Mutations in human DNA polymerase γ confer unique mechanisms of catalytic deficiency that mirror the disease severity in mitochondrial disorder patients

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Human mitochondrial DNA polymerase γ (pol γ) is solely responsible for the replication and repair of the mitochondrial genome. Unsurprisingly, alterations in pol γ activity have been associated with mitochondrial diseases such as Alpers syndrome and progressive external ophthalmoplegia. Thus far, predicting the severity of mitochondrial disease based on the magnitude of deficiency in pol γ activity has been difficult. In order to understand the relationship between disease severity in patients and enzymatic defects in vitro, we characterized the molecular mechanisms of four pol γ mutations, A957P, A957S, R1096C and R1096H, which have been found in patients suffering from aggressive Alpers syndrome to mild progressive external ophthalmoplegia. The A957P mutant showed the most striking deficiencies in the incorporation efficiency of a correct deoxyribonucleotide triphosphate (dNTP) relative to wild-type pol γ, with less, but still significant incorporation efficiency defects seen in R1096H and R1096C, and only a small decrease in incorporation efficiency observed for A957S. Importantly, this trend matches the disease severity observed in patients very well (approximated as A957P ≫ R1096C ≥ R1096H ≫ A957S, from most severe disease to least severe). Further, the A957P mutation conferred a two orders of magnitude loss of fidelity relative to wild-type pol γ, indicating that a buildup of mitochondrial genomic mutations may contribute to the death in infancy seen with these patients. We conclude that characterizing the unique molecular mechanisms of pol γ deficiency for physiologically important mutant enzymes is important for understanding mitochondrial disease and for predicting disease severity.

INTRODUCTION

Human mitochondrial DNA polymerase γ (pol γ) is fully responsible for the replication and repair of the circular, 16.5 kb human mitochondrial genome, which contains 37 genes involved primarily in oxidative phosphorylation (1). The pol γ holoenzyme is a heterotrimer made up of a catalytic subunit, which includes DNA polymerase, 3′ to 5′ exonuclease and 5′-deoxyribose phosphate lyase activities, and an accessory dimer, which is important for processivity, and contains two magnesium ions (2–6). Deficiencies in the activity of pol γ have been linked to mitochondrial disorders, which are known to affect one in 5000 Americans (7). Thus far, more than 160 mutations in pol γ and many single-nucleotide polymorphisms have been found in patients with mitochondrial disease (http://tools.niehs.nih.gov/polg/) (8,9). Point mutations in pol γ are commonly linked to Alpers syndrome (10), a disorder characterized by seizures, neurological deterioration and liver failure and usually causes death in early childhood, and progressive external ophthalmoplegia (PEO) (11), which is associated with ptosis, myopathy and exercise intolerance with an onset in adulthood. Pol γ mutations have also been implicated in parkinsonism (12), breast cancer (13), male infertility (14), depression (15) and mitochondrial toxicity.

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upon treatment with anti-HIV therapy (16–18). Thus far, no treatment is available for mitochondrial disorders. The molecular mechanism of how pol γ mutation affects catalysis has been characterized for only a few physiologically important mutations (19–25) and prediction of disease severity based on modeling mutation location has been proposed (26). Characterizing these molecular mechanisms can aid in elucidating the cause and progression of these mitochondrial disorders and may help predict mitochondrial disease severity.

We have focused on four physiologically important mutations found in the polymerase domain of the pol γ catalytic subunit, R1096H, R1096C, A957S and A957P. These mutants allow us the unique opportunity to examine point subunit, R1096H, R1096C, A957S and A957P. These mutants allow us the unique opportunity to examine point mutations (19–24), this study is important because all four mutations have been identified, supporting clinical findings, but dedicated studies probing the mode of catalytic deficiencies have not yet been carried out.

To understand how these individual mutations affect pol γ catalysis, we undertook a series of steady-state and pre-steady-state studies examining the ability of pol γ to bind and incorporate a dNTP into a DNA substrate. Interestingly, the defects in the efficiency of dNTP incorporation for each of the four mutants corresponded well to the observed disease severity (A957P ≫ R1096C ≥ R1096H ≫ A957S, most to least severe disease), and a significant loss of fidelity was observed with the A957P mutant. Thus, we have elucidated how each of these four mutations uniquely alter pol γ catalysis, and these resulting molecular mechanisms can be used to help explain the observed range of disease severity. While others have used similar methods to characterize pol γ mutants (19–24), this study is important because all four mutations have been identified in mitochondrial disorders with varying severity (despite affecting two proximal amino acids), and because we show significant and unique alterations in enzymatic activity which allow us to draw general conclusions about the types of kinetic mechanisms capable of propagating disease. Thorough characterization of such mechanisms can be useful in predicting mitochondrial disease severity.

RESULTS

A modest change in affinity for a DNA template is seen for the pol γ mutants

Since both A957 and R1096 are close to the strand of template DNA (Fig. 1), steady-state kinetics were used to determine the dissociation constant, $K_d$, for DNA binding to the pol γ catalytic subunit. Using an electrophoretic mobility shift assay (EMSA), varying concentrations of the WT and mutant pol γ catalytic subunits were incubated with a radiolabeled human pol γ holoenzyme (3IKM) (2) and another A family DNA polymerase member, the bacteriophage T7 DNA polymerase, which was solved in complex with DNA and ddATP (1SKR) (36). The A957 residue in particular is close to the incoming ddATP and is just opposite Y955 (Fig. 1), which is known to be part of a hydrophobic pocket that aids incoming dNTP binding (19,22,37). Since both R1096 and A957 are proximal to the DNA template strand in this model (Fig. 1) (38), it is easy to conceive that mutations at either of these positions could result in deficiencies in incorporating the incoming dNTP and binding the DNA substrate.

Kinetic characterization, which is vital to understand the molecular mechanism of mitochondrial disease, has not been undertaken for the R1096C, R1096H or A957P mutations. Our previous work on the A957S mutant using steady-state kinetics showed ~1.7-fold loss of incorporation efficiency when compared with wild type pol γ (WT pol γ) but little change in processivity (25). In contrast, when expressing constructs in Saccharomyces cerevisiae that contained each of these four mutations in its pol γ homolog, Mip1, we observed 100% petite colony formation (indicative of high mitochondrial dysfunction) for all but the A957S mutation, which showed 13% petite colony formation (39). Thus, some impairment of polymerase function due to each of the four mutations has been identified, supporting clinical findings, but dedicated studies probing the mode of catalytic deficiencies have not yet been carried out.

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D34/D38 primer/template substrate. Native polyacrylamide gel electrophoresis was used to separate the free and bound DNA, and the larger molecular weight shift, indicative of the DNA/enzyme complex, was quantified using phosphorimaging. Both the A957P and A957S mutants showed essentially no change in affinity for the DNA primer/template substrate relative to WT pol γ, while the R1096H and R1096C mutants showed only 1.8- and 1.5-fold lower affinities (Table 1).

The pol γ mutants show catalytic defects through different mechanisms

The molecular mechanisms of catalytic deficiencies of the mutants relative to WT pol γ were characterized using pre-steady-state kinetics. In a pre-steady-state burst experiment, pol γ holoenzyme and an excess of a radiolabeled D23/D45 primer/template substrate were mixed with MgCl₂ and dCTP (the next correct nucleotide) using a rapid chemical quench apparatus. Pre-incubation steps prior to the rapid mixing removed complications from rates associated with the binding of the accessory and catalytic pol γ subunits, and the DNA primer/template substrate binding to the pol γ holoenzyme. The products were separated on a denaturing 20% polyacrylamide gel and quantified using phosphorimaging. A plot of the amount of product versus time generated

<table>
<thead>
<tr>
<th>Catalytic subunit</th>
<th>Kₐ (nm)</th>
<th>Kₐ (WT pol γ)/Kₐ (mutant pol γ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>65 ± 3</td>
<td>—</td>
</tr>
<tr>
<td>R1096H</td>
<td>120 ± 11</td>
<td>0.54</td>
</tr>
<tr>
<td>R1096C</td>
<td>95 ± 18</td>
<td>0.68</td>
</tr>
<tr>
<td>A957S</td>
<td>61*</td>
<td>1.1</td>
</tr>
<tr>
<td>A957P</td>
<td>67 ± 1</td>
<td>0.97</td>
</tr>
</tbody>
</table>

*From Graziewicz et al. (25).

a distinctive biphasic burst curve, since the overall rate-limiting step for the WT pol γ holoenzyme is the release of pyrophosphate that occurs after chemical catalysis. The burst phase is dependent on nucleotide concentration and indicates the rate constant of a single-nucleotide incorporation, and the second phase signifies the steady-state rate constant which is product release. Like WT pol γ, all four mutant holoenzymes displayed burst kinetics (data not shown), indicating that the overall rate-limiting step of the catalytic cycle still occurs after polymerization.

Since both A957 and R1096 are located in the polymerase domain of the catalytic subunit of pol γ (Fig. 1), we focused on the effects of the mutations on the dissociation constant of the incoming nucleotide, Kₐ, and the maximum rate of incorporation, kₙ. Using single turnover experiments in which enzyme concentration is in excess to the radiolabeled D23/D45 primer/template substrate, the ability of each enzyme to incorporate a single, correct nucleotide (dCMP opposite dG) was assayed. The pol γ holoenzyme and the radiolabeled DNA primer/template substrate were mixed with MgCl₂ and varying concentrations of dCTP using a rapid chemical quench apparatus, and the products were separated and quantified as described for the burst experiments. The plots shown in Figure 2 were either fit to single exponential equations in the case of the WT pol γ, R1096H, and A957S, or double exponential equations for R1096C and A957P. The A957P mutant misincorporated a second dCTP opposite dT at significant rates, so the sum of the first nucleotide incorporation (N⁺¹) and the second nucleotide incorporation (N⁺²) products were used to determine the total amount of product formed (Fig. 2E). The resulting kobs values obtained from these fits were plotted against nucleotide concentration (Fig. 3) in order to obtain kₙ and Kₐ values. The R1096H mutant displayed the largest change in the incorporation rate, with a 32-fold decrease in kₙ relative to WT pol γ, while the A957P mutant showed a 2.1-fold decrease and the R1096C and A957S mutants indicated minimal changes (1.2- and 1.3-fold increases, respectively) (Table 2). Changes in incoming nucleotide affinity ranged in magnitude, with the A957S, R1096C and A957P mutants showing 2.4-, 4.3- and 29-fold increases in Kₐ. Interestingly, the R1096H mutant, which had the largest loss in incorporation rate, had a 6.8-fold decrease in Kₐ, indicating an increased affinity for the incoming nucleotide. Overall, catalytic deficiencies in the A957S and R1096C mutants came from a loss of affinity of the incoming nucleotide, while R1096H showed a significantly slower maximum rate of nucleotide incorporation, and A957P showed deficiencies in both kₙ and Kₐ.
The magnitude of effects on correct nucleotide incorporation efficiencies are relative to disease severity

While the individual rate constants do not indicate an apparent pattern of catalytic deficiency for the mutants, the most informative rate constant, \( k_{\text{cat}}/K_d \), which is a measure of enzyme efficiency, indicated a clear trend of the ability of each mutant to incorporate a correct nucleotide. The A957S mutant holoenzyme showed the least deficiency in catalysis, with only a 1.8-fold loss in incorporation efficiency compared with WT pol \( \gamma \) holoenzyme, while the R1096C and R1096H mutant holoenzymes had 3.4- and 4.7-fold lower incorporation efficiencies (Table 2). The A957P mutant showed the most severe catalytic defects, with a 60-fold lower nucleotide incorporation efficiency relative to WT pol \( \gamma \) (Table 2). Importantly, this ranking of in vitro catalytic defects in incorporation efficiency, A957P \( \gg \) R1096H \( \geq \) R1096C \( \geq \) A957S (from most defective to least defective), corresponds well to the relative disease severity observed in patients, which can be ranked as A957P \( \gg \) R1096C \( \geq \) R1096H \( \gg \) A957S from most severe to least severe disease (vide supra).
The A957P mutant is capable of incorporating a second, incorrect nucleotide

The ability of A957P to incorporate a second, incorrect nucleotide was observed in the single-nucleotide incorporation studies (Fig. 2E). To ensure this was not an artifact of the higher concentrations of dNTP needed due to the high $K_d$ measured for A957P, we performed single incorporation experiments using identical conditions for all the WT pol and four mutant holoenzymes. Using the single turnover experimental conditions described for the correct incorporation experiments, the ability of the pol holoenzymes to incorporate multiple dCTPs into the radiolabeled D23/D45 primer/template substrate was assayed. In this case, the first incorporation ($N^{+1}$) is correct (dCMP opposite dG), and the second incorporation ($N^{+2}$) is a dCMP opposite dT misincorporation. The gel analysis showed that despite the extended duration of the incubations and the presence of high dCTP concentrations, only A957P was able to form the $N^{+2}$ mismatch in significant amounts. To quantify the relative abilities of the enzymes to generate the $N^{+2}$ mismatch, the amount of the $N^{+2}$ mismatch was expressed as a percentage of the amount of the $N^{+1}$ match. In this case, the $N^{+2}$ product was 11, 11, 18 and 23% of the $N^{+1}$ product for R1096H, A957S, R1096C and WT pol $\gamma$, respectively, indicating measurable, but limited ability of incorporating a mismatch (Supplementary Material, Fig. S2). However, for the A957P mutant, the $N^{+2}$ mismatch was 110% of the $N^{+1}$ match, indicating that more of the misincorporation product is formed relative to the correct incorporation product, implying a severe loss of fidelity for this mutant.

A significant loss of fidelity is seen for the A957P mutant

To further probe the loss of fidelity with the A957P mutant, misincorporation was directly assayed using pre-steady-state kinetics. Under single turnover conditions, both pyrimidine-pyrimidine mismatches and pyrimidine:purine mismatches...
were explored. Similar to the single, correct nucleotide incorporation assays, an excess of WT pol γ or A957P holoenzyme relative to a radiolabeled DNA primer/template substrate was rapidly mixed with MgCl₂ and varying concentrations of dTTP. For the pyrimidine:purine mismatch studies, the pre-steady-state rate constants of misincorporation of dTTP into the radiolabeled D23/D45 primer/template substrate were measured. Representative gels are shown in Figure 4A and B, showing the relative formation of the first misincorporation product (N²,dTTP opposite dG), and the formation of a second misincorporation product (N³,dTTP opposite dT) by WT pol γ and A957P under similar conditions. Even though WT pol γ holoenzyme was able to form the N¹⁺ mismatch, no further misincorporation products were seen. This is in contrast to the A957P mutant, and here the sum of the N¹⁺ and N²⁺ products was used to determine the amount of total products formed. The results of these experiments at multiple dTTP concentrations is shown in Figure 5A and B, where the individual traces are fit to single exponential equations. The kobs values obtained from these equations were plotted against dTTP concentration to obtain kpol and Kd values for misincorporation (Fig. 6A and B, Table 3). While there is little change in the rate of misincorporation, the A957P mutant has a 5.2-fold lower affinity for the incoming nucleotide, resulting in a 4.8-fold lower efficiency of incorrect nucleotide incorporation for the A957P mutant relative to WT pol γ (Table 3). While this initially seems to contradict the findings of lower fidelity for the A957P mutant (Fig. 4), it is important to recall the 60-fold loss of efficiency for the incorporation of a correct nucleotide by the A957P mutant. Thus, in order to compare the propensity of an enzyme to incorporate an incorrect nucleotide, the fidelity, which is the efficiency of incorporation of a correct nucleotide divided by the efficiency of incorporation of an incorrect nucleotide, must be compared. Using the previously determined efficiency of correct incorporation of dCTP opposite dG for this particular radiolabeled DNA primer/template substrate (Fig. 3, Table 2), a 12-fold loss of fidelity was seen for the A957P mutant relative to WT pol γ (Table 3).

To determine the rate of incorporation of a pyrimidine:pyrimidine mismatch, a similar experiment was undertaken in which the rate of insertion of a dTTP into the radiolabeled D21/D36 primer/template substrate was measured for A957P and WT pol γ holoenzymes. Representative gels of one concentration of dTTP are shown in Figure 4C and D. In this case, the A957P holoenzyme incorporated the first dTTP opposite dT mismatch (N¹⁺), followed by a second dTTP opposite dG mismatch (N²⁺) at significant rates (Fig. 4D), while the WT pol γ formed primarily the first dTTP opposite dT mismatch (N¹⁺, Fig. 4C) in addition to trace amounts of an N³⁺ product, which is the result of the two mismatches followed by a correct dTTP opposite dA match. Plots of the amount of total products versus time are shown in Figure 5C and D, and the resulting kobs values were plotted against dTTP concentration to generate kpol and Kd values (Fig. 6C and D). Here, due to favorable changes in both kpol and Kd, there was a modest 1.7-fold increase in the efficiency of incorporation of an incorrect nucleotide by A957P relative to WT pol γ (Table 3). In order to compare relative fidelities of the dTTP:dT misincorporation, the incorporation efficiencies of dATP insertion opposite dT for this radiolabeled DNA primer/template substrate were measured for both WT pol γ and A957P (data not shown). Values for kpol and Kd for WT pol γ were 220 ± 16 s⁻¹ and 3.2 ± 0.7 μM, respectively, for an incorporation efficiency of 69 μM⁻¹ s⁻¹ for dAMP:dT. The A957P mutant had kpol and Kd values of 56 ± 3 s⁻¹ and 190 ± 38 μM for dAMP:dT, respectively, for an efficiency of 0.30 μM⁻¹ s⁻¹. Using these data, a striking 390-fold loss of fidelity was seen for the A957P mutant when compared with WT pol γ (Table 3). For both pyrimidine:pyrimidine and pyrimidine:purine mismatches, A957P shows a significant loss of fidelity, ranging from a 12- to 390-fold change in discrimination.

**DISCUSSION**

We used steady-state and pre-steady-state kinetics to examine the molecular mechanisms of catalytic dysfunction of four pol γ catalytic subunit mutants, A957S, A957P, R1096C and R1096H, and determined how the range of catalytic dysfunction related to the spectrum of disease severity seen in patients with these mutations. Molecular mechanisms of deficiency for polymerase domain mutations such as these can include alteration in the affinity for a DNA substrate or the incoming dNTP, defects in the maximum rate of incorporation and the loss of fidelity. Such mechanisms for mutations in other pol γ domains can include modifications in the exonuclease rate, kexo, for both correct and incorrect incorporations as seen in the exonuclease domain mutant R232G (24), or changes in accessory subunit affinity like the linker domain (critical for subunit interactions) mutant A467T (20).

Despite the proximity of A957 and R1096 to the DNA substrate (Fig. 1), the affinity of the mutant pol γ catalytic subunits for a DNA primer/template varied at most 1.8-fold (Table 1). It is likely that the DNA strand makes numerous contacts with the enzyme, and perhaps any disruption in contacts with the template strand due to point mutation is mitigated by these other interactions. Similarly, minimal changes in affinity for DNA have been seen with residues near A957 and R1096; for example, various mutations at Y955, which is just opposite A957 (Fig. 1), resulted in only 1.8- to 2-fold changes in affinity for DNA relative to WT pol γ (40). Even lower changes in affinity for DNA were seen for other polymerase domain mutants, including G517V (41), T851A, R853Q, Q897H and T885S (21). These findings further support our hypothesis that the multiple sites of contact of

Table 2. Kinetic parameters for the correct nucleotide incorporation by WT pol γ and mutant holoenzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>kpol s⁻¹</th>
<th>Kd μM</th>
<th>Efficiency, μM⁻¹ s⁻¹</th>
<th>Fold loss of efficiency relative to WT pol γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>72 ± 3</td>
<td>2.1 ± 0.2</td>
<td>34</td>
<td>1.0</td>
</tr>
<tr>
<td>R1096H</td>
<td>2.22 ± 0.08</td>
<td>0.31 ± 0.05</td>
<td>7.2</td>
<td>4.7</td>
</tr>
<tr>
<td>R1096C</td>
<td>89 ± 5</td>
<td>9 ± 2</td>
<td>9.9</td>
<td>3.4</td>
</tr>
<tr>
<td>A957S</td>
<td>97 ± 5</td>
<td>5.0 ± 0.8</td>
<td>19</td>
<td>1.8</td>
</tr>
<tr>
<td>A957P</td>
<td>35 ± 3</td>
<td>61 ± 16</td>
<td>0.57</td>
<td>60</td>
</tr>
</tbody>
</table>
the DNA with the pol γ catalytic subunit can help overcome some disruption resulting from a point mutation.

Pre-steady-state kinetics were used to determine the ability of each mutant to incorporate a correct nucleotide into a DNA primer/template substrate. The incorporation efficiency rate constant is most informative for reporting on overall catalytic defects since it takes into account changes in both kcat and Kd. Importantly, the magnitude of defects in the efficiency of incorporation of a correct nucleotide mirrored the relative disease severity observed in patients (ranked as A957P ≫ R1096C ≫ R1096H ≫ A957S from most severe to least severe disease), suggesting that characterizing the molecular mechanisms of mutants can be useful in predicting disease severity. Once caveat is that some mutations are known to affect dNTP selectivity, as this mutant does not have altered fidelity (Supplementary Material, Fig. S2.) Since R1096 should not directly interact with the incoming dNTP (Fig. 1), any structural changes from accommodating a cysteine or histidine at R1096 are probably propagated to the active site to affect dNTP binding. Overall, these two particular mutations show that even when the same amino acid is affected, the molecular mechanism used to confer catalytic defects may be quite different. This is in contrast to an interesting pre-steady-state study on the H932Y pol γ mutant, which is associated with PEO and sensory ataxic neuropathy dysarthria and ophthalmoparesis, and H932A, which has not been observed in humans. The H932A and H932Y mutants behaved similarly, showing no change and a 1.3-fold change in kcat, respectively, with the significant changes in efficiency coming from 150-fold and 56-fold changes in Kd, respectively (23).

When comparing the A957 mutants, A957S showed essentially no change in kcat and a modest loss of dNTP affinity, while the A957P mutant showed defects in both kcat and Kd, although the loss of affinity was much more significant than the slower rate of incorporation. The 29-fold change in affinity for A957P is unsurprising due to the structural alteration caused by a proline mutation, and due to its proximity to the hydrophobic pocket (formed by Y955, Y951 and E895) which is critical for binding the incoming dNTP (10). Similarly, a more significant change in Kd relative to kcat was also seen for the nearby Y955C mutant, generating a 1.5-fold decrease in kcat and a 77-fold decrease in affinity for the insertion of dTTP opposite dA (37). Also, steady-state studies on Y955C showed a large increase in Km that dwarfed the deficiencies observed in kcat (19,22).

This hydrophobic pocket formed by Y55, Y51 and E895 has been shown to be important for fidelity in addition to binding the incoming dNTP (10,22). It is thus not surprising that the structurally disruptive proline mutation at A957, which is just opposite this hydrophobic pocket, would result in a drastic loss of fidelity relative to the other mutants (Fig. 4 and Supplementary Material, Fig. S2). Here, fidelity decreased 12-fold (for a pyrimidine:purine mismatch, compare 16 000 to 1300) and 390-fold (for a pyrimidine:pyrimidine mismatch, compare 18 000 to 460) for the A957P mutant relative to WT pol γ (Table 3), and multiple insertions of mismatches were possible (Fig. 4 and Supplementary Material, Fig. S2). This indicates that while WT pol γ is better at discriminating against pyrimidine:pyrimidine mismatches, A957P is better at discriminating against pyrimidine:purine mismatches relative to pyrimidine:pyrimidine mismatches (Table 3). In the absence of a crystal structure, it is difficult to speculate on the cause for this change in discrimination types. It is possible that structural changes caused by the proline alter the space available for an incoming purine dNTP relative to a pyrimidine dNTP. Indeed, the pol γ A957P mutation was able to correctly incorporate a dCTP (Table 2) relative to a correct dATP (Table 3) with ~2-fold higher efficiency. Interestingly, studies on the Y955C mutation, which is associated with early-onset PEO and parkinsonism, showed a similar pattern in fidelity as A957P. The Y955C mutant showed a 6.4-fold change for a pyrimidine:purine

Figure 4. Tendency of A957P to incorporate additional nucleotides relative to WT pol γ. The corresponding primer/template (with each primer/template incorporation labeled as N+1, N+2, N+3) is shown for clarity. For (A) and (B), N+3 and N+2 signify the misincorporation of dTTP opposite dG and dTTP opposite dT, respectively. For (C) and (D), N+1 corresponds to the misincorporation of dTTP opposite dT, N+2 is the misincorporation of dTTP opposite dG and N+3 is the correct incorporation of dTTP opposite dA. Timepoints at each lane are shown in seconds. (A) Incorporation of 1 mM dTTP by WT pol γ. Two skipped lanes were removed. The only product is dTTP opposite dG. (B) Incorporation of 1 mM dTTP by A957P. The primary product is dTTP opposite dG. (C) Incorporation of 0.65 mM WT pol (R1096C), to a 32-fold decrease (R1096H). An even larger discrepancy between the mutants is seen when measuring dNTP affinity; the R1096H mutant shows a 6.8-fold increase, while the R1096C mutant shows a 4.3-fold decrease. (However, the strong affinity seen in the R1096H mutant does not affect dNTP selectivity, as this mutant does not have altered fidelity (Supplementary Material, Fig. S2.) Since R1096 should not directly interact with the incoming dNTP (Fig. 1), any structural changes from accommodating a cysteine or histidine at R1096 are probably propagated to the active site to affect dNTP binding. Overall, these two particular mutations show that even when the same amino acid is affected, the molecular mechanism used to confer catalytic defects may be quite different. This is in contrast to an interesting pre-steady-state study on the H932Y pol γ mutant, which is associated with PEO and sensory ataxic neuropathy dysarthria and ophthalmoparesis, and H932A, which has not been observed in humans. The H932A and H932Y mutants behaved similarly, showing no change and a 1.3-fold change in kcat, respectively, with the significant changes in efficiency coming from 150-fold and 56-fold changes in Kd, respectively (23).

When comparing the A957 mutants, A957S showed essentially no change in kcat and a modest loss of dNTP affinity, while the A957P mutant showed defects in both kcat and Kd, although the loss of affinity was much more significant than the slower rate of incorporation. The 29-fold change in affinity for A957P is unsurprising due to the structural alteration caused by a proline mutation, and due to its proximity to the hydrophobic pocket (formed by Y955, Y951 and E895) which is critical for binding the incoming dNTP (10). Similarly, a more significant change in Kd relative to kcat was also seen for the nearby Y955C mutant, generating a 1.5-fold decrease in kcat and a 77-fold decrease in affinity for the insertion of dTTP opposite dA (37). Also, steady-state studies on Y955C showed a large increase in Km that dwarfed the deficiencies observed in kcat (19,22).

This hydrophobic pocket formed by Y55, Y51 and E895 has been shown to be important for fidelity in addition to binding the incoming dNTP (10,22). It is thus not surprising that the structurally disruptive proline mutation at A957, which is just opposite this hydrophobic pocket, would result in a drastic loss of fidelity relative to the other mutants (Fig. 4 and Supplementary Material, Fig. S2). Here, fidelity decreased 12-fold (for a pyrimidine:purine mismatch, compare 16 000 to 1300) and 390-fold (for a pyrimidine:pyrimidine mismatch, compare 18 000 to 460) for the A957P mutant relative to WT pol γ (Table 3), and multiple insertions of mismatches were possible (Fig. 4 and Supplementary Material, Fig. S2). This indicates that while WT pol γ is better at discriminating against pyrimidine:pyrimidine mismatches, A957P is better at discriminating against pyrimidine:purine mismatches relative to pyrimidine:pyrimidine mismatches (Table 3). In the absence of a crystal structure, it is difficult to speculate on the cause for this change in discrimination types. It is possible that structural changes caused by the proline alter the space available for an incoming purine dNTP relative to a pyrimidine dNTP. Indeed, the pol γ A957P mutation was able to correctly incorporate a dCTP (Table 2) relative to a correct dATP (Table 3) with ~2-fold higher efficiency. Interestingly, studies on the Y955C mutation, which is associated with early-onset PEO and parkinsonism, showed a similar pattern in fidelity as A957P. The Y955C mutant showed a 6.4-fold change for a pyrimidine:purine
mismatch, and a 26- to 120-fold change in fidelity for pyrimidine:pyrimidine mismatches relative to WT pol γ, although unlike A957P, the Y955C mutant was still overall able to discriminate against pyrimidine:pyrimidine mismatches better than pyrimidine:purine mismatches (37). Still, the loss of fidelity seen in A957P for a pyrimidine:pyrimidine mismatch was up to about 3.3-fold higher than in the Y955C mutant. The cause for the striking changes in pyrimidine:pyrimidine mismatches for these two polymerase domain mutants warrants further investigation. Other work on the fidelity of pol γ mutations shows much less severe discrimination alterations relative to WT pol γ, such as the H932Y mutant which yielded a range of essentially no change to a 2.9-fold change for various mismatches (23), and G923D and R943H (both associated with PEO) which indicated 2.4-fold and 3.5-fold change in fidelity, respectively, in steady-state studies (25). Thus to our knowledge, the defects in fidelity seen in the A957P mutant are some of the most significant observed to date. The absence of introns in the mitochondrial genome means such mutations are likely to affect the protein. Further, there currently is no evidence for mismatch repair activity or nucleotide excision repair in mammalian mitochondria. Such a drastic drop in fidelity can lead to the transcription and translation of defective enzymes of oxidative phosphorylation, the primarily protein products of the mitochondrial genome. As described previously, patients with the A957P mutation typically die in early infancy (28–30), and this may be due in part to a buildup of oxidative phosphorylation defects resulting from a loss of pol γ fidelity. Indeed, severe defects in ATP production were observed in patients (29) as well as elevated lactate levels in the brain (30). The infidelity combined with the severe defects in correct nucleotide incorporation efficiency (Table 2) seen in the A957P mutant provide a mechanism for explaining why this mutation yields the most severe mitochondrial disease relative to the other three mutations.

In this work, we selected four pol γ mutants based on their clinical importance; all four have been associated with mitochondrial disorders of varying severity. Importantly, the four mutations affect the same or nearby amino acids, allowing us to kinetically probe the range of differences in molecular mechanism of disease at a single site. The incorporation efficiency of the A957S, A957P, R1096C and R1096H mutants showed defects that followed the trend of disease severity seen in patients with these mutations, with the A957P mutant also showing a dramatic loss of fidelity. By characterizing these unique mechanisms of pol γ deficiency, we were able to clarify why the observed disease states are so varied despite affecting the same or nearby amino acids. Our work indicates that determining these molecular mechanisms of disease may be useful in predicting disease severity.
Figure 6. Concentration dependence of incorrect nucleotide incorporation on the observed rate. The observed rate constants obtained from Figure 5 were fit to a hyperbolic equation to generate $k_{pol}$ and $K_d$ values, and the standard error is the deviance from the hyperbolic fits. (A) WT pol γ, dTTP opposite dG misincorporation. $k_{pol} = 0.34 ± 0.01 \text{s}^{-1}$, $K_d = 165 ± 15 \mu M$, efficiency = 0.0021 $\mu M^{-1} \text{s}^{-1}$. $K_d = 860 ± 110 \mu M$, efficiency = 0.00044 $\mu M^{-1} \text{s}^{-1}$. (C) WT pol γ, dTTP opposite dT misincorporation. $k_{pol} = 0.074 ± 0.004 \text{s}^{-1}$, $K_d = 193 ± 36 \mu M$, efficiency = 0.00038 $\mu M^{-1} \text{s}^{-1}$. (D) A957P, dTTP opposite dT misincorporation. $k_{pol} = 0.157 ± 0.004 \text{s}^{-1}$, $K_d = 240 ± 19 \mu M$, efficiency = 0.00065 $\mu M^{-1} \text{s}^{-1}$.

MATERIALS AND METHODS

Reagents

The nucleotides dTTP, dATP and dCTP were purchased from GE Healthcare. The DNA oligonucleotides D21 (5′-TCA GGT CCC TGT TCG GGC GCC-3′), D36 (3′-CGA AAG TCC AGG GAC AAG CCC GCG GTG ACG ATC TCT-5′), D23 (5′-GCC TCG CAG CCG FCC AAG CT-3′) and D45 (3′-CGG AGC GTC GGC AGG TTG GTT GAG TTG GAG CTA GGT TAC GGC AGG-3′) were labeled at the 5′ terminus and were used without further purification. The D38 was confirmed by sequencing the pol γ catalytic subunit cDNA, a pQVSL11.4 baculoviral transfer vector encoding p140 exo- without its mitochondrial targeting sequence (44). The oligonucleotides containing the point mutations (underlined sequence) for introducing mutations in pol γ catalytic subunit cDNA, a pQVSL11.4 baculoviral transfer vector encoding p140 exo- were purchased from GE Healthcare. The DNA oligonucleotides D21 (5′-TCA GGT CCC TGT TCG GGC GCC-3′), D36 (3′-CGA AAG TCC AGG GAC AAG CCC GCG GTG ACG ATC TCT-5′), D23 (5′-GCC TCG CAG CCG FCC AAG CT-3′) and D45 (3′-CGG AGC GTC GGC AGG TTG GTT GAG TTG GAG CTA GGT TAC GGC AGG-3′) were labeled at the 5′ terminus with [γ-32P] ATP (Perkin Elmer) using T4 polynucleotide kinase (New England Biolabs), and annealed to the D36 and D45 templates, respectively, as described previously (42). For the EMSA studies, the DNA oligonucleotides D34 (5′-GTA TGT TCG CCT GCT ATC ATG AGC GTA GTT GCC GCC A-3′) and D38 (3′-TCA TAC ACG GCC ACA TTA AAT CTT GCA TCC ACC CTA TT-5′) were obtained from IDT and were used without further purification. The D38 was labeled at the 5′ terminus with [γ-32P] ATP using T4 polynucleotide kinase and annealed to the D34.

Expression and purification of proteins

The WT accessory subunit of pol γ was expressed and purified in Escherichia coli as described previously (43). The exonuclease-deficient (exo-) pol γ catalytic subunit (denoted in this work as WT pol γ), which was previously characterized for WT catalytic fidelity (5), was expressed and purified as described elsewhere, except a single nickel column using a 20–400 mM imidazole linear gradient for pol γ elution was used for purification (25). The enzymes were analyzed for purity using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) (Supplementary Material, Fig. S1). The mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene) to introduce changes into the pol γ catalytic subunit cDNA, a pQVSL11.4 baculoviral transfer vector encoding p140 exo- without its mitochondrial targeting sequence (44). The oligonucleotides containing the point mutations (underlined sequence) for introducing mutations in pol γ catalytic subunit cDNA, a pQVSL11.4 baculoviral transfer vector encoding p140 exo- were purchased from GE Healthcare. The DNA oligonucleotides D21 (5′-TCA GGT CCC TGT TCG GGC GCC-3′), D36 (3′-CGA AAG TCC AGG GAC AAG CCC GCG GTG ACG ATC TCT-5′), D23 (5′-GCC TCG CAG CCG FCC AAG CT-3′) and D45 (3′-CGG AGC GTC GGC AGG TTG GTT GAG TTG GAG CTA GGT TAC GGC AGG-3′) were labeled at the 5′ terminus and were used without further purification. The D38 was confirmed by sequencing the pol γ insert in the baculovirus transfer vector. We have described the generation of the A957S mutant elsewhere (25). Purification of the mutant catalytic subunits was performed as described previously (5,40,45).

DNA/enzyme-binding affinity assays

The $K_d$ of a DNA primer/template substrate for WT pol γ and the mutant catalytic subunits were determined using an EMSA as described previously (21,40). The 5′-32P-labeled D38 was annealed to D34, and a mixture of 10 mM Tris–HCl (pH 8.0), 0.2 mg/ml acetylated BSA, 2 mM dithiothreitol, 1 pmol
of DNA primer/template substrate and 0, 0.5, 1, 1.5, 2, 3, 4, and 5 pmol of the various pol γ catalytic subunits was incubated for 5 min at room temperature. After adding a buffer containing 10 mM Tris–HCl (pH 8.0), 0.1% bromophenol blue and 50% glycerol, the incubations were separated via electrophoresis for 1 h on an 8% TBE polyacrylamide gel (Invitrogen) in 45 mM Tris, 45 mM boric acid and 1 mM EDTA. The gels were dried, exposed on a phosphorimaging storage screen, and imaged on a Typhoon 9400 PhosphorImager (GE Healthcare). NIH Image software was used to quantitate the bands.

**Correct nucleotide incorporation assays**

Single incorporation assays were performed in pre-steady-state time frames using a Kinetek Instruments RQF-3 rapid chemical quench apparatus (Kinetek). All concentrations are final after mixing. For the burst experiments, a pre-incubated solution of 50 nm pol γ catalytic subunit (based on total protein rather than active site concentration), 250 nm accessory subunit and 180 nm radiolabeled D24/D45 primer/template substrate in reaction buffer (50 mM Tris, 100 mM NaCl, pH 7.8 at 37°C) was mixed with 2.5 mM MgCl₂ and 20 μM dCTP in reaction buffer at 37°C using a rapid chemical quench apparatus. Following quenching the incubations with 0.3 M EDTA (pH 8.0) after various timepoints, the products were separated from the substrate on a 20% denaturing polyacrylamide gel (8 M urea) and quantitated using a Bio-Rad Molecular Imager FX. Using Kaleidagraph software, plots of [product] versus time were fit to either a single exponential equation, [product] = A[1 − exp(−k_{obs}t)], or double exponential equation, [product] = A₁[1 − exp(−k_{obs1}t)] + A₂(1 − exp(−k_{obs2}t)), where A is the amplitude, k_{obs} is the observed first-order rate constant for dNTP incorporation and t is the time. When more than one product was present, the sum of all products formed was used in the [product] versus time plots. Plotting the k_{obs} values versus [dCTP] and fitting this the hyperbolic equation k_{obs} = (k_{pol} × [dNTP])/(K_d + [dNTP]) yielded k_{pol}, the maximum rate of nucleotide incorporation, and K_d, the dissociation constant of the nucleotide for the enzyme–DNA complex.

**Incorrect nucleotide incorporation assays**

The same conditions and data analysis were used as described in the correct nucleotide incorporation assays, although the slower reactions were mixed manually instead of using the rapid chemical quench apparatus. For the dTTP opposite dG experiments, the radiolabeled D23/D45 primer-template was used, and for the dTTP opposite dT experiments, the radiolabeled D21/D36 primer-template was used.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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**Conflict of Interest statement.** None declared.

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