Modification of the 4Fe-4S Cluster Charge Transport Pathway Alters RNA Synthesis by Yeast DNA Primase

Lauren E. Salay+,1,2, Alexandra M. Blee+,1,2, Md Kausar Raza+,3,4, Kaitlyn S. Gallagher1,2, Huiqing Chen1,5, Andrew J. Dorfueille2,6, Jacqueline K. Barton3,*, Walter J. Chazin1,2,7,*

1Department of Biochemistry, Vanderbilt University, Nashville, TN 37240
2Center for Structural Biology, Vanderbilt University, Nashville TN 37240
3Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125
4Present address: Department of Chemistry, Pennsylvania State University, University Park, PA 16802
5Present address: Institute of Life Science, Jiangsu University, Zhenjiang, China
6Present address: Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139
7Department of Chemistry, Vanderbilt University, Nashville, TN 37235

Abstract

DNA synthesis during replication begins with the generation of a ~10 nucleotide primer by DNA primase. Primase contains a redox-active 4Fe-4S cluster in the C-terminal domain of the p58 subunit (p58C). The redox state of this 4Fe-4S cluster can be modulated via transport of charge through the protein and the DNA substrate (redox switching); changes in the redox state of the cluster alter the ability of p58C to associate with its substrate. The efficiency of redox switching in p58C can be altered by mutating tyrosine residues that bridge the 4Fe-4S cluster and the nucleic acid binding site. Here, we report the effects of mutating bridging tyrosines to phenylalanines in yeast p58C. High-resolution crystal structures show that these mutations, even with six tyrosines simultaneously mutated, do not perturb the three-dimensional structure of the protein. In contrast, measurements of the electrochemical properties on DNA-modified electrodes of p58C containing multiple tyrosine to phenylalanine mutations reveal deficiencies in their ability to engage in DNA charge transport. Significantly, this loss of electrochemical activity correlates with decreased primase activity. While single site mutants showed modest decreases in activity compared to the wild-type primase, the protein containing six mutations exhibited more than a 10-fold decrease. Thus, many possible tyrosine-mediated pathways for charge transport in yeast p58C exist but...
inhibiting these pathways together diminishes the ability of yeast primase to generate primers. These results support a model in which redox switching is essential for primase activity.

**Graphical Abstract**

![Graphical Abstract](image)

**INTRODUCTION**

Accurate, efficient DNA replication is essential for all organisms and relies on the concerted function of several key enzymes. Replicative DNA polymerases, such as polymerases δ or ε, synthesize daughter strands in the 5’ to 3’ direction but require a pre-existing 3’-OH for addition of a nucleotide. To satisfy this requirement, a DNA-dependent RNA polymerase, DNA primase, generates a 7-12 nucleotide RNA primer de novo. DNA primase then hands off the RNA primer to DNA polymerase α (pol α) to extend the RNA primer by approximately 20 nucleotides before hand-off to the more processive polymerases, δ or ε. In eukaryotes, DNA primase forms a heterotetramer with DNA polymerase α termed pol-prim.

Primase and pol α both contain a catalytic and a regulatory subunit. RNA primer synthesis occurs in the p48 catalytic subunit, although the C-terminal domain of the p58 regulatory subunit (p58C) is required to bind template DNA, initiate the primer efficiently, and generate products of the appropriate length. After a primer is generated by primase, it is transferred to the flexibly tethered p180 pol α catalytic subunit. Despite in-depth biochemical analysis and determination of multiple structures, the mechanism of hand-off between DNA primase and DNA pol α remains enigmatic.

Three-dimensional structures of the complex give information on possible mechanisms of priming. The p48 subunit and the p58 N-terminal domain (p58N) form an integrated
structural unit. The globular p58C is linked to p58N by an unstructured 20 amino acid linker\(^9\,10\). The linker allows p58C to occupy many different positions relative to the rest of the protein, corresponding to different configurations of the p48-p58 complex ranging from direct positioning over the p48 active site to fully extended in solution\(^10\,11\). During primer synthesis, it has been hypothesized that the p58C subunit remains bound to the 5’ terminus of the primer while p48 adds nucleotides to the 3’ end. Thus, it has been proposed that the length of the linker between the N- and C-terminal domains of the p58 regulatory subunit limits the size of the primer (primer length counting)\(^9\,12\). Furthermore, the positioning of the p48 and p58 subunits relative to each other is thought to vary during each catalytic stage\(^13\). It has been proposed that the stochastic reorganization of subunits may drive primer handoff\(^10\). However, all hypotheses so far have either been disproven or lack functional validation. What all models agree upon is that the p58 C-terminal domain (p58C) is required to initiate RNA priming and terminate RNA synthesis at a unit length of 7-10 nts\(^8\,14\).

The p58C domain has been identified as a 4Fe-4S cluster-containing protein in both human and \textit{Saccharomyces cerevisiae} (yeast)\(^15\)-\(^18\). We have shown that the redox state of the 4Fe-4S cluster can be modulated through transport of charge through the protein and the DNA/RNA substrates\(^19\)-\(^22\). Importantly, the redox state of the cluster influences the ability of p58C to associate with its substrate\(^19\)-\(^22\). When 4Fe-4S clusters are in the oxidized 3+ state, binding to the DNA polyanion is significantly tighter than when the cluster is in the reduced 2+ state, as we have seen previously with the binding of DNA repair proteins containing 4Fe-4S clusters (>50x for Endonuclease III)\(^23\).

Polymerases interact with the newly generated duplex portion of DNA primed templates, while p58C is understood to remain bound to the 5’ triphosphate of the daughter strand for the duration of the catalytic cycle\(^12\). Thermodynamically appropriate signaling partners are abundant in the replisome; both pol \(\delta\)\(^24\),\(^25\) and \(\epsilon\)\(^26\) contain 4Fe-4S clusters and we have obtained evidence that the constitutively bound pol \(\epsilon\) also contains a 4Fe-4S cluster. Furthermore, there is evidence for multiple pol-prim molecules\(^27\) interacting with the replisome. When bound to a duplex, the potential of the Fe-S cluster in each of these proteins (primase\(^21\),\(^22\), pol \(\delta\)\(^28\), and pol \(\epsilon\)\(^29\)) shifts to similar DNA-bound potentials and exhibit similar redox properties. These proteins can engage in a self-exchange redox reaction\(^21\),\(^28\) or with other DNA bound signaling partners\(^29\). Since polymerases are potential signaling partners, one model involves charge from the 4Fe-4S cluster in pol \(\alpha\), \(\delta\), or \(\epsilon\) being transported through the duplex portion of the newly synthesized RNA to the 4Fe-4S cluster in p58C.

The ensemble of these data have lead us to suggest a model for handoff involving a redox switch between the cluster in p58C and an expected 4Fe-4S cluster in pol \(\alpha\)\(^25\), with electron transfer through the two proteins and their DNA-RNA substrate\(^21\),\(^22\). In this model, a primase molecule with an exogenously oxidized 4Fe-4S cluster begins primer synthesis. The p58C domain remains bound to the 5’ triphosphate of the daughter strand while the p48 subunit extends the primer. When the primer is extended to form a 7-11 base pair duplex, the p58C 4Fe-4S is reduced via DNA charge transfer by the expected 4Fe-4S cluster in pol \(\alpha\)\(^25\) or possibly by another replisome component such as another primase/pol-prim molecule\(^27\), pol \(\delta\)\(^24\), or pol \(\epsilon\)\(^26\). The reduced 4Fe-4S cluster in p58C has decreased affinity
for its substrate, promoting dissociation from the product. This redox switch facilitates the hand off of the substrate to pol α.

The p58C 4Fe-4S cluster is highly insulated in the core of the protein, making direct electron transfer from a neighboring protein unlikely. A crystal structure of human p58C in complex with a RNA/DNA product substrate shows that the duplex portion of the substrate and 4Fe-4S are approximately 25 Å apart, a distance too far for direct electron transfer from DNA to the p58C cluster. Instead, amino acid side chains such as tyrosine or tryptophan can act as intermediate donors and acceptors to facilitate electron hopping over distances ranging between 10-15 Å through the protein to reduce or oxidize the cluster. Investigation of human p58C has revealed that mutation of any one of three conserved tyrosine residues Y309, Y345, or Y347 to a residue less efficient at electron transfer such as phenylalanine, is sufficient to disrupt charge transport between the DNA binding surface and the 4Fe-4S cluster, thus inhibiting redox switching. Furthermore, in vitro studies with human primase reveal that the regulation of primer initiation and termination can be disrupted by such mutations in human p58C.

These previous studies have led to the hypothesis that pol-prim hand-off is mediated in part by 4Fe-4S cluster redox-dependent regulation of the DNA binding affinity of pol-prim subunits.

The property of DNA redox switching first observed in human p58C is conserved in yeast. The structure of the yeast p58C domain is very similar to the human p58C domain. Furthermore, an overlay of human p58C in complex with an RNA/DNA product substrate (PDBID 5F0S) and the wildtype yeast p58C (PDBID 6DI6) shows they are remarkably similar (0.88 Å backbone RMSD) (Fig. 1C). Moreover, at the site where the RNA/DNA substrate binds, the electrostatic surface representation of yeast p58C reveals a highly basic cleft (Fig. 1D). Despite the remarkable structural similarity, yeast p58C is divergent from human in sequence, with only ~40% identity in a local alignment using BLAST. Of note, the yeast protein contains more tyrosine and tryptophan residues than the human protein, yet human Y309, an essential residue in mediating redox switching, is not conserved in yeast. Instead, the homologous residue is a leucine, which energetically cannot participate in hole hopping. These observations suggest that the pathway mediating redox switching differs between the human and yeast proteins and that yeast p58C may have multiple pathways. The question of multiple pathways for charge transport in primase is important to consider, especially if the redox switch is essential for proper priming of replication.

The structure of yeast p58C shows there are several tyrosine and tryptophan residues that bridge the 4Fe-4S cluster and the DNA binding surface, which could mediate charge transport (Figure 1B). In our previous study of the yeast protein, we showed that two conserved tyrosine residues (Y395, Y397) and one tyrosine positioned nearby (Y431) can form an electron transfer pathway in yeast p58C from the cluster to the DNA (Figure 1B, red residues). Mutation of a single tyrosine to phenylalanine within this predicted path diminished the ability of the 4Fe-4S cluster to engage in electron transfer. Unlike human p58C, a single mutation in yeast p58C reduces electron transfer by ~4 fold compared to ~10 fold reduction in the human protein. Moreover, we found that incorporation of single tyrosine to phenylalanine mutations along the predicted pathway does not decrease cell
viability in yeast, even under various stressors. An exception occurs in a yeast strain where PRI2 Y397 is mutated to leucine and confers lethality, which we attribute to this protein mutant being more susceptible to oxidative degradation.

The potential for multiple pathways for charge transport through the yeast protein suggests that mutation of many if not all of these residues may be required to inhibit charge transport sufficiently to prevent efficient redox switching of the 4Fe-4S cluster. To address this possibility, we prepared yeast p58C mutants with five or all six tyrosine residues substituted with phenylalanine. These mutants were subjected to biophysical, structural, and electrochemical analysis, which showed that while the biophysical properties and structure were not substantially affected, the ability to engage in charge transport was significantly diminished. Moreover, importantly, p58C mutants exhibited reduced RNA priming activity that correlates with the ability to carry out charge transport. These results support our model in which DNA redox switching promotes termination of RNA primer synthesis and handoff to pol α.

METHODS

Expression plasmids.

Preparation of the pRSF-duet expression vector for WT yeast primase and generation of Y395F and Y397F mutants have been described previously. Site-directed mutagenesis was performed for Y412F, Y431F, Y352F/Y353F/Y395F/Y397F/Y412F (5YF412) and Y352F/Y353F/Y395F/Y431F (5YF431) mutants using a Q5 mutagenesis kit (New England Biolabs) with the primers listed in Supplemental Table 1 and annealing temperatures of 58-60 °C. The gene for the Y352F/Y353F/Y395F/Y397F/Y412F/Y431F (6YF) mutant yeast primase PRI2 was synthesized (Genewiz Inc.) and inserted along with WT PRI1 into the pRSF-duet vector. The genes for yeast PRI1 and PRI2 can be accessed at Uniprot with the accession IDs of P10363 and P20457, respectively.

Protein Expression and Purification.

The protocol for expression and purification for yeast p58C and mutants have been previously described. Full-length yeast primase was prepared similarly. Plasmids were transformed into Rosetta2 DE3 PlySs cells and cultured in Terrific Broth (TB) at 37 °C with shaking until the OD$_{600}$ reached approximately 1-1.2. The cells were cooled to 18 °C and induced with 0.5 mM IPTG. The cells were then allowed to grow for 16 hours at 18 °C before harvesting. The cells were flash-frozen and stored at −80 °C.

Yeast primase was purified using a four-step approach: Ni-NTA affinity chromatography, 6xHis tag cleavage and Ni-NTA repass, heparin affinity chromatography, and size exclusion chromatography. All steps were performed at 4 °C unless otherwise specified. Briefly, cell pellets were thawed, resuspended in a phosphate buffer, and lysed by sonication. The supernatant was passed over an Ni-NTA column (Cytiva). The 6xHis tag was cleaved with H3C protease overnight. The protein was repassed over the Ni-NTA column and flow-through and wash fractions were collected and dialyzed into Heparin Buffer A: 20 mM HEPES (pH 6.8), 50 mM NaCl, 2 mM dithiothreitol (DTT). Dialyzed protein was filtered.
and passed over a 5 mL Hi-Trap HP heparin column (GE Healthcare) pre-equilibrated with Heparin Buffer A. Protein was typically eluted at ~25% Heparin Buffer B: 20 mM HEPES (pH 6.8), 1 M NaCl, 2 mM DTT. Fractions containing yeast primase were pooled, concentrated, and passed over an S200 size exclusion chromatography column in 20 mM HEPES (pH 6.8), 200 mM NaCl, 1 mM TCEP, 3% glycerol. The purest fractions were pooled, diluted to ~3 μM, flash-frozen, and stored at −80 °C until use.

**Circular Dichroism Spectroscopy.**

CD spectra were collected as described. Prior to analysis, WT or mutant p58C protein samples were stored in 20 mM HEPES (pH 6.8), 200 mM NaCl, 2 mM DTT at −80 °C. Protein was thawed and transferred to 10 mM KH₂PO₄ (pH 7.2) using Amicon ultra centrifugal filters. Dialyzed and filtered p58C was diluted to 0.5-1 mg/mL. The far-UV spectrum was measured from 260–190 nm in increments of 0.5 nm at a rate of 50 nm/min with a response time of 2 s and a bandwidth of 1 nm, using a Jasco J-810 Spectropolarimeter and quartz cuvette. A buffer blank was collected prior to the protein spectra. Three spectra were averaged and smoothed for each protein.

Thermal denaturation monitored by CD was performed as previously described. Briefly, the protein was dialyzed into 10 mM KH₂PO₄ (pH 7.2) using Amicon ultra centrifugal filters. The circular dichroism was monitored at every half degree at 222 nm as the temperature was increased from 20 °C to 80 °C for 90 minutes. The apparent Tm was determined by taking the first derivative of the resulting curve.

**Fluorescence Polarization Anisotropy.**

Prior to analysis, WT or mutant p58C protein samples were stored in 20 mM HEPES (pH 6.8), 200 mM NaCl, 2 mM DTT at −80 °C. Protein was thawed and transferred to 20 mM HEPES (pH 6.8), 75 mM NaCl, 2 mM DTT using Amicon ultra centrifugal filters. The fluorescence polarization anisotropy assay was performed as described. Briefly, the 6-carboxyfluorescein labeled DNA substrate was annealed by adding both substrates together in a 1.1:1 ratio, heating at 95 °C for 10 minutes, and allowed to cool slowly. The substrate used for the fluorescence anisotropy assay is a duplex of the following oligos: 5’-[6FAM]-TCTCTCTCTAAA-3’ and 5’-TTTGAGAG-3’. After annealing and cooling, protein at various concentrations was added to solutions containing 50 nm DNA substrate and loaded onto a black 384-well plate. Polarized fluorescence intensities were measured using excitation and emission wavelengths of 485 nm and 520 nm using a Synergy H1 Hybrid Reader. Data represent the average of three replicates normalized to anisotropy at 0 μM protein. Final apparent Kᵰ values were calculated using the one-site total binding equation in GraphPad Prism 8 and represent the mean ± one standard deviation.

**X-Ray Crystallography.**

Prior to crystallization, proteins were stored in 20 mM HEPES (pH 6.8), 200 mM NaCl, 2 mM DTT at −80 °C. Protein was then thawed and transferred to 20 mM HEPES (pH 6.8), 75 mM NaCl, 2 mM DTT using Amicon ultra centrifugal filters. Crystals of multi-tyrosine mutant p58C were grown by hanging drop vapor diffusion at 18 °C from a drop composed of equal volumes of p58C mutant (~5.5 mg/mL) and reservoir solution containing 100 mM
TRIS (pH 8.5), 55-70% MPD. Crystals formed after 2 days and were then looped and transferred to a cryo-protectant containing reservoir solution and 20% glycerol. Loop crystals were flash frozen in liquid N$_2$. X-ray data were collected at Sector 21 (Life Sciences Collaborative Access Team) of the Advanced Photon Source at Argonne National Laboratory. All data were indexed and scaled using HK.L2000$^{37}$. Phasing of the diffraction data was done by molecular replacement using Phaser$^{38}$ and yeast p58C Y397F as the search model (PDB ID: 6DTZ). The models were refined iteratively using Phenix$^{39}$ and Coot$^{40}$. Backbone RMSD compared to WT was calculated for each mutant using Coot LSQ Superpose for backbone atoms. Further structure visualization and analysis was carried out in Chimera$^{41}$. Access to these programs was provided by SBGrid$^{42}$. Structures are deposited in the PDB under the accession codes 7TL2 (p58C 5YF412), 7TL3 (p58C 5YF431), and 7TL4 (p58C 6YF).

**Oligonucleotide Preparation.**

All oligomers were procured from Integrated DNA Technologies (IDT). DNA sequences for electrochemistry assays are shown in Supplemental Table 2. Both thiol-protected single-stranded DNA (p-s-ssDNA) and complementary and abasic site ssDNA were dissolved in Milli-Q water and analyzed on a reverse-phase PLRP-S 300 Å column (Agilent) by HPLC. Pure fractions of oligos were collected, flash frozen in liquid nitrogen, and lyophilized. The dried oligomers were resuspended in 20 mM Tris-HCl pH 7.2, 75 mM NaCl. Concentrations were measured by UV-Vis absorption spectroscopy. The thiol-modified oligonucleotide was deprotected using 50-fold excess of tris(2-carboxyethyl) phosphine (TCEP) while vortexing for 3 h. Deprotection of p-s-ssDNA was verified by HPLC and mass spectrometry using an Autoflex matrix-assisted laser desorption ionization time-of-flight mass spectrometer (Bruker). The complementary ssDNA and deprotected thiolated ssDNA were mixed in equimolar concentrations (60 μM), vortexed for 2 min, degassed with Ar (1 s of sparging per 1 μL of solution), and sealed with Teflon tape. The DNA was then annealed using a thermocycler (Beckman Instruments) by initial heating to 90 °C, followed by slow cooling to 20 °C for 90 minutes. Annealed duplex DNA solutions were stored at −20 °C until used.

**DNA-modified Electrode Assembly/Preparation.**

Multiplex chips$^{43, 44}$ were dried thoroughly with argon gas and ozone-cleaned for 20 minutes in an Uvo brand ozone cleaner at 20 mW. As per the previously defined protocols, clean chips were assembled on the surface of polycarbonate holders with an acrylic clamp and a Buna-N rubber gasket. The four quadrants of the chip were separated by the fastened gasket and clamp$^{44}$. Annealed 5'-thiol modified duplex DNA substrates (25 μL of 30 μM) were added to each quadrant of the multiplex chip. Substrates incubated for 18-20 hours on the gold surface to allow the formation of a DNA monolayer. DNA monolayers were washed with 20 mM Tris-HCl, 75 mM NaCl, pH 7.2, and subsequently backfilled with 0.1 mM 6-mercaptohexanol (Sigma) for 15-20 minutes. Monolayers were then washed 8-10 times per quadrant with 20 mM Tris-HCl, 75 mM NaCl, pH 7.2.

**Sample Preparation for Electrochemistry.**

Wild type and mutant p58C were stored in 20 mM HEPES, pH 6.8, 200 mM NaCl, 2 mM DTT. Before performing the electrochemistry experiments, mutant p58C was transferred to

---

*Biochemistry. Author manuscript; available in PMC 2022 October 12.*
20 mM Tris-HCl, 75 mM NaCl, pH 7.2. After buffer exchange, the concentrations of 4Fe-4S cluster-containing p58C or multi-mutants were determined via UV-Visible spectroscopy, using the absorbance of the 4Fe-4S cluster at 410 nm with an extinction coefficient = 17000 M$^{-1}$ cm$^{-1}$. Samples were then taken into the anaerobic chamber (Coy Laboratory Products). Prior to the deposition at the surface of the gold electrode, p58C/multi-mutant samples were diluted to 38 μM 4Fe-4S p58C variants with deoxygenated buffer (20 mM Tris-HCl, 75 mM NaCl, pH 7.2).

**DNA Electrochemistry Measurements of p58C Variants**

Multiplex gold electrodes were a component of a three-electrode system with an external Ag/AgCl reference electrode (Bioanalytical Systems) and an auxiliary platinum electrode. All electrochemistry experiments were performed in a deoxygenated buffer containing 20 mM Tris-HCl, pH 7.2, 75 mM NaCl. Cyclic voltammograms were recorded to confirm the formation of a DNA monolayer on the surface of each electrode. Each quadrant of the electrode was washed. 200 μL of protein sample was then deposited on the surface of the chip to ensure a complete three-electrode circuit. Cyclic voltammetry scans were produced at 100 mV/s rates, over the redox potential window of +412 mV to −188 mV vs. NHE. Bulk electrolysis for all electrochemical oxidation and reduction reactions on DNA was conducted at an applied voltage potential of +412 mV vs. NHE and −188 mV vs NHE, respectively. The single oxidation reaction on a surface was carried out with an applied oxidation potential +412 mV for 500 s. Likewise, the reduction potential −188 mV vs NHE was also applied for 500 s for the single reduction reactions on a surface. Variants of p58C were compared for charge transfer efficiency, with at least three trials of oxidation reactions at an applied potential of +412 mV vs. NHE for 500s. Charge transfer was measured for oxidized samples using CHI software, assessing the area under the reductive peak in CV after electrochemical oxidation.

**Activity Assay.**

The activity of WT and mutant yeast primase was measured using a modified malachite green assay. Native purifications of primase contain a population of both reduced (large majority) and oxidized (small minority) protein. The primase 4Fe-4S cluster is extremely sensitive to addition of oxidizing and reducing agents and rapidly degrades causing the protein to precipitate, so these assays report results from these ‘majority-reduced’ protein mixtures. Reactions were initiated by incubating 75 nM primase (WT or mutant) at 37 °C for 15 minutes in buffer containing 37.5 mM HEPES (pH 8.0), 62.5 mM NaCl, 12.5 mM MgCl$_2$, 1.25 mM TCEP, 0.25 mg/mL BSA, 125 μM ATP, 1.25 μM dT$_{50}$, 1.25 units of thermostable inorganic pyrophosphatase (New England BioLabs), and RNase free water. Aliquots (80 μL) of the reaction mixture were added to a 96-well clear bottom plate and quenched by the addition of 20 μL of malachite green prepared following the protocol from the Malachite Green Phosphate Assay Kit (Sigma-Aldrich). Absorbance at 620 nm was measured after incubation for 30 minutes at ambient temperature. Phosphate standards (0, 4, 8, 12, 16, 24, 32, and 40 μM) were prepared using the reaction buffer and stock phosphate standard from the kit. Assays were performed in biological triplicates consisting of two to three technical replicates each. Phosphate values were calculated using the linear trend line extrapolated from the standard curve.

*Biochemistry. Author manuscript; available in PMC 2022 October 12.*
RESULTS

Structure-based yeast p58C mutations designed to inhibit charge transport from the 4Fe-4S cluster

Inspection of yeast p58C shows that six tyrosine residues and one tryptophan bridge the DNA binding surface and 4Fe-4S cluster of yeast p58C. Four tyrosines (Y395, Y397, Y412, Y431) and the tryptophan are conserved between the two proteins (Figure 1). Two additional tyrosines, Y352 and Y353 are positioned close to the 4Fe-4S cluster and the DNA binding site and could be involved in 4Fe-4S cluster protection and 4Fe-4S to DNA electron transfer. All seven of these residues are within feasible distances (10–15 Å) to contribute to a network of possible electron transfer pathways within the protein, as measured by the distances between centroids of the residues in PDB 6D16 (Supplemental Table 3). These residues may all contribute to a redundant set of electron transfer pathways through the protein.46-48

In order to investigate the roles of these residues in electron transport, multiple site mutations in yeast p58C were prepared with the goal of completely suppressing the conduit through the protein between the 4Fe-4S cluster and the DNA binding site, including: Y352F/Y353F/Y395F/Y412F (5YF412), Y352F/Y353F/Y395F/Y397F/Y431F (5YF431), and Y352F/Y353F/Y395F/Y397F/Y412F/Y431F (6YF). We began by characterizing seven single- and multi-site tyrosine to phenylalanine mutations (Y395F, Y397F, Y412F, Y431F, 5YF412, 5YF431, 6YF) using CD measurements of thermal stability to assay the effect of the mutations. For the single site mutations, we found that all have apparent Tm values within 1-2 °C of the value measured for WT p58C (67 °C). Moreover, even multiple site Tyr-Phe mutations have only a modest effect on stability, with apparent Tm values of 63 ± 1 °C. We also prepared and tested the conserved tryptophan W376, which was also mutated to phenylalanine. Remarkably, the apparent Tm for the W376F mutant is 54°C, a full 13° lower than the wildtype protein. The disparity in the effect on thermal stability is likely due to the large number of hydrophobic contacts to the conserved tryptophan in the hydrophobic core. Thus, W367 seems to play a critical role in maintaining the structural integrity of the protein and thus was not included in any further experiments. These results suggested that generating constructs with multiple Tyr-Phe mutations would allow us to inhibit charge transport through the protein without significantly destabilizing the protein.

Multiple Tyr-Phe mutations retain the physical properties of yeast p58C.

While the Tyr residues are more peripheral to the hydrophobic core of yeast p58C and the substitution is relatively conservative, the combination of so many mutations risks causing significant perturbations in the protein. Importantly, in order for accurate electrochemical characterization, the mutant proteins must bind nucleic acid substrates similarly to the wild-type protein. Furthermore, structural alterations may lead to spurious results in electrochemistry by altering distances between residues key to the charge transport pathway. Structural perturbations might also complicate biochemical or cellular properties. In order to address these potential concerns, three key physical properties of the 5YF412, 5YF431, and 6YF multi-site mutants were compared to the wild type protein. CD spectroscopy was used to assess the distribution of secondary structural elements in solution. High resolution X-ray crystal structures were determined to identify any effects on the tertiary structure.
Fluorescence anisotropy assays were performed to determine if the affinity for DNA was appreciably perturbed.

The CD spectra of the three multi-tyrosine mutants revealed they all have similar global secondary structure distribution relative to the wild-type (WT) protein, with a high population of helical secondary structure elements (Figure 2A). The mutants were then crystallized using the same conditions as the WT protein\(^\text{22}\) and X-ray crystal structures were refined to 1.53 Å, 2.05 Å and 1.80 Å for 5YF412, 5YF431, and 6YF, respectively (Supplemental Table 4). The structures show that multiple tyrosine to phenylalanine mutations do not significantly alter the structure of p58C, as reflected, for example, in the very small backbone root-mean-square deviations (RMSD) compared to WT of 0.42 Å, 0.24 Å, and 0.47 Å, respectively (Figure 2B, Supplemental Table 4). The structural similarity is so significant that it extends even to the orientations of the phenylalanine side chains, which are the same as the tyrosine side chains for which they are substituted. Interestingly, the most significant effect on the structure is on the loop region near residue 412 (Figure 2B). Comparison of the 6YF mutant and WT structures reveal that a hydrogen bond is lost between H401 and Y412, which presumably correlates with the subtle perturbation of the loop (Supplemental Figure S1). It is possible that the effect on the structure in the crystal is suppressed and may be significantly larger in solution, as the subsequent turn and alpha helix are stabilized by crystal packing interactions. However, given the similarities of the structures in solution and in the crystal, it is likely that these mutations do not significantly disrupt the structure of the domain.

Fluorescence polarization anisotropy assays were carried out to measure the DNA binding affinities of 5YF412, 5YF431, and 6YF using a standard FITC labelled substrate as described previously\(^\text{21, 22, 35}\). These assays were performed aerobically, with protein containing primarily reduced 4Fe-4S cluster. Assays measuring the DNA binding affinity of the protein with an oxidized cluster are not possible because yeast p58C is highly sensitive to chemical oxidation, and electrochemical oxidation experiments use DNA as a means to oxidize the cluster. Since the oxidized protein binds tightly to the DNA, recovery of sufficient quantities of the purely oxidized protein from our electrochemical chips for DNA binding experiments is not feasible. In the reduced state, the protein binds DNA substrates with modest micromolar affinity. The mutants bind only slightly less well than the wild-type protein with K\(_D\) values of 13 ± 1.3 μM for 5YF412, 8.9 ± 0.8 μM for 5YF431, and 8.9 ± 1.6 μM for 6YF relative to the value of 4.0 ± 0.6 μM for the wild-type protein (Supplemental Table 5) Although the DNA binding affinity was slightly decreased compared to wild-type under these conditions, the proteins still bind DNA in the same, low micromolar regime. Together these results indicate that the tyrosine to phenylalanine substitutions in the hydrophobic core had only very minor effects on the structure, stability and DNA binding properties of p58C.

**Multiple Tyr-Phe mutations inhibit the electrochemical properties of yeast p58C**

To interrogate the effect of the 6YF mutant on the electron transfer from DNA to the 4Fe-4S cluster, we conducted an anaerobic electrochemical analysis using DNA-modified electrodes\(^\text{44}\) (Figures 3, S1, S2). The protein was subjected to bulk electrolysis and electron
transfer was monitored by cyclic voltammetry. All electrochemistry was performed in parallel with wild type p58C using the same multiplexed DNA-modified Au electrode. 

As expected from our previous studies, the redox state of the yeast wild-type p58C 4Fe-4S cluster was able to be modified by bulk electrolysis on the DNA-modified electrode. Oxidized yeast p58C engages in DNA charge transport, as evident from the electrochemical signal apparent from the cluster on the DNA-modified electrode following bulk oxidation (Figure 3). Following reduction of the oxidized p58C, however, the 4Fe-4S cluster becomes uncoupled from the DNA-modified electrode, which we attribute to dissociation of the 4Fe-4S protein from the DNA due to the lower DNA affinity of the reduced form \(^{21, 22}\). In contrast, we observe that bulk oxidation and reduction are inefficient for the multi-mutant proteins 5YF412, 5YF431, and 6YF, consistent with their inhibiting charge transfer through the protein (Figures 3, 4). Furthermore, for these mutants, severely attenuated charge transfer is evident after bulk oxidation.

It is worth noting that the 4Fe-4S cluster in our first 6YF mutant sample degraded over time. Electrochemical analysis with this aged sample indicated appreciable signal on the DNA-modified electrode, even though fresh samples of the 5YF412, 5F431, and 6YF mutants had shown little electrochemical signal (Figure 4). To establish whether the signal was DNA-mediated, we then tested the aged sample in parallel on DNA-modified electrodes, either containing or lacking an intervening abasic site. This test is essential to ensure whether one is examining a DNA-mediated reaction or signal associated with direct contact of the redox moiety with the electrode \(^{44}\). We found for this sample that an equivalent redox signal was found in the presence and absence of the intervening abasic site (Figure 5), consistent with the CV signal not being DNA-mediated. Importantly, this result was not observed for a fresh sample of 6YF or 5YF mutants. We suspect that bulk electrolysis of the older 6YF p58C sample destabilized the 4Fe-4S \(^{3+}\) state and degraded the cluster, releasing free iron into solution. To obtain further insights, we also performed EPR spectroscopy on WT, older 6YF and fresh 6YF samples (Supplemental Figure S3). For the intact samples we observed the trace appearance at \(g = 2.026\), which lies within the range reported for \([4Fe-4S]^{3+}\) forms of HiPiP clusters \(^{47, 48}\). However, in the EPR spectrum of the oxidized form of the degraded 6YF sample, a signal also appears at \(g = 3.12\); this signal is not characteristic of high spin Fe(III) and may reflect cluster degradation with formation of free Fe \(^{49}\). These observations suggest that the potential for degradation should be carefully considered when investigating the electrochemical properties of DNA-binding proteins containing 4Fe-4S clusters \(^{28, 49, 50}\).

**The 6YF mutant has reduced catalytic efficiency of RNA synthesis**

To determine if the reduced ability of multi-site p58C mutants to engage in redox switching correlate with an effect on the biochemical function of yeast primase, we compared the RNA priming activity of the maximally mutated 6YF mutant to wild-type yeast primase. Previous studies from our laboratory have shown that mutating Tyr395 and Tyr397 to phenylalanine reduces the ability of yeast p58C to switch between the oxidized and reduced states \(^{22}\). To assess the effect of these single- and the multi-site mutants relative to the wild-type protein, we compared their catalytic activity in an RNA primer initiation assay monitoring with a
coupled-synthesis phosphatase (malachite green) reporter that measures the quantity of free orthophosphate released by the nucleotide polymerization reaction.

As shown in Figure 6, the Y395F and Y397F mutants show a 2-3-fold lower release of orthophosphate relative to wild type primase (17.0 ± 0.5 μM, 11.8 ± 1.1 μM, 36.8 ± 0.8 μM, respectively). The amount of orthophosphate released in the reaction by the 6YF mutant (0.9 ± 0.2 μM), however, revealed a much more dramatic decrease in activity. Since the value is at the lower detection limit of the assay, we estimate that the decrease is at least 40-fold. Here, we find that there are only very small differences in the structure and DNA binding affinity for wild type versus 6YF p58C. The one significant difference between the two proteins is their ability to engage in charge transfer. Thus, the trend seen in these data is consistent with our proposal that there are many possible tyrosine-mediated pathways for charge transport in yeast p58C and that inhibiting these pathways in total reduces the ability of yeast primase to bind DNA and generate primers.

DISCUSSION

DNA-mediated redox switching of the 4Fe-4S cluster in the DNA primase p58C domain alters its association with DNA substrates. The 4Fe-4S cluster is buried within the domain, at its closest, 6-7 Å away from the protein surface. However, the pathway through the protein to the surface is highly insulated with aliphatic sidechains, which inhibits direct charge transport. In contrast, we have shown that changes in the 4Fe-4S charged state can occur via DNA charge transport. Electron transfer/hole hopping via charge transport-competent tyrosine and tryptophan side chains act as a bridge between the 4Fe-4S cluster and the DNA binding surface. Unlike human p58C, inspection of the yeast p58C structure reveals there are multiple potential charge transport pathways between the 4Fe-4S cluster and the DNA substrate (Figure 1). We surmised these pathways in yeast p58C are redundant and all can support charge transport and enable redox-dependent alteration of DNA binding.

Are the time scales of charge transport and primer synthesis properly aligned? Primase must undergo several conformational changes to initiate primer synthesis. As such, de novo primer synthesis is very slow; calf thymus primase synthesizes products on the order of 0.0027 s⁻¹. Charge transport through DNA and proteins occur on ps and μs timescales, respectively. In contrast, conformational changes in proteins typically occur on the 10 μs to ms timescale. Thus, the timescale of electron transfer through the protein is sufficiently fast to allow redox switching during the catalytic cycle, even with the multi-step, long distance electron hopping paths in p58C. In fact, there are two slow steps in RNA primer synthesis: the initiation step and primer release after product formation. These two slow steps are where we hypothesize electron transfer to occur. The very rapid timescale of electron/hole hopping relative to the slow rate of primer synthesis is therefore consistent with our model of primase function.

To test our hypothesis that the multiple pathways through yeast p58C can all support charge transport, mutants with multiple Tyr-Phe substitutions between the protein surface and the 4Fe-4S cluster were prepared and their charge transfer properties compared to the wild-type.
protein. Despite the fact that as many as six mutations were incorporated, the mutants were found to be remarkably stable and unperturbed in their ability to bind DNA. Moreover, their structures were nearly identical to the wild-type protein, even to the point of the side chains that are mutated having the same orientation (Figure 2B). Furthermore, although six hydroxyl groups were removed in 6YF, only three hydrogen bonds were lost.

We had previously shown that charge transfers through human and yeast p58C are robust\textsuperscript{21, 22}. Human p58C has a limited number of tryptophans and tyrosines that contribute to a well-defined pathway through which electrons can travel. In contrast, yeast p58C has many such residues that may participate in charge transfer through the protein. As a result, multiple pathways for charge transport to the cluster exist. Indeed, our previous results for single-site yeast primase mutants suggested that charge transfer through p58C is essential for proper primase function, but that alternate pathways through p58C likely exist. Thus, the ability of the 4Fe-4S cluster to support redox reactions in yeast primase depends more on the integrity of the cluster than the specific pathway the electron takes through the protein.

The 6YF mutant, lacking the six tyrosines bridging the 4Fe-4S cluster to the DNA binding site, shows little charge transfer efficiency compared to the wild-type protein (Figures 3, 4). This defect is more severe than for single site Tyr mutants\textsuperscript{22}. Moreover, for the mutants containing 5 tyrosine mutations, the defect is also severe. These results support the hypothesis that there are multiple, redundant electron transfer pathways through the protein; the single-site mutants can compensate for the loss of one tyrosine residue by “redirecting” charge through an alternate pathway. However, as the number of mutations increases, leading to fewer paths, a threshold is reached where charge transfer is greatly attenuated. No single pathway is expected to be dominant. Instead, transport of charge is anticipated to be stochastic, dependent on the dynamic “breathing” of the protein and the particular electronic environment of each tyrosine side chain.

Charge transport through human p58C has been explored through molecular dynamics (MD) simulations, where multiple electron transfer pathways have been described using hole hopping among tyrosine and tryptophan residues\textsuperscript{47, 48}. In that study, the MD calculations predict a change in DNA binding affinity upon cluster oxidation, as required for redox switching. Moreover, analysis of the contribution of various tyrosine residues to charge transport pathways led to the proposal that mutations along the pathway render the use of alternative “routes” through the protein more favorable, potentially at the cost of a decrease in rate of transfer\textsuperscript{47, 48}. In this model, “breathing” of the protein also promotes destabilization of the 4Fe-4S\textsuperscript{3+} cluster and its subsequent degradation into [3Fe4S]\textsuperscript{+}. Extrapolating from this model, the observation of degradation of the 4Fe-4S\textsuperscript{3+} state into [3Fe4S]\textsuperscript{+} state in the aged sample of 6YF may be associated with its slow rate of electron transfer and longer lifetime in the oxidized state.

We find a modest two-fold reduction in the intrinsic substrate binding affinity by fluorescence anisotropy for 6YF mutant that has not been chemically or electrochemically oxidized and thus, is primarily in the lower affinity reduced form. We have previously shown that oxidation of the 4Fe-4S cluster enhances the association of cluster-containing proteins with template DNA\textsuperscript{23}. The 6YF mutant was designed to short circuit the electron
transport pathways and the electrochemistry results indicate that the design was successful, as the 6YF mutant is less efficient at carrying out electron transfer to the 4Fe-4S cluster than the wild-type protein. The lower efficiency means that in comparison to the wild-type protein, less protein will be oxidized under a given set of conditions and consequently fewer molecules bind to substrate, which in turn results in ~10-fold less total protein being oxidized (Figure 4). Thus, the electrochemistry experiments show that the small reduction in intrinsic binding affinity observed for the 6YF mutant is far outweighed by the combined effects of the mutations on the ability to undergo redox switching.

Our measurements of primase activity are consistent with the electrochemical data. Single-site substitution of Tyr residues somewhat affects electron transfer and catalytic activity but alternate electron transfer pathways are possible, so significant activity is retained (Figure 5). However, when all pathways are substantially inhibited, the higher DNA binding affinity associated with oxidation is lost and we find that the primase activity of the 6YF mutant is substantially diminished. Importantly, activity is lost even though the mutations are far from the catalytic site and do not directly affect catalysis. Instead, the loss of activity associated with the ensemble of mutations can be attributed to the inability of the protein to carry out DNA-mediated redox switching.

In summary, although many tyrosine-mediated pathways for charge transport are available in yeast p58C, inhibiting these pathways inhibits redox switching and hence the ability of primase to initiate RNA primer synthesis. The results reported here provide support for our model for DNA primase function involving redox switching of the 4Fe-4S cluster.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This research was supported by National Institutes of Health grants R35 GM118089 (W.J.C.), R01 GM126904 (J.K.B.), T32 GM0320 (L.E.S.) and T32 CA009582 (A.M.B.). Further funding was provided by the Vanderbilt University Leadership Alliance FYRE program to AJD and the Jiangsu Univ Study-Abroad Program to HC. We thank Professor Jason Slinker for providing multiplexed chips. We would also like to acknowledge Professor Mike Hill, Dr. Rebekah Silva, and Dr. Adela Nano for help with initial electrochemistry training and valuable discussions. We also thank Dr. Paula H. Oyala for performing EPR data collection and for valuable discussions. We would like to acknowledge the LS-CAT beamline scientists and staff. This research used resources of the Advanced Photon Source, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory under Contract No. DE-AC02-06CH11357. Use of the LS-CAT Sector 21 was supported by the Michigan Economic Development Corporation and the Michigan Technology Tri-Corridor (Grant 085P1000817).

REFERENCES


Figure 1.
Comparison of conserved tyrosine residues and substrate binding site in human and yeast p58C. (a), Conserved tyrosine side chains in human p58C (PDBID 5F0Q) are colored red. A conserved tryptophan is colored yellow. The substrate binding interface is approximated by the curved black line. (b), Conserved tyrosine side chains in yeast p58C domain (PDBID 6DI6) are colored red. A conserved tryptophan residue is colored yellow. Three additional tyrosine side chains in this region that presumably also contribute to electron hopping are colored purple. The substrate binding interface is approximated by the curved black line. (c), Superposition of the yeast p58C (red, PDBID 6DI6) and human p58C in complex with an RNA/DNA product substrate (protein in light grey, RNA/DNA substrate in dark grey; PDBID 5F0Q). These structures are highly similar as reflected in the backbone RMSD of 0.88 Å. (d), The structure of yeast p58C in the same orientation as (c) rendered as an opaque surface colored by electrostatic field at the surface with blue for positive charge and red for negative charge. The polyanionic RNA/DNA substrate from the 5F0Q structure of human p58C fits very well in the highly basic surface of yeast p58C, which closely aligns with the substrate binding site in the human structure.
Figure 2.
Multiple tyrosine to phenylalanine mutations do not significantly disrupt yeast p58C structure or DNA binding. (a), Circular dichroism spectroscopy of yeast p58C wild-type (WT) and tyrosine to phenylalanine mutants (5YF412, 5YF431, and 6YF) measured at 20 °C). Each curve represents the average of three smoothed measurements. Ellipticity for buffer and cuvette alone were subtracted from each data point. Data were scaled to the WT spectrum. (b), X-ray crystal structures of yeast 5YF412, 5YF431, and 6YF multi-tyrosine to phenylalanine p58C mutants (color) overlaid with WT p58C (grey, PDB ID code 6DI6). Mutated residue side chains shown. Structure for yeast 5YF412 determined at 1.53 Å, yeast 5YF431 at 2.05 Å, and yeast 6YF at 1.80 Å. Detailed data collection and refinement statistics in Supplemental Table 4 (c), Fluorescence polarization anisotropy (FA) of yeast p58C WT and tyrosine to phenylalanine mutants 5YF412, 5YF431, and 6YF, performed on ice and measured at room temperature (19-21 °C). A FITC-labeled 8 base pair duplex fluorescent substrate that contains a single-stranded/double-stranded DNA junction was used. K ds are reported in Supplemental Table 5.
Figure 3.
Anaerobic electrochemical analysis of the yeast p58C mutants. Bulk electrolysis (left) and CV after oxidation (middle) of (a) p58C WT and (b) 6YF. (c) Illustration of change in DNA binding associated with redox switching on a multiplex chip (right). All scans were performed on 38 μM 4Fe-4S p58C in a buffer containing 20 mM Tris-HCl and 75 mM NaCl at pH 7.2, with a 100-mV/s scan rate for CV.
Figure 4.
Quantification of total charge transferred for yeast p58C and multi-mutants. Total charge transferred (nC) after electrochemical bulk oxidation conditions (8.33 min) for wild-type, 5YF412, 5YF431, and 6YF were 51.0 ± 17.8 nC, 8.5 ± 5.3 nC, 7.1 ± 4.4 nC, 4.0 ± 1.4 nC, respectively. All scans were performed on 38 μM 4Fe-4S p58C variants in a buffer containing 20 mM Tris-HCl and 75 mM NaCl at pH 7.2, with a 100-mV/s scan rate for CV. Error bars indicate the standard deviation for each protein (N = 3).
Figure 5.
Characterization of degradation of the 4Fe-4S cluster in p58C. Pictorial representation of multiplex Au chip (top). Two chips were prepared, with one half of each chip consisting of well-matched (WM) DNA (dark blue) and the other containing DNA with an abasic site (red). Anerobic bulk electrolysis and CV for oxidation an electrode modified with well-matched DNA duplexes (blue) and abasic site (abasic DNA signal in red) of p58C WT and 6YF (degraded) mutant. All scans were performed on 38 μM 4Fe-4S p58C in a buffer containing 20 mM Tris-HCl and 75 mM NaCl at pH 7.2 with a 100-mV/s scan rate for CV.
Figure 6.
Phosphate (μM) released from initiation assays of wild-type, Y395F, Y397F, and 6YF primase. The mean and standard deviation of the amount of phosphate released from 3 independent initiation assays of wild-type, Y395F, Y397F, and 6YF primase were 36.8 ± 0.8 μM, 17.0 ± 0.5 μM, 11.8 ± 1.1 μM, 0.9 ± 0.2 μM, respectively (± SE).