Disease Mutations in the Human Mitochondrial DNA Polymerase Thumb Subdomain Impart Severe Defects in Mitochondrial DNA Replication* 

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Forty-five different point mutations in POLG, the gene encoding the catalytic subunit of the human mitochondrial DNA polymerase (pol γ), cause the early onset mitochondrial DNA depletion disorder, Alpers syndrome. Sequence analysis of the C-terminal polymerase region of pol γ revealed a cluster of four Alpers mutations at highly conserved residues in the thumb subdomain (G848S, c.2542g→a; T851A, c.2551a→g; R852C, c.2554c→t; R853Q, c.2558g→a) and two Alpers mutations at less conserved positions in the adjacent palm subdomain (Q879H, c.2637g→t and T885S, c.2653a→t). Biochemical characterization of purified, recombinant forms of pol γ revealed that Alpers mutations in the thumb subdomain reduced polymerase activity more than 99% relative to the wild-type enzyme, whereas the palm subdomain mutations retained 50–70% wild-type polymerase activity. All six mutant enzymes retained physical and functional interaction with the pol γ accessory subunit (p55), and none of the six mutants exhibited defects in misinsertion fidelity in vitro. However, differential DNA binding by these mutants suggests a possible orientation of the DNA with respect to the polymerase during catalysis. To our knowledge this study represents the first structure-function analysis of the thumb subdomain in pol γ and examines the consequences of mitochondrial disease mutations in this region.

As the only DNA polymerase found in animal cell mitochondria, DNA polymerase γ (pol γ) bears sole responsibility for DNA synthesis in all replication and repair transactions involving mitochondrial DNA (1, 2). Mammalian cell pol γ is a heterotrimeric complex composed of one catalytic subunit of 140 kDa (p140) and two 55-kDa accessory subunits (p55) that form a dimer (3). The catalytic subunit contains an N-terminal exonuclease domain connected by a linker region to a C-terminal polymerase domain. Whereas the exonuclease domain contains essential motifs I, II, and III for its activity, the polymerase domain comprising the thumb, palm, and finger subdomains contains motifs A, B, and C that are crucial for polymerase activity. The catalytic subunit is a family A DNA polymerase that includes bacterial pol I and T7 DNA polymerase and possesses DNA polymerase, 3′ → 5′ exonuclease, and 5′-deoxyribose phosphate lyase activities (for review, see Refs. 1 and 2). The 55-kDa accessory subunit (p55) confers processive DNA synthesis and tight binding of the pol γ complex to DNA (4, 5).

Depletion of mtDNA as well as the accumulation of deletions and point mutations in mtDNA have been observed in several mitochondrial disorders (for review, see Ref. 6). mtDNA depletion syndromes are caused by defects in nuclear genes responsible for replication and maintenance of the mitochondrial genome (7). Mutation of POLG, the gene encoding the catalytic subunit of pol γ, is frequently involved in disorders linked to mtDNA depletion (8, 9). Presently, more than 150 point mutations in POLG are linked with a wide variety of mitochondrial diseases, including the autosomal dominant (ad) and recessive forms of progressive external ophthalmoplegia (PEO), Alpers syndrome, parkinsonism, ataxia-neuropathy syndromes, and male infertility (tools.niehs.nih.gov/Polg) (9).

Alpers syndrome, a hepatocerebral mtDNA depletion disorder, and myocerebrohepatopathy are rare heritable autosomal recessive diseases primarily affecting young children (10–12). These diseases generally manifest during the first few weeks to years of life, and symptoms gradually develop in a stepwise manner eventually leading to death. Alpers syndrome is characterized by refractory seizures, psychomotor regression, and hepatic failure (11, 12). Mutation of POLG was first linked to Alpers syndrome in 2004 (13), and to date 45 different point mutations in POLG (18 localized to the polymerase domain) are associated with Alpers syndrome (9, 14, 15). However, only two Alpers mutations (A467T and W748S, both in the linker region) have been biochemically characterized (16, 17).

During the initial cloning and sequencing of the human, Drosophila, and chicken pol γ genes, we noted a highly conserved region N-terminal to motif A in the polymerase domain that was specific to pol γ (18). This region corresponds to part of the thumb subdomain that tracks DNA into the active site of both Escherichia coli pol I and T7 DNA polymerase (19–21). A high concentration of disease mutations, many associated with Alpers syndrome, is found in the thumb subdomain.

Here we investigated six mitochondrial disease mutations clustered in the N-terminal portion of the polymerase domain of the enzyme (Fig. 1A). Four mutations (G848S, c.2542g→a; T851A, c.2551a→g; R852C, c.2554c→t; R853Q, c.2558g→a)
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reside in the thumb subdomain and two (Q879H, c.2637g→t and T885S, c.2653a→t) are located in the palm subdomain. These mutations are associated with Alpers, PEO, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS), ataxia-neuropathy syndrome, Leigh syndrome, and myocerebrohepatopathy (Table 1). Purified, recombinant forms of pol γ bearing these amino acid substitutions were biochemically characterized in vitro to evaluate polymerase activity, DNA binding, steady state kinetics of nucleotide insertion, primer extension activity, misinsertion fidelity, and association with the accessory subunit. Kinetic analysis revealed both nucleotide binding and catalytic defects for the thumb domain mutants, and mutations affecting the most highly conserved residues exhibited the most severe defects in overall catalysis. Nevertheless, none of the mutations reduced the in vitro nucleotide selectivity, and both physical and functional interactions with the accessory subunit were unaffected for each enzyme. Interestingly, DNA binding was impaired in only two of the four thumb domain mutants, suggesting a specific orientation of DNA within the polymerase active site.

EXPERIMENTAL PROCEDURES

Construction of Substituted p140 Proteins—Mutations in the cDNA encoding the exonuclease-deficient (Exo−) pol γ (POLG) were generated using the QuikChange site-directed mutagenesis kit (Stratagene) with the pQVSL11.4 baculoviral transfer vector encoding p140 Exo−/H9253 without its mitochondrial targeting sequence (22) as template. This exonuclease deficient pol γ without an additional mutation is denoted wild-type (WT) in this study. The oligonucleotides containing the point mutations (underlined nucleotide) for introducing the G848S mutation in POLG are 5′-CAA GTG GTG ACT GCC ACC ATC ACT CGC CGG-3′ and 5′-CCG GCG AGT GAT GGT GCT GCC AGT CAC CAC TGG-3′, for the T851A mutation are 5′-CTG CCC GGA CCA TTC TGG GTG AG-3′ and 5′-CTC ACC AGC CCC GCG AGC GAT GGT GCC GCC AGC-3′, for the R852C mutation are 5′-GCC GGC ACC ATC ACT TGC CCG GCT GTA GAG CCC-3′ and 5′-GGG CTC CAC AGC CCG GCC AGT GAT GGT GGC GCC GGC-3′, for the R853Q mutation are 5′-GGC ACC ATC ACT CGC CAG GTG GAG CCC AC-3′ and 5′-GGT GCC TCC ACA GCC TTG CA-3′, for the Q879H mutation are 5′-GTT GAA GAC ATG GTG GCC ACC ATC ACT CGC CAG GTG GAG CCC AC-3′ and 5′-GTT GCC ACC TCC ACA GCC TTG CA-3′, and for the Q879H mutation are 5′-GTT GAA GAC ATG GTG GCC ACC ATC ACT CGC CAG GTG GAG CCC AC-3′ and 5′-GTT GCC ACC TCC ACA GCC TTG CA-3′, for the Q879H mutation are 5′-GTT GAA GAC ATG GTG GCC ACC ATC ACT CGC CAG GTG GAG CCC AC-3′ and 5′-GTT GCC ACC TCC ACA GCC TTG CA-3′, and for the T885S mutation are 5′-GCC CCA CCT GCC TAC TCC CT TGT GGT GCT GAT G-3′ and 5′-CAT CAG CAC CCA CAA AGG GTG AGC CAG GTG GGC G-3′. All six mutations were confirmed by DNA sequencing of the pol γ insert.

Expression and Purification—The WT and mutant forms of the His6 affinity-tagged recombinant catalytic subunit of human pol γ were produced in baculovirus-infected Sf9 cells, and the proteins were purified to homogeneity as described previously (23, 24). The His6 affinity-tagged p55 accessory subunit was expressed in E. coli and purified to homogeneity as described previously (25). After purification the proteins were frozen in small aliquots in liquid nitrogen and stored at −80 °C.

Enzymatic Assays—DNA polymerase activity was determined using the standard pol γ assay with poly(dA)-oligo(dT)12–18 (Amersham Biosciences) as the primer-template substrate. For steady state kinetic values the same assay was performed in the presence and absence of the p55 accessory subunit using poly(dA)-oligo(dT)12–18 as substrate, with reactions containing 25 mM NaCl as previously described (4). The two-subunit form of pol γ was reconstituted as previously described (4).

DNA polymerase assays were also performed using WT and mutant pol γ proteins with activated calf thymus DNA as substrate. The 50-μl reaction contained 50 mM HEPES-KOH (pH 8.0), 2 mM 2-mercaptoethanol, 10 mM MgCl2, 0.1 mg/ml heat-treated BSA, 50 μM dNTPs, 13.3 nm (α-32P)dTTP, 5 μg of the activated calf thymus DNA, and 100 ng of purified WT or mutant p140 enzyme. Reactions were incubated at 37 °C for 10 min and processed as previously described (4).

The primer extension analysis of WT and mutant forms of pol γ proteins utilized a 5′-32P-end-labeled 35-mer oligonucleotide (5′-CCA GTG CCA AGC TTG CAT GCC TGC AGG TCG ACT CT-3′) singly primed M13 ssDNA substrate as described (23) without the preincubation step. Briefly, the 10-μl reaction contained 25 mM HEPES-KOH (pH 7.6), 5 mM 2-mercaptoethanol, 5 mM MgCl2, 0.05 mg/ml heat-treated BSA, 0 or 75 mM NaCl, 25 μM dNTPs, 20 fmol of the labeled oligonucleotide, 50 fmol of purified WT or mutant p140 enzyme in the presence or absence of 100 fmol of the p55 accessory subunit, as indicated. After incubation at 37 °C for 20 min, reactions were terminated, and products were analyzed using denaturing polyacrylamide gel electrophoresis as described (23). Gels were dried, exposed to a phosphor screen, and visualized with a Typhoon 9400 PhosphorImager (Molecular Dynamics).

Nucleotide Misincorporation Kinetics—The fidelity of nucleotide selection by pol γ was determined with a polycrylamide gel-based, single nucleotide extension assay utilizing a 22-mer oligonucleotide (5′-ACC ATG ACC ATG TAC ATC AGA G-3′ (primer)) annealed to a 40-mer oligonucleotide (3′-TTG TAC TGG TAC ATG TAC TCT CAG CCA TAT GTG CACT-5′ (template)) (24, 26). Briefly, reaction mixtures (10 μl) contained 25 mM HEPES-KOH (pH 7.6), 2 mM 2-mercaptoethanol, 0.1 mM EDTA, 50 μg/ml acetylated BSA, 5 mM MgCl2, 1 pmol of primer-template, 50 fmol of exonuclease-deficient p140 with 100 fmol of p55 and one of the four common deoxynucleoside triphosphates. As the next correct nucleotide, dTTP concentration was varied from 0 to 640 nM, whereas the three incorrect nucleotide concentrations were varied from 0 to 3 mM. After incubation at 37 °C for 10 min, reactions were terminated by the addition (10 μl) of 95% deionized formamide and 10 mM EDTA. Samples (2 μl) were boiled for 5 min at 95 °C and resolved by electrophoresis on 12% polyacrylamide gels containing 6 M urea. Gels were dried as before, and radioactive bands were detected with a Typhoon 9400 PhosphorImager (Molecular Dynamics) and quantified with NIH Image software. Km and Vmax values were determined by fitting the data to the steady state Michaelis-Menten model using KaleidaGraph (Version 4.0, Synergy).
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DNA Binding Assay—The dissociation constant ($K_d$) for DNA binding for the WT and mutant forms of p140 was determined by electrophoretic mobility shift assay, essentially as previously described (27). Briefly, double-stranded primer-template molecules were constructed by hybridizing a 5'-32P-end-labeled 38-mer (5'-TTA TCG CAC CTA CGT TCA ATA TTA CAG GCG AAC ATA CT-3') to a 1.2-fold molar excess of an unlabeled, complementary 34-mer (5'-GTA TGT TCG CCT GTA ATA TGT AAC GTA GGT GCG A-3'). Reaction mixtures (20 μl) were assembled at room temperature and contained 10 mM Tris-HCl (pH 8.0), 0.2 mg/ml acetylated BSA, 2 mM dithiothreitol, 1 pmol of primer-template, and 0, 0.5, 1, 1.5, 2, 3, 4, or 5 pmol of each pol γ protein. After 5 min of incubation, 5 μl of 5× loading buffer (10 mM Tris-HCl (pH 8.0), 0.1% bromphenol blue, 50% glycerol) was added, and aliquots of the reaction mixture were subjected to electrophoresis for 1 h at 180 V at 4 °C through an 8% TBE polyacrylamide gel (Invitrogen) in 0.5× TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA). The gels were dried and exposed on a phosphor storage screen. Radioactivity was imaged on a Typhoon 9400 PhosphorImager (Jasco, Inc., MD) following the method described in Chan et al. (16, 25), permitting visualization of co-immunoprecipitated proteins by immunoblot analysis.

Circular Dichroism Measurements and Analysis—Circular dichroism (CD) studies were performed with a Jasco 810 Spectropolarimeter equipped with a Peltier thermal controller (Jasco, Inc., MD) following the method described in Chan et al. (17) with p140 proteins at 20 μg/ml.

Immunoprecipitation Assay—Rabbit polyclonal antibodies raised against recombinant human p55 accessory subunit (4) were immobilized on protein G-Sepharose beads (GE Healthcare), and the beads were then equilibrated in phosphate-buffered saline Nonidet P-40-BSA buffer consisting of 50 mM potassium phosphate (pH 7.5), 150 mM NaCl, 0.1% Nonidet P-40, and 0.1 mg/ml BSA. Prepared protein G-Sepharose beads (20 μl) were mixed as indicated with purified p55 accessory subunit (3 μg) and p140 WT or mutant proteins (3 μg) in a 1.5-ml polypropylene microcentrifuge tube, and the reactions were brought to a final volume of 400 μl with phosphate-buffered saline Nonidet P-40-BSA buffer. Samples were then processed as previously described (16, 25), permitting visualization of co-immunoprecipitated proteins by immunoblot analysis.

RESULTS

Alpers Syndrome pol γ Mutations Located in the Thumb Subdomain of the Polymerase Domain Are Highly Conserved—Six POLG mutations associated with Alpers syndrome or myocerebrohepatopathy were chosen for analysis because of their location in the thumb and early palm subdomains of the polymerase active site. The G848S, T851A, R852C, R853Q, Q879H, and T885S mutations were identified in trans with other POLG mutations in mitochondrial disease patients with variable clinical presentations (Table 1). These six mutations occurred in a region of pol γ with high amino acid homology among several organisms (Fig. 1B). Four of these six residues, Gly-848, Thr-851, Arg-852, and Arg-853, reside in close proximity to one another at the N terminus of the polymerase region and are invariant from yeast to humans. However, the Gln-879 and Thr-885 residues are only moderately conserved through evolution in the pol γ amino acid sequences. To gain insight into the consequences of these mutations in the associated mitochondrial diseases, all six mutant proteins were constructed in a pol γ Exo− background (D198A/E200A) (23) to abolish 3′→5′ exonuclease activity that can interfere with biochemical assays involving nucleic acids. The WT and the mutant proteins were purified to homogeneity after overproduction in baculovirus-infected insect cells as previously described (23, 24).

Alpers Mutant p140 Proteins Retained Their Secondary Structure but Exhibited Decreased Stability—Six Alpers pol γ mutations on p140 secondary structure were assessed by CD spectroscopy. As shown in Fig. 2A, the spectrum of each mutant protein followed a similar pattern to that of the WT enzyme, suggesting that they are properly folded and retain secondary structure similar to the WT enzyme. Also, the melting temperature ($T_m$) and the change in enthalpy for protein folding ($\Delta H_m$) were calculated for each mutant protein by monitoring the thermal denaturation of α-helices over a 30–60 °C temperature range. This analysis revealed that all mutant proteins had similar $T_m$ values ranging between 45 and 49 °C (Fig. 2B). However, all mutant proteins except T885S had a lower $\Delta H_m$ value compared with WT enzyme (Fig. 2B), suggesting that these

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TABLE 1

| POLG mutation | Disease | Genetics | Reference |
|---------------|---------|----------|-----------|
| G848S        | Alpers syndrome | In trans with A467T, Q497H, T251I-P587L, or W748S-E1143G in Alpers syndrome | 15, 35, 43–50 |
|              | Leigh syndrome | In trans with R232H in Leigh syndrome | 49 |
|              | MELAS       | In trans with R627Q in MELAS | 38 |
|              | PEO with ataxia-neuropathy | In trans with G746S and E1143G in PEO with ataxia | 50 |
|              | PEO         | In trans with T251I and P487L in PEO | 51, 52 |
| T851A        | Alpers syndrome | In trans with R1047W | 48, 53 |
|              |             | In trans with H277C | 14, 48, 50 |
| R852C        | Alpers syndrome | In cis with G11D and in trans with W748S-E1143G or A467T | 15 |
| R853Q        | Ataxia-neuropathy | In trans with G11D-R627Q | 15 |
| Q879H        | Myocerebrohepatopathy | In trans with T251I-P587L | 35, 54 |
| T885S        | Alpers syndrome with valproate-induced hepatic failure | In cis with A467T and in trans with Q879H-E1143G | 35, 54 |
mutations might affect the overall stability and, therefore, the biochemical properties of these enzymes.

**Mutation of Highly Conserved Residues Has Drastic Effects on Enzyme Activity**—To determine the effect of the six mutations on overall enzyme activity, polymerase assays were performed using poly(dA)-oligo(dT)\textsubscript{12–18} as substrate, as previously described (24). The Gly-848, Thr-851, Arg-852, and Arg-853 amino acid residues are highly conserved and clustered in the thumb domain (Fig. 1B), suggesting their significance in the overall function of the enzyme. Indeed, the specific activity of the WT enzyme was 210 units/ng, whereas the specific activities of the G848S-, T851A-, R852C-, and R853Q-substituted proteins were less than 1 unit/ng (Fig. 3A). In contrast, mutation of the moderately conserved residues only reduced polymerase activity of the Q879H and T885S proteins to 53 and 68% of WT p140 activity, respectively (110 and 140 units/ng, respectively) (Fig. 3A).

Polymerase activity was also determined using activated calf thymus DNA as substrate, which revealed a 15-fold reduction in WT p140 activity compared with the poly(dA)-oligo(dT)\textsubscript{12–18} substrate (14 versus 208 units/ng) (Fig. 3B). The mutant p140 proteins showed a similar substrate preference, although the activities of the T851A and R852C proteins were not as fully reduced on the natural DNA substrate.

**DNA Binding Affinity of Mutant Proteins Suggests a Possible Orientation of the Enzyme with Primer-Template during Catalysis**—The reduced enzyme activity of these mutant p140 proteins can be because of their impaired binding to DNA. Hence, the DNA binding affinity of the WT and mutant p140 enzymes was estimated by electrophoretic mobility shift assay. Radiolabeled 34/38-mer oligonucleotide primer-template was incubated with various concentrations of WT or mutant p140, and native polyacrylamide gel electrophoresis was used to separate protein-DNA complexes from free DNA. The apparent $K_d$ (DNA) values were subsequently calculated from the reciprocal plots of the fraction of DNA shifted at various enzyme concentrations. This analysis revealed that the WT p140 enzyme had strong affinity to DNA ($K_d$(DNA)/H1100531 nM, Fig. 4A). Although most mutant proteins retained DNA binding affinity at the level of WT protein, the G848S ($K_d$(DNA)/H11005152 nM) and R852C ($K_d$(DNA)/H11005114 nM) p140 proteins exhibited 5- and 4-fold reductions, respectively, in DNA binding affinity compared with the WT enzyme (Fig. 4A). Gly-848 and Arg-852 are four residues apart in the primary sequence. Because the thumb subdomain in family A DNA polymerases is predominantly $\alpha$-helical (19–21), these two residues could be located on the same face of an $\alpha$-helix that interacts with the DNA substrate. Using a helical wheel drawing program we determined that these residues would likely reside on the same helical face (Fig. 4B). Hence, mutations of these critical residues had greater effects on DNA binding than the other Alpers mutant proteins presented in this study.
Alpers Mutant Proteins Physically and Functionally Interact with the p55 Accessory Subunit—Previous studies have shown that interaction of the accessory subunit (p55) with the p140 catalytic subunit enhances the processivity and DNA binding properties of the catalytic subunit (4), and the most common POLG mutation, namely the A467T, has been demonstrated to impair this association (16). Hence, the physical association of p55 with WT and mutant p140 enzymes was assessed with co-immunoprecipitation experiments. The p55 protein was linked to protein G-Sepharose beads with polyclonal antibodies raised against recombinant p55 protein, and the ability of this immobilized p55 to capture WT and mutant forms of p140 was screened. This analysis revealed that WT and all six mutant p140 enzymes interacted physically with p55 (Fig. 5, lanes 4–10). The interaction was clearly dependent on p55, as no appreciable amount of WT p140 was immunoprecipitated in the absence of p55 (Fig. 5, lane 2).

Next, to check whether this physical interaction of the p140 mutant proteins with p55 translates into functional interaction, an in vitro primer extension assay was performed using WT and mutant p140 enzymes in the presence or absence of p55, as previously described (4). The processivity of the catalytic subunit is stimulated as much as 50-fold upon interaction with the accessory subunit (4). The assay revealed that WT p140 extended about 100 nucleotides in the absence of NaCl under conditions that permitted multiple binding events, and the activity was mildly inhibited in the presence of 75 mM NaCl. The addition of p55 to the reaction enhanced the primer extension both in the presence and absence of NaCl as complete extension of substrate was observed (Fig. 6, compare lanes 3 and 4 to lanes 5 and 6). G848S p140 extended only a few nucleotides (Fig. 6, lanes 7 and 8), and the addition of p55 stimulated...
The polymerase in a salt-dependent manner (Fig. 6, compare lanes 7 and 8 to lanes 9 and 10). As expected, the lengths of these end-labeled products were proportional to overall polymerase activity. The T851A p140 extended the primer efficiently in the presence of NaCl (Fig. 6, compare lanes 11 and 12); however, in the presence of p55 the primer extension ability of the T851A p140 was moderately inhibited, and the activity of the mutant protein was rescued with the addition of 75 mM NaCl (Fig. 6, compare lane 11 to lane 13 and lane 12 to lane 14). The R852C p140 had complete primer extension in the presence of p55 in a salt-dependent fashion (Fig. 6, lanes 15–18). The primer extension ability of the R853Q enzyme was most drastically affected, as it could not extend more than seven bases even in the presence of salt and p55 (Fig. 6, lanes 19–22). Both Q879H and T885S p140 enzymes generated high molecular weight products (Fig. 6, lanes 23 and 27). These reactions were mildly inhibited by salt (Fig. 6, lanes 24 and 28), and the p55 rescued the salt inhibition (Fig. 6, lanes 26 and 30), which is characteristic of the WT p140 enzyme (4). These assays revealed functional interaction of the p55 accessory subunit with all six mutant variants of the p140 catalytic subunit.

The true processivity of each mutant protein was studied under identical conditions, albeit in the presence of a DNA trap that binds the polymerase once it has extended the end-labeled primer-template substrate and dissociated. This single binding assay revealed that the extended primers had similar lengths to those in the primer extension assay, although the overall quantity of extended primers was significantly reduced both in the presence and absence of p55 (data not shown).

**Kinetic Parameters of Mutant Proteins**

To better understand the role of the p55 accessory subunit on the WT and mutant p140 catalytic subunits, the kinetic analysis was also performed with reconstituted WT and mutant holoenzymes (17, 24). This analysis revealed that the addition of p55 had only a modest effect on the catalytic efficiency of the Q879H and T885S p140 enzymes (Table 2). However, the Q879H and T885S p140 enzymes generated high molecular weight products (Fig. 6, lanes 19–22). Including p55 only slightly enhanced the activity of the Q879H and T885S p140 enzymes compared with the WT enzyme. These results translated to less than 1% of WT activity for the four proteins with mutations in the thumb domain (Table 2). However, the Q879H and T885S p140s displayed 46 and 86% of WT enzyme activity, respectively, suggesting that mutations in the palm subdomain can retain activity close to WT values (Table 2).

*Errors in Nucleotide Selection Are Not Found in These p140 Mutant Proteins—*Rearrangement of mtDNA after replication errors can lead to deletion or depletion of mtDNA, and depletion of mtDNA is a hallmark of Alpers syndrome and myocerebrohepatopathy. Accordingly, we evaluated the effect of these six Alpers mutations on the fidelity of replication using a single
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TABLE 3
Misinsertion fidelity of DNA synthesis by the Alpers syndrome p140 mutants

| Enzyme | dATP/dTTP | dCTP/dTTP | dGTP/dTTP |
|--------|-----------|-----------|-----------|
|        | f         | f         | f         |
|        | WT        | G848S     | T851A     | R852C     | R853Q     | Q879H     | T885S     |
|        | ×10⁻³     | ×10⁻³     | ×10⁻³     |
|        | 14        | 12        | 1.9       | 14        | 1.8       | 11        | 7.1       |
|        | 1         | 0.84      | 0.14      | 1.0       | 0.13      | 0.77      | 0.51      |
|        | 0.65      | 0.90      | 0.11      | 0.11      | 0.81      | 0.54      | 0.36      |
|        | 1         | 1.4       | 0.17      | 0.16      | 1.2       | 0.82      | 0.56      |
|        | 0.28      | 0.16      | 0.11      | 0.07      | 0.92      | 0.26      | 0.28      |
|        | 1         | 0.57      | 0.39      | 0.24      | 3.3       | 0.93      | 1.0       |

The kinetic parameters (K_m and k_cat) for incorporating a single correct (dTTP) or incorrect (dATP, dCTP, and dGTP) nucleotide onto a 3′-terminal matched primer-template were measured for the WT and mutant p140 enzymes in the presence of the p55 accessory subunit as described under “Experimental Procedures.” The error frequencies were then determined from the ratio f = (k_d/K_m) incorrect nucleotide/(k_cat/K_m) correct nucleotide. The ratio of error frequencies for mutant enzymes relative to WT is expressed as f_MUT/F_WT.

DISCUSSION

Of the more than 150 disease mutations in the POLG gene, we have previously focused on adPEO mutations and other common disease mutations (16, 17, 24). Nearly all of the adPEO mutations in POLG are located in the polymerase region of pol γ, and our study of four adPEO mutant pol γs revealed a wide range of polymerase activities ranging from 0.03% to nearly 30% of WT activity (24). Biochemical analysis of two of the common POLG disease mutations, A467T and W748S, revealed similar defects in polymerase activity but also indicated defective subunit association for the A467T p140 and significantly reduced DNA binding by the W748S p140 (16, 17). For the current report we noted a cluster of mutations associated with Alpers disease and other early mtDNA depletion syndromes that was located in a unique sequence within the putative thumb domain and early palm domain of the polymerase active site. We have analyzed the biochemical consequences of six of these mutations to begin to explain the underlying molecular mechanisms leading to mtDNA depletion. This study expands the repertoire of biochemically characterized pol γ enzymes that harbor disease alterations (for a complete review, see Ref. 9).

Gly-848 and Arg-852 Are Located at the Face of the Thumb Helix That Interacts with DNA—The DNA polymerase activities of the G848S and R852C p140 proteins were much less than 1%, which is consistent with mtDNA deletions observed in Alpers patients with these two mutations. After A467T and W748S, G848S is the third most common mutation found in POLG, and G848S is associated not only with Alpers syndrome but also with ataxia-neuropathy syndromes, MELAS, PEO, and Leigh syndrome (14, 15). Both human genetic data (tools.niehs.nih.gov/polg) and yeast data on the equivalent G651S mutation in Mip1 (30) indicate that the G848S mutation is a recessive mutation. Presentation of symptoms and progression of disease appear to depend, in part, on the identity of the mutation in the other allele (Table 1). Our biochemical analysis indicated that G848S as well as R852C pol γ retained less than 1% of WT polymerase activity, but the structure of these proteins remained intact as judged by predictable chromatographic behavior during purification, CD analysis, and interaction with the accessory subunit. The equivalent change in the analogous position in the yeast Mip1 protein, G651S, causes a drastic increase in petite frequency and point mutations in mtDNA (30). One significant difference between this mutant protein and the others in this study was the 5-fold increase in K_d for DNA binding. The Gly-848 residue is expected to be on the same face of an α-helix with Arg-852 in the thumb subdomain (Fig. 4B). Because alterations in either residue affected DNA binding, we propose that this side of the α-helix interacts with the DNA. Available tertiary structures of family A polymerases show interaction of the thumb domain with the minor groove of double-stranded DNA (21, 31).

T851A and R853Q pol γ Mutations—Mutation at Thr-851 and Arg-853 also caused a substantial decrease in polymerase activity, consistent with a mtDNA depletion phenotype, but these two proteins did not exhibit altered affinity for DNA. Alanine-scanning mutagenesis of the thumb α-helix of HIV-1 reverse transcriptase showed that V261A and L264A substitutions inactivated the enzyme although neither side chain on the α-helixH was facing the DNA substrate (32). In the absence of structural information for the human pol γ, the reason for this polymerase defect by the T851A and R853Q mutations is unclear.

We were surprised by the profound biochemical defect displayed by the R853Q substituted pol γ, as mutation of this same codon to encode a tryptophan residue is associated with much later age of onset POLG diseases. The R853W mutation is associated with autosomal recessive PEO when found in trans with P587L (33), and compound heterozygotes pairing R853W in trans with G737R can cause parkinsonism in the absence of PEO (34). The R853Q mutation in trans with T251I-P587L is associated with myocerebrohepatopathy in a patient that presented at 2–3 months of age. The allelic T251I-P587L mutation is recessive and has been found in PEO and Alpers syndrome with G848S. The absence of significant polymerase activity displayed by the R853Q pol γ is consistent with mtDNA depletion in the patients and helps to explain the early childhood myocerebrohepatopathy.

Q879H and T885S pol γ Mutations—Mutation of the moderately conserved Q879 and T885 residues only caused moder-
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Recessive Versus Dominant POLG Mutations—Attempts to correlate a given POLG mutation with its mode of inheritance, the severity of the induced clinical phenotype, and the biochemical defects conferred on the mutant pol γ protein are complex but instructive. Many mutations following a dominant mode of inheritance also appear to show dominant negative biochemical behavior in vitro. For example, several POLG mutations co-segregate with the adult-onset adPEO and encode enzymes with amino acid substitutions in polymerase motif B of pol γ. These proteins exhibited reduced DNA polymerase activity and low nucleotide selectivity in vitro, and the observed biochemical defects were generally proportional to the severity of clinical symptoms (24). One simple interpretation of this trend is that heterozygous adPEO mutations create a competition for the mtDNA replication fork between a dysfunctional and WT pol γ. Because both copies of POLG are expressed in human cells, and mono-allelic expression of a single WT copy of POLG is sufficient to avoid disease (36), we presume that pol γ produced from an adPEO allele actively interferes with mtDNA replication in vivo. By extension, this model predicts that recessive missense mutations in POLG produce dysfunctional polymerases that minimally interfere with ongoing mtDNA replication catalyzed by WT pol γ. For example, the recessive A467T mutation of POLG produces an inefficient enzyme with reduced DNA binding and defective association with the pol γ accessory subunit (16). This prediction is supported by the observation that a number of mitochondrial disorders result when A467T is heterozygous with other POLG mutations, and the clinical severity is largely determined by the nature of the mutation in the other POLG allele (14). Biochemical analysis of six recessive Alpers mutations in the current study presents challenges to this simple model. The early age of onset and the devastating severity of Alpers disease suggest profound biochemical defects in pol γ enzymes derived from POLG alleles with mutations linked to Alpers. Indeed, all four proteins with amino acid substitutions in the putative thumb subdomain were found to be non-functional. Like A467T pol γ, the G848S and R852C proteins also displayed a significant reduction in DNA binding. Although the recessive mode of inheritance implies that the mutant proteins are non-competitive at the replication fork, these mutations appear to be more complex than simple loss of function mutations that predispose heterozygous individuals to mitochondrial disorders, because the clinical phenotype is not entirely controlled by the other allele. For example, A467T POLG in trans with R627Q POLG can result in ataxia-neuropathy (37), whereas compound heterozygotes bearing G848S POLG in trans with R627Q POLG can present with MELAS (38). Does the residual DNA polymerase activity of A467T pol γ contribute to a milder phenotype? Although the Alpers proteins may not directly compete with WT p140, they may compete indirectly through interaction with other proteins at the mtDNA replication fork. Unlike A467T p140, each of the Alpers proteins retains physical and functional interaction with the pol γ accessory subunit. Does the enhanced DNA binding conferred by the accessory subunit reduce dissociation of mutant pol γ from mtDNA, thereby interfering with the ability of a WT enzyme to complete replication? Clearly, knowledge of the complex interactions of all the proteins in the mitochondrial replisome is needed to refine the model to address these more difficult questions.

Thumb Subdomains in Other DNA Polymerases—In prototypical family A polymerases, such as the E. coli pol I, the thumb subdomain consists of two flexible α-helices of ~50 amino acids that interact with the incoming primer-template (31). Consistent with these findings, deletion of 24 amino acids in the Klenow fragment corresponding to part of the tip of the thumb subdomain causes a 100-fold reduction in DNA binding affinity, reduces processive synthesis, and increases the rate of frameshift errors (39). G848S pol γ (and to a lesser extent R852C pol γ) also displayed a decreased DNA binding affinity. None of the mutant proteins studied displayed a significant decrease in misinsertion fidelity. Consistent with this observation, alanine scanning mutagenesis of conserved residues in the tip of the Klenow thumb also did not display an increase in misinsertion (point mutations) or frameshift errors (40). As in most DNA polymerases, the thumb subdomain is structurally conserved in HIV-1 reverse transcriptase (41). Alanine scanning mutagenesis of amino acid residues in the HIV-1 reverse transcriptase thumb structure revealed that mutations in three residues, Gln-258, Gly-262, and Trp-266, in α-helixH had a significant decrease in primer-template binding, base substitutions, and frameshift fidelity (32, 42). Interestingly, the periodicity of these residues in the α-helixH shows that they are all on the same helical face that interacts with minor groove of the DNA (32, 42). Although none of our Alpers mutant p140 enzymes displayed any significant change in fidelity, based on the defect in DNA binding we predict that the Gly-848 and...
Arg852 are also on the same helical face that interacts with the DNA.

The structure-function studies presented here indicate that the four amino acids, Gly-848, Thr-851, Arg-852, and Arg-853, are critical for the polymerase activity of pol γ. We propose that these four amino acids form part of the helical region of the thumb subdomain, which is known from other polymerase structures to interact with the minor groove of the incoming DNA. Our biochemical results predict insufficient mtDNA replication in vivo, consistent with the mtDNA depletion and the presentation of Alpers or myocerebrohepatopathy in patients with these POLG mutations. Although further three-dimensional structural information is needed to fully interpret how these structural alterations may change the polymerase function, this study represents the first analysis of polymerase thumb domain mutations in the pol γ polymerases.

Acknowledgments—We thank Dr. Robert Petrovich of the NIEHS Protein Expression Core Facility for help with the CD studies, Farida Sharief for help with tissue culture of Sf9 insect cells, and Drs. Katarzyna Bebenek and Rajendra Prasad for critically reading this manuscript.

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