complement the growth defect of recA718 pol A12 (12). Many of the substitutions in the O-helix that do not reduce the ability of Taq pol I to carry out functional complementation reduce the fidelity of DNA synthesis in vitro.

**Purification of Mutant Polymerases**—To demonstrate that the reduction in fidelity exhibited by crude extracts is due to the mutant Taq pol I, we purified the wild-type enzyme as well as the polymerase from the three single mutants Ala-661→Glu (A661E), Ala-661→Pro (A661P), and Thr-664→Arg (T664R). As a control, we also purified enzyme from the single mutant Ile-665→Thr (I665T), which the screen suggested would have no alteration in fidelity. As shown in Fig. 3, each polymerase was purified to apparent homogeneity and had mobility corresponding to the expected molecular weight of 99,000. The specific activities of the purified enzymes, determined in assays with activated calf thymus DNA, were 66,000, 45,000, 19,000, 23,000, and 30,000 units/mg protein for the wild-type, A661E, A661P, T664R, and I665T, respectively. That the mutated enzymes retain at least 29% of wild-type activity in vitro is in accord with their ability to complement the growth defect caused in E. coli by temperature-sensitive host pol I, and ensures that analysis of fidelity will not be complicated by major impairments of catalytic efficiency.

**Primer Elongation by Purified Polymerases**—Primer elongation assays were carried out with the homogenous mutant polymerases. Fig. 4A shows reactions incubated for 5 min at 42 °C. Wild-type Taq pol I extended most of the primer to one nucleotide before the template position opposite the missing complementary dNTP. Only about 30% of the primers were elongated further. In reactions containing equivalent activity, the mutant polymerases A661E, T664R, and A661P extended a larger proportion of the primers past the sites where the wild-type polymerase ceased synthesis. The control enzyme I665T yielded an elongation pattern similar to that of the wild-type enzyme. Elongation reactions with the three polymerases were also carried out for 60 min (Fig. 4B). Again, A661E and T664R synthesized a greater proportion of longer products than obtained with the wild-type and I665T polymerases. Notably, A661E, T664R, and A661P synthesized longer products in 5 min (Fig. 4A) than the wild-type did in 60 min (Fig. 4B).

To further document the reduced fidelity illustrated in Fig. 4, A and B, we carried out a time course of primer elongation. Fig. 4C shows results for the minus dCTP reaction catalyzed by the wild-type and T664R polymerases. Wild-type Taq pol I extended 9% of the primers past the first deoxyguanosine template residue within the 60-min incubation period, but elongation past the second deoxyguanosine was not detected (lane 6). In the same interval, T664R extended 93% of the primer past the first template deoxyguanosine, and elongation proceeded past as many as five template deoxyguanosines (lane 12). Importantly, a comparable proportion of primers was extended at all time points, despite the striking difference in the length of the products. These time course data indicate that greater elongation reflects increased ability to utilize non-complementary substrates and primer termini, rather than a putative difference in the amount of activity present. Such a difference might be hypothesized, because activity of the individual polymerases was based on assays utilizing a gapped DNA template-primer (i.e., activated calf thymus DNA).

Interestingly, very little primer was extended to a length greater than 21 nucleotides in any of the minus dATP reactions (Fig. 4). Cessation of elongation may be due to the run of two Ts in the template at positions corresponding to a 21- and 22-mer. Similarly, extension may have ceased at the template Gs corresponding to 33- and 34-mer products in minus dCTP reactions, and at template As corresponding to 25- and 26-mer products in minus dTTP reactions. Apparently, extension of primers terminating with two non-complementary nucleotides is much less efficient than extension of primers terminating with a single mismatched nucleotide. These results suggest that extension of mismatched primer termini is catalyzed by a template-dependent mechanism characteristic of DNA polymerases, rather than by a template-independent mechanism such as that of terminal deoxynucleotidyl transferase.

**Forward Mutation Assay**—To quantitate the fidelity of DNA synthesis by the purified polymerases, we measured the frequency of mutations produced by copying a biologically active template in vitro (25). The target sequence was the lacZa gene located within a single-stranded region in gapped circular double-stranded M13mp2 DNA (26). The gapped segment was filled by synthesis with the wild-type or mutant enzymes. The double-stranded circular product was transfected into E. coli, and the mutation frequency was determined by scoring white and pale blue mutant plaques. A comparison of the specific activities and mutation frequencies of the purified enzymes is presented in Table I. After synthesis by wild-type Taq pol I, the mutation frequency was not greater than that of the uncopied control. Synthesis by A661E and T664R gave rise to mutation frequencies more than 7- and 25-fold greater, respectively, than that of the wild-type polymerase.

A sample of independent, randomly chosen mutants produced by T664R was characterized by DNA sequence analysis. Both base substitutions and frameshifts were found throughout the targeted lacZa gene and its regulatory sequence. Of the 64 independent plaques, 57 had mutations in the target. Other mutations presumably occurred outside the target region. Some had more than one base substitution and a total of 66 mutations were observed (Fig. 5). Among them, 61 were base substitutions. Transitions (38/61) were more frequent than transversions (23/61). T→C transitions accounted for 31 of 61 base substitutions, while T→A (9/61), A→T (8/61), and G→A (5/61) substitutions were less frequent. This base substitution spectrum is essentially the same as that reported for wild-type Taq pol I (27). From these data, the base substitution fidelity of T664R can be calculated according to Tindall and Kunkel (27) as 8.6×10⁻⁴ or 1 error per 1200 nucleotides. On the basis of the five frameshift mutations detected, the frameshift error can be calculated as 4.9×10⁻⁵ or 1 error per 20,000 nucleotides.

**DISCUSSION**

We have utilized random sequence mutagenesis and a genetic complementation system to obtain a large series of catalytically hardy mutants in the O-helix of Taq pol I (12). In the present work, we screened preparations of 67 of the mutant enzymes for alterations in the fidelity of DNA synthesis by
Fig. 4. Primer extension by purified Taq DNA polymerases. Primer elongation was catalyzed by purified wild-type Taq pol I or one of the mutant derivatives A661E, A661P, T664R, or I665T. A, incubation was for 5 min at 45 °C in reactions containing all four complementary dNTPs.
TABLE I
Mutation frequency in the lacZa forward mutation assay

| Taq pol I | Specific activity | Plaques scored | Mutation frequency |
|-----------|-------------------|----------------|--------------------|
|           | units/mg          | Total Mutant   | ×10^-7             |
| WT        | 66,000            | 8,637 22       | 2.5                |
| A661E     | 45,000            | 6,782 116      | 17.1               |
| T664R     | 23,000            | 5,148 324      | 62.9               |

Measuring elongation of DNA primers in reactions lacking one dNTP, as indicated. B, incubation was for 60 min at 45°C in reactions containing all four complementary dNTPs, and in reactions lacking one dNTP as indicated. C, incubation was at 45°C for the times indicated in the absence of dCTP. Open and filled triangles represent the positions of the primer and the fully extended product, respectively. The sequence of the incorporated complementary nucleotides is given at the right. Only the primer was loaded in lanes 1 and 7.
either side of the essential (10, 11, 41, 42) and non-mutable Lys-663 (12) (Figs. 2 and 6). The most frequent substitutions among our low fidelity mutants occurred at Ala-661 and Thr-664 (Fig. 2). These amino acids are not on the face of the helix that interacts with the incoming dNTP, as judged from the crystal structure of E. coli pol I (8), nor are they essential for catalytic activity in vivo, based on the ability of other amino acids to replace them (12). In fact, we have reported that six different amino acids could replace either Ala-661 or Thr-664 and yield active DNA polymerases, in the absence of mutations at other positions that could compensate (12). A variety of amino acids are also found in the corresponding positions in other DNA polymerases within the pol I family. Ser, Asn, Lys, or Glu is found at the position corresponding to Ala-661, and Ala, Val, Lys, or Ile is found at the position corresponding to Thr-664 (3). Thus, many substitutions are tolerated adjacent to the essential Lys-663 and Arg-659 in Taq pol I and homologous residues in other DNA polymerases.

Among the 13 low fidelity mutants we identified (Fig. 2), only two contain substitutions in the five C-terminal amino acids of the O-helix (Phe-667 through Tyr-671). This segment interacts with the terminal region of the template-primer duplex as judged from the crystal structure of Taq pol I in the presence of a blunt-ended oligonucleotide (43). The double-stranded oligonucleotide could represent the product of the polymerase reaction. Moreover the two substitutions we observed occurred in the presence of other replacements that could alter fidelity.

We offer two explanations for how the diverse substitutions we identified in the O-helix could reduce the fidelity of DNA synthesis. First, these substitutions might alter the local conformation around the catalytic pocket and thereby reposition critical residues that interact with either the template-primer or the incoming dNTPs. Among the substitutions in the 13 low fidelity Taq pol I mutants were five prolines, which disrupt helical structure, and three glycines, which could relax the conformation around critical residues at the active site.

A second explanation for the reduced fidelity of our mutants involves a pyrophosphate error prevention mechanism (44, 45). It might have been predicted that amino acid substitutions adjacent to the Phe and Tyr (Fig. 6) would alter the fidelity of DNA synthesis since they interact with the template-primer binding site (38). However, most of the substitutions that alter fidelity are located upstream from these residues in proximity to Lys-663. The assignment of individual amino acid residues that interact with specific atoms in the incoming dNTPs is tentative, at best (46). It is based primarily on inferences from kinetic studies (45), binding studies with binary complexes (44), and cross-linking studies with wild-type and mutant DNA polymerases (11). Nevertheless, evidence suggests that both Lys-663 and Arg-659, and the homologous residues in other DNA polymerases, interact with the \(\beta\)- and \(\gamma\)-phosphates in the incoming dNTP and could thus facilitate removal of pyrophosphate during catalysis (44). The incoming dNTP may initially interact with the carboxylate region of the palm domain (46, 47). The carboxylates could anchor a pair of divalent metal ions to facilitate deprotonation of the \(3'-\)hydroxyl terminus of the primer strand (46). As judged from the structure of Taq pol I with a double-stranded oligonucleotide, Lys-663 and Arg-659 in the O-helix in the fingers domain are distant from the carboxylate residues in the palm region and thus a conformational change may be required to facilitate their interaction with the \(\beta\)- and \(\gamma\)-phosphates in the incoming dNTP. Support for the involvement of Lys-663 and/or Arg-659 in pyrophosphate release is largely indirect. The corresponding Arg-754 and Lys-758 in E. coli pol I have been shown to interact with phosphate residues in the incoming dNTP (8, 9). Substitution of Ala at Lys-758 in E. coli pol I reduced \(k_{cat}\) 330–3,500-fold with poly(dA) poly(dT) \(_{15}\) as template-primer, while substitution of Ala at Arg-754 reduced \(k_{cat}\) 19–30-fold (10, 48). These data suggest that Arg-754 and Lys-758 in E. coli pol I are involved in catalysis but they do not establish a direct interaction with the dNTP or involvement in pyrophosphate release. More direct evidence stems from crystallographic data that showed that the Klenow fragment is complexed with pyrophosphate near the conserved Lys and Arg residues (42). Thus, our low fidelity mutants containing substitutions near Lys-663 or Arg-659 may exhibit attenuated pyrophosphate release. A two-step chemical mechanism for pyrophosphate release that effects the fidelity of DNA synthesis has been suggested. The first step involves binding of the substrate via the \(\gamma\)-phosphoryl group and the second step involves verification of complementary base pair interaction by coordination with the \(\beta\)-phosphoryl group (44). This error preventing step follows binding of the substrate and precedes primer elongation. Enhancement of misincorporation by the addition of pyrophosphate has been demonstrated for DNA polymerases (49) and has been attributed to a kinetic proofreading step in a multi-step proofreading mechanism (45).

The essentiality of individual amino acids in DNA polymerases has been inferred from sequence comparisons (1) that identify evolutionarily conserved and presumably critical residues. Chemical modification, site-specific mutagenesis, and kinetic studies follow, and provide quantitative data on which to assess detailed catalytic function. We have taken a different approach to evaluating the role of amino acids in the O-helix of Taq pol I. We constructed a library of active mutants by using random mutagenesis and genetic selection and identified immutable/essential residues (12). We then screened these active mutants for alterations in the fidelity of DNA synthesis. Our results suggest that many substitutions within the polymerase site can alter fidelity while preserving near wild-type activity. The mutants we have identified may be of value in ascertaining the mechanisms of substrate discrimination by DNA polymerases. In addition, they may have utility in applied molecular evolution (50) by increasing mutagenesis during amplification of genes by the polymerase chain reaction (51, 52).

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