A Conservative Isoleucine to Leucine Mutation Causes Major Rearrangements and Cold Sensitivity in KlenTaq1 DNA Polymerase

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ABSTRACT: Assembly of polymerase chain reactions at room temperature can sometimes lead to low yields or unintentional products due to mispriming. Mutation of isoleucine 707 to leucine in DNA polymerase I from Thermus aquaticus substantially decreases its activity at room temperature without compromising its ability to amplify DNA. To understand why a conservative change to the enzyme over 20 Å from the active site can have a large impact on its activity at low temperature, we solved the X-ray crystal structure of the large (5′-to-3′ exonuclease-deleted) fragment of Taq DNA polymerase containing the cold-sensitive mutation in the ternary (E–DNA–ddNTP) and binary (E–DNA) complexes. The I707L KlenTaq1 ternary complex was identical to the wild-type in the closed conformation except for the mutation and a rotamer change in nearby phenylalanine 749, suggesting that the enzyme should remain active. However, soaking out of the nucleotide substrate at low temperature results in an altered binary complex made possible by the rotamer change at F749 near the tip of the polymerase O-helix. Surprisingly, two adenosines in the 5′-template overhang fill the vacated active site by stacking with the primer strand, thereby blocking the active site at low temperature. Replacement of the two overhanging adenosines with pyrimidines substantially increased activity at room temperature by keeping the template overhang out of the active site, confirming the importance of base stacking. These results explain the cold-sensitive phenotype of the I707L mutation in KlenTaq1 and serve as an example of a large conformational change affected by a conservative mutation.

When comparing homologous proteins, changes to another member of a group of amino acids with similar chemical side chain characteristics (e.g., hydrophobic, polar and uncharged, polar and charged, aromatic) are often dismissed as inconsequential. Two hydrophobic amino acids, leucine and isoleucine, have the same molecular weight, atomic composition, and volume. These two amino acids differ only in the position of a branching methyl group in the side chain, leucine at the γ carbon and isoleucine at the β carbon.

It is thus remarkable that mutation from isoleucine to leucine at position 707 in DNA polymerase I from Thermus aquaticus resulted in an unusual cold-sensitive phenotype. DNA polymerase I from T. aquaticus, or Taq DNA polymerase, is one of the best characterized DNA polymerases and the first thermostable enzyme to be employed for the polymerase chain reaction (PCR).3 One problem with Taq DNA polymerase as a PCR enzyme is that it remains active at low temperatures, leading to nonspecific priming.4,5 The amplification of nontarget sequences due to mispriming can lead to spurious products and low yields of the target sequence. This can be avoided by decreasing the activity of Taq DNA polymerase at low temperatures during reaction assembly and prior to heating. Kermekchiev and colleagues3 randomly mutated the large (5′-to-3′ exonuclease-deleted) fragment of Taq DNA polymerase (Klenow fragment of Taq DNA polymerase, or KlenTaq1) and screened mutants for low activity at 37 °C while retaining high activity at 68 °C. One mutation, isoleucine 707 to leucine, resulted in a large increase in the ratio of activity at high versus low temperature and a dramatic improvement in amplification of difficult PCR targets.6 According to the crystal structure of the wild-type KlenTaq1,7 this conservative mutation was located near the exterior of the enzyme and over 20 Å away from the polymerase active site (Figure 1A). It was not clear how this mutation, termed Cs3C, could selectively alter the polymerase mechanism at low temperature.

DNA polymerase I enzymes employ a complex induced-fit mechanism to bind and select the complementary nucleotide during DNA synthesis. DNA polymerase I enzymes belong to the A family of DNA polymerases and are shaped like a human hand, with a thumb subdomain that grasps the DNA, a palm subdomain that contains the active site, and a fingers

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Table 1. Crystallographic Statistics

|                          | I707L–DNA–ddCTP | I707L–DNA(AAA) | I707L–DNA(TTT) |
|--------------------------|-----------------|----------------|---------------|
| space group              | P3,21           | P3,21          | P3,21         |
| cell dimensions a, b, c (Å) | 107.68, 107.68, 89.68; α = β = 90°, γ = 120° | 110.04, 110.04, 91.29; α = β = 90°, γ = 120° | 110.03, 110.03, 90.67; α = β = 90°, γ = 120° |
| resolution (Å)           | 32.80–1.67 (1.70–1.67) | 47.65–2.20 (2.26–2.20) | 47.64–2.50 (2.56–2.50) |
| no. of reflections       | 67,931 (2624)   | 32,282 (2330)  | 21,497 (1557) |
| Rwork/Rfree              | 0.161/0.192     | 0.223/0.273    | 0.223/0.286   |
| I/σ(I)                   | 30.2 (2.78)     | 12.0 (2.75)    | 11.6 (2.56)   |
| percent completeness     | 97.3 (76.3)     | 98.5 (96.8)    | 98.4 (97.4)   |
| redundancy               | 3.6 (2.7)       | 3.6 (3.7)      | 4.6 (4.0)     |
| Rsym                     | 0.047 (0.376)   | 0.060 (0.681)  | 0.120 (0.842) |
| atoms in asymmetric unit | 5441            | 4431           | 4723          |
| Root-Mean-Square Deviation |              |                |               |
| bond lengths (Å)         | 0.011           | 0.013          | 0.009         |
| bond angles (deg)        | 1.6             | 1.7            | 1.4           |
| Ramachandran outliers    | 0.2%            | 1.0%           | 1.5%          |
| Protein Data Bank code   | 4N55            | 4N56           | 4XIU          |

"Data for the highest-resolution shell are in parentheses. From Molprobity."
Kinetics of Single Nucleotide Addition. The fluorescent pre-steady-state kinetics assay for nucleotide addition was performed essentially as previously described. Briefly, the fluorescent 6FAMPrimer was mixed with AAGTemplate, TTGTemplate, and CGGTemplate at equimolar concentrations in separate tubes and annealed by heating to 85 °C and slowly cooling to room temperature. To measure the nucleotide incorporation rate of deoxycytosine triphosphate (dCTP) opposite a 5′-XXG-3′ (where XX represents AA, CC, or TT) overhang on a 6-FAM tagged template and primer DNA complex in the I707L mutant and wild-type KlenTaq1 polymerases, separate quench flow assays were performed. The assays were conducted by mixing the enzyme (4 μM)–DNA (0.2 μM) complex with equal volumes of 20 μM dCTP and quenching the reaction at four time points using four reaction volumes of a 95% formamide, 20 mM ethylenediaminetetraacetic acid solution. The enzymes, fluorescent DNA, and dCTP were all diluted from their original concentrations with reaction buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl2). All room-temperature reactions were executed manually on the benchtop. The primer lengths and amounts were measured by capillary electrophoresis with fluorescence detection (Duke University DNA Analysis Facility, Durham, NC), and the results were analyzed using PeakScanner software (Applied Biosystems) to determine the fraction of fluorescent primer extended. KaleidaGraph software (Synergy Software, Reading, PA) was used to fit plots of fraction primer extended versus time to the equation y = (1 − e−kt).

Molecular Dynamics Simulation. The structure of KlenTaq1 DNA polymerase was obtained from the Protein Data Bank (PDB code: 4KTQ). In silico changes were made to modify residue 707 from an isoleucine to a leucine and to match the template sequence to that of the Template oligonucleotide. Explicit hydrogen atoms were added using the tleap module of AmberTools18 followed by the addition of Na+ counterions to neutralize the system. The system was solvated with a truncated octahedron unit cell with TIP3P water molecules19 using a 12.0 Å solvent buffer between the solute and the closest edge of the unit cell for a total atom count of 73 778 atoms. The Amber ff12SB force field19 was applied to the protein and DNA residues. The GPU-accelerated pmemd code19 of Amber 12 was used to perform all minimizations and molecular dynamics (MD). The initial structure was minimized using a seven-step procedure involving 1000 steps of steepest descent minimization followed by 4000 steps of conjugate gradient minimization at each step. Positional restraints on all solute heavy atoms were initially set at 10.0 kcal/mol/Å2 and subsequently reduced at each step until the last minimization was performed without restraints. From the final minimized structure, five simulations were started using different initial random seeds to set the initial velocities. Because of the unknown complexity of the conformational change we were trying to simulate, we decided that using different initial velocities would increase our conformational sampling during MD20 and more quickly lead to the proper orientation of the I707L mutant. Each of the five simulations were separately heated linearly from 10 to 335 K over 2.0 ns while maintaining 10 kcal/mol/Å2 positional restraints on all heavy atoms of the protein and DNA. Once each simulation reached the desired 335 K, they each underwent a 3.5 ns equilibration process where the positional restraints were reduced in logical intervals until the final 500 ps of equilibration was performed without restraints. Unrestrained MD was performed on each simulation for 2.0 μs (a total of 10 μs) at constant pressure (1 atm) and temperature (335 K) maintained with a Langevin thermostat21 using periodic boundary conditions, saving the coordinates, velocities, and energies every 100 ps. The SHAKE algorithm22 was used to fix all covalent bond distances involving hydrogen, allowing a 2 fs time step for each simulation. All simulations were visualized using VMD v.1.9.1,23 and analysis was performed with the cpptraj module24 of AmberTools 13.

I707L KlenTaq1 Simulation. The final structure from the simulation of 4KTQ with the in silico I707L mutation was used to model in the missing residues of the fingers domain in the I707L mutant binary complex crystal structure. To accomplish this, the final MD structure from the 4KTQ-I707L simulation was aligned to the I707L mutant binary complex crystal structure, and the coordinates of the missing regions from simulated structure were saved and inserted into the crystal structure PDB. Next, we followed the same procedure for the 4KTQ-I707L to add hydrogen, neutralize, and solvate to a total of 81 212 atoms using AmberTools. After solvating, the same procedure (as stated for the 4KTQ-I707L simulation) was followed for performing minimization, heating, equilibration, and a total of 2.2 μs of unrestrained MD at 298 K using the GPU-accelerated pmemd code of Amber 12.

RESULTS

To determine how the I707L mutation causes a cold-sensitive phenotype, we co-crystallized the mutant KlenTaq1 DNA polymerase with DNA and a substrate nucleotide using similar procedures as those for wild-type KlenTaq1. The incorporation of a 2′,3′-dideoxyctydine triphosphate (ddCTP) into the 3′-end of the 11 nucleotide primer by the polymerase prevents further elongation of the primer strand and traps the enzyme in a ternary (enzyme–DNA–dNTP) complex with excess ddCTP. To facilitate comparisons between wild-type KlenTaq1 and the I707L mutant, we used an identical DNA template and primer sequence as that in previous studies with wild-type KlenTaq1 that produced 3KTQ.pdb and 4KTQ.pdb, and our crystals, grown under the same conditions, were isomorphous to those crystals. A crystal diffracted to 1.67 Å resolution, and its crystal structure was solved by molecular replacement using 3KTQ.pdb (Table 1). The crystal structure of the I707L mutant polymerase ternary complex is nearly identical to the wild-type ternary structure, 3KTQ.pdb, except at higher resolution (Figure 1A). The improved resolution allowed us to model some external side chains missing in the wild-type KlenTaq1 structure.

While the mutant polymerase ternary complex has an identical backbone structure as that of the wild type, its side chain rotamers differ at two notable residues. Kermekchiev and colleagues noted that isoleucine 707 in the P helix was packed closely with nearby phenylalanine 749 in the Q helix and that a leucine placed in the most similar rotameric state as isoleucine 707 would result in a steric clash with F749. The crystal structure of the I707L mutant KlenTaq1 revealed that the leucine 707 side chain is not in a similar rotameric state as isoleucine 707. The leucine side chain vacates a space occupied by isoleucine’s γ1 and δ carbons, and that space is filled by a rotation of the phenylalanine 749 side chain (Figure 1B). Thus, the change in the location of a side chain methyl group from the γ to δ carbon via mutation changes the conformation of a single neighboring residue in the polymerase ternary complex.
The 2Clantivalent cation with a red dashed line corresponding to complex (3KTQ; yellow). Residue 707 is connected to an active site complex (cyan) superimposed on the wild-type KlenTaq1 ternary complex. (A) The crystal structure of the I707L mutant ternary complex reveals little which is associated with strong anomalous di−ference density map showed the identity was manganese(II) from the crystallization and cryoprotectant solutions. No major deviations were observed between the active sites of the mutant ternary complex and wild-type KlenTaq1 ternary complex in the closed conformation.

To gain additional insight into the cold sensitivity of the I707L mutant, we attempted to determine the structure of the binary polymerase–DNA complex. The substrate ddCTP was removed by soaking a ternary complex crystal in the mother liquor, as previously described for the wild-type KlenTaq1 binary “open” complex (4KTQ.pdb).4 The resultant crystal structure was solved to 2.2 Å using 4KTQ.pdb as a molecular replacement model (Table 1). As expected, most of the polymerase 3′-to-5′ exonuclease domain (inactive), thumb domain, and palm domain retain their structures (Figure 2A). The fingers subdomain and the DNA, however, underwent major rearrangements.

The conservative mutation of isoleucine 707 to leucine resulted in a large conformation change in the polymerase. Unlike the I707L KlenTaq1 ternary complex, the leucine side chain adopts a rotamer pointing inward toward the protein interior in the binary complex, but phenylalanine 749 rotates away (Figure 2B). Phenylalanine 749 lies near the mobile O and O1 helices of the fingers subdomain. In this case, it also appears to function as a fulcrum for the fingers subdomain. Without the stability afforded by the presence of the dNTP substrate in the active site and phenylalanine 749, the fingers subdomain appears to be generally disordered in the I707L KlenTaq1 binary complex. While the electron density for most of the protein is easily interpretable, the electron density for the fingers, particularly portions distal from the protein, is weak and discontinuous (Figure 2B). Some parts could not be modeled (Figure 2B). The temperature factors (B-factors) for the fingers subdomain are much higher than the rest of the protein (Figure 2A). Together, these results suggest that, in the I707L mutant, the fingers subdomain either is destabilized relative to the rest of the protein or adopts multiple conformations.

In addition to alterations in the fingers, the DNA bound to the I707L mutant also adopts a dramatically different structure than two different wild-type binary complexes containing an 5′-AAAG overhang (4KTQ.pdb and 3SZ2.pdb).5,25 Structures of two A family DNA polymerase enzymes show that the template DNA is fed into the active site from a cleft made by the fingers and palm subdomains.5,8 In the I707L mutant binary complex, we observed strong electron density in the active site that appeared to be nucleotides with bases stacked. When modeled into the electron density, the template guanosine to be copied (G) is flapped out away from the active site while the previous two adenosines in the template 5′-overhang fit inside and stack neatly against the cytosine at the primer 3′-terminus (Figure 2C). This unusual DNA conformation coincides with the appearance of a manganese(II) ion near the flipped template guanosine (Figure 2C). The importance of this manganese ion is not clear. In the I707L mutant, the adenosines in the overhang are sandwiched between the primer terminus and phenylalanine 667 in the O helix. This residue plays a role in discrimination against ddNTPs in A family DNA polymerases.26–28 The active site cleft is occupied by the two adenosines in the overhang (A+1 and A+2) and is made larger by a shift in the O helix position away from the palm domain (Figure 2C). This is associated with the alternate rotameric state of phenylalanine 749 (Figure 2B).

In the I707L mutant binary complex, two adenosines blocked the active site by stacking against the primer terminus and phenylalanine 667. The adenine base is much better able to

Figure 1. Crystal structure of I707L KlenTaq1–DNA–dNTP ternary complex. (A) The crystal structure of the I707L mutant ternary complex (cyan) superimposed on the wild-type KlenTaq1 ternary complex (3KTQ; yellow). Residue 707 is connected to an active site divalent cation with a red dashed line corresponding to ~24 Å long. (B) The 2Fobs − Fcal map of the mutant ternary complex near the residue 707 mutation is shown at 1.0σ. (C) The active site, consisting of conserved acidic residues and a ddCTP, contains two metal ions, one of which is associated with strong anomalous difference density (displayed at 4.0σ in magenta) around a divalent cation site. The manganese(II) atom is connected to nearby oxygen atoms with purple dashed lines corresponding to ~2.1 Å long. The interatomic distances between the magnesium(II) atom and surrounding oxygen atoms are also ~2.1 Å.
The addition of 10 μM dCTP to the reaction results in the incorporation of a single nucleotide to the fluorescent primer chain opposite the template G, which is detectable using a capillary electrophoresis sequencer. The wild-type KlenTaq1 showed no substantial change to the rate of nucleotide incorporation at room temperature for all three templates (Table 2), but the I707L mutant KlenTaq1 was substantially slowed by the AA overhang. These results support the hypothesis that base stacking by adenines aids in blocking the active site, but not permanently. In fact the I707L mutant appeared to be slightly faster than wild-type KlenTaq1 for templates with overhanging pyrimidines (Table 2), suggesting that blockage may occur only near pairs of adenines or purines more generally. We did not test a GG overhang in our single-hit assay because multiple deoxyctydines would be incorporated, creating multiple peaks in the electropherogram. These solution kinetics data are consistent with the hypothesis that aromatic stacking contributes to blockage in the I707L KlenTaq1 active site. No manganese was added to the reaction, suggesting that the unusual base stacking in the active site does not require the manganese ion bound near the template guanosine.

On the basis of these results, we hypothesized that pyrimidines in S’-overhangs in the template would base stack poorly and not cause blockage in the active site of I707L KlenTaq1 like adenosines. To test this hypothesis, we co-crystallized I707L KlenTaq1 using a similar template with TTT instead of AAA in the S’-overhang under the same conditions and solved its structure using molecular replacement (Table 1). The resulting TTT-overhang ternary crystal structure with the TTT-overhang is located in a cleft between the fingers and palm domains (Figure 2C), as has been described previously in other A family DNA polymerase I enzymes. We observed no electron density in the active site cleft corresponding to stacked bases from the overhang. Conserved tyrosine 671 is stacked against the last template nucleotide in the duplex, and the overall structure, including that of the fingers subdomain, is nearly identical to that of the open, binary wild-type KlenTaq1 complex (4KTQ.pdb).

Our results indicate that the I707L mutant has a higher tendency than wild-type KlenTaq1 to adopt the blocked conformation in the presence of at least two adenosines in the S’-overhang. We mutated isoleucine 707 of the open, wild-type KlenTaq1 binary complex (4KTQ.pdb) to leucine in silico and ran molecular dynamics (MD) simulations at a high temper-
ature (62 °C) to see if I707L KlenTaq1 would transition to the blocked conformation. Of the five simulations starting from different random initial atomic velocities, one showed a substantial change in structure during the 2 μs simulation time. Although the template overhang does not swing in to block the active site in this simulation, the fingers subdomain moves substantially away from the rest of the polymerase to a position similar to the I707L mutant binary complex (Figure 3A,B). This conformational change opens the active site further and disrupts the last C:G base pair in the n-1 position. The template G along with G_{n-1} base stack with the last C in the primer strand and tyrosine 667 (Figure 3C). The quadruple stack of aromatic rings is similar to the stacking observed in the I707L mutant binary complex (Figure 3A,B). This conformational change opens the active site further and disrupts the last C:G base pair in the n-1 position. The template G along with G_{n-1} base stack with the last C in the primer strand and tyrosine 667 (Figure 3C). The quadruple stack of aromatic rings is similar to the stacking observed in the I707L mutant binary complex (Figure 3A,B). This conformational change opens the active site further and disrupts the last C:G base pair in the n-1 position.

**DISCUSSION**

We employed X-ray crystallography to solve a biochemical mystery. How can a conservative mutation located over 20 Å away from the active site affect activity selectively at low temperature? The I707L KlenTaq1 binary complex structure reveals a plausible explanation for its relatively low activity at low temperature. The conservative mutation of isoleucine 707 to leucine, while distant from the active site, occurred near a critical fulcrum for the mobile O helix of the fingers subdomain. The side chain methyl group, when bonded to the β carbon (isoleucine), allows its neighbor, phenylalanine 749, to adopt an alternate rotamer. The space vacated by the phenylalanine 749 side chain is filled by a movement of the O helix, the O1 helix, and the loop between the two helices. This chain reaction of small movements creates a larger active site cleft that can be occupied by two stacked adenosines from the template overhang, which block the incoming nucleotide and template base from accessing the active site. Pyrimidines, in contrast, base stack relatively poorly compared to adenosines and do not block the active site, according to the I707L mutant crystal structure containing the TTT-overhang (Figure 2C). If the free energy of the blocked state is not substantially lower than that of the unblocked state in the I707L mutant, then both states could be occupied at low and high temperatures. If, however, the transition state energy between the two states is substantial, then the enzyme might be transiently trapped in the blocked state at low temperatures, but less so at high temperatures. This model is supported by the observed slow rate of

![Figure 3.](image_url)
dCTP incorporation by the I707L mutant at room temperature for a template with an AAG 5′-overhang (Table 2) and by the stability of the blocked I707L mutant complex in our room temperature molecular dynamics simulation (Figure 3D). The speed bumps presented by consecutive adenosines or possibly purines could slow the enzyme at low temperatures during PCR reaction assembly to the point that all bound primers produce only short extension products.

The blocked conformation may constitute an alternate conformational state for KlenTaq1. This conformation has been previously observed in a wild-type KlenTaq1 binary complex with 5′-AAAT, 5′-AAA-tetrahydrofuran, and a 5′-AAA-dNaM overhangs, showing the same two adenosines stacked against a primer ddC and F667. Although their crystallization conditions were different than ours and Li and colleagues, these crystals had nearly identical unit cell dimensions in the same space group. The appearance of the blocked conformation in multiple contexts and under multiple conditions suggests that the fingers subdomain of KlenTaq1 is more dynamic than previously thought. Our molecular dynamics simulations of I707L KlenTaq1 also indicate that the fingers subdomain is highly mobile. Although the template did not swing into the active site to block it during the 10 μs of total MD simulation time, the simulations do suggest that the blocked conformation is energetically accessible by I707L KlenTaq1. They also support our hypothesis that blockage is, in part, driven by base stacking in the polymerase active site. These structural studies point to the possibility that blockage of the polymerase active site is dependent on not only the enzyme sequence (as in the I707L mutation) but also the template sequence to be copied. To our knowledge, the blocked conformation has been observed only in KlenTaq1 and not other DNA polymerase I crystal structures.

In conjunction with Kermekchiev et al., our results suggest a complex network of effects from polymerase conformation, primer–template annealing, thermodynamic states, and temperature produced the cold-sensitive phenotype from a template with a 74°C activation for low and high temperatures, conservative mutation distant from the polymerase active site.

A 74°C activity ratio between 4 and 7 and produces much higher yields of difficult-to-amplify target PCR products. We propose that, at low temperature, the I707L mutant is slowed each time it encounters pairs of adenosines (or possibly purines generally) due to the blockage of the active site by the template DNA.

Our molecular dynamics simulation of in silico I707L mutant suggests that pairs of G’s can also stack in the active site like pairs of A’s (Figure 3C). While some primers still attach to nontarget sequences and are extended, this process is slow, resulting in shorter unintended products. These products are less likely to serve as templates for the next round of amplification; thus, fewer spurious PCR products are made. Once the temperature is raised to the annealing temperature for the primer, the primer is more specific for the target sequence (i.e., few primers are bound to incorrect sites). At this point, the short, unintended products made at room temperature during reaction assembly become dead ends for the PCR.

Because only the target sequence is initially copied into a long product, yields are much higher for the I707L mutant KlenTaq1 than that for wild-type KlenTaq1.

We note that, for CCG and TTG template overhangs, the I707L mutant polymerase appears to be substantially faster than wild-type KlenTaq1 (Table 2). This result suggests that, when unblocked, the I707L mutant can copy DNA at a faster rate. Kermekchiev and colleagues also observed higher activity at 74°C for the I707L mutant compared to wild-type KlenTaq1. Together, these data suggest that, when unblocked, the I707L KlenTaq1 is faster than wild type. Our crystal structure of the I707L KlenTaq1 ternary complex showed no major difference to the wild-type ternary complex that could explain the mutant’s faster rate. When I707L KlenTaq1 encounters pairs of adenosines at high temperatures, the enzyme can still become temporarily blocked, but we hypothesize that high temperatures allow the enzyme to return back to the open conformation quickly. Our molecular dynamics simulations suggest that the fingers subdomain of 1707L KlenTaq1 is quite labile at high temperature. The slower rate of DNA synthesis when encountering adenosines is compensated by the faster rates when not encountering adenosines, resulting in a higher overall activity at 74°C.

These results not only explain a cold-sensitive phenotype for a mutant polymerase but also demonstrate large, long-range effects from a conservative mutation. In this specific case, the subtle change in location of a methyl group by one carbon atom with no change in atomic composition caused a chain reaction of effects that destabilized an entire polymerase subdomain and altered the DNA conformation. Because the change is located near a fulcrum for a critical mobile region of the polymerase, the mutation can affect the structure and function of the
enzyme despite the large distance between the mutation and the enzyme active site.

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**ABBREVIATIONS**
KlenTaq1, Klenow fragment of DNA polymerase I from Thermus aquaticus; F, phenylalanine; I, isoleucine; L, leucine; PCR, polymerase chain reaction; dNTP, 2′-deoxynucleoside triphosphate; dDTTP, 2′,3′-dideoxythidine triphosphate; 6FAM, 6-carboxyfluorescein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PEG, poly(ethylene glycol); A, adenosine; C, cytidine; G, guanosine; T, thymidine; B-factor, temperature factor; nm, nanometer

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