DNA Polymerase α Subunit Residues and Interactions Required for Efficient Initiation Complex Formation Identified by a Genetic Selection*

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Background: A genetic selection revealed interactions important for replication initiation.

Results: A PHP mutation ablated interaction with the ε subunit and distorted the active site. C-terminal mutations decreased binding by the clamp loader τ subunit.

Conclusion: The β binding domain functions in both β and τ binding.

Significance: This work advances our knowledge of replicase interactions critical for function.

Biophysical and structural studies have defined many of the interactions that occur between individual components or subassemblies of the bacterial replicase, DNA polymerase III holoenzyme (Pol III HE). Here, we extended our knowledge of residues and interactions that are important for the first step of the replicase reaction: the ATP-dependent formation of an initiation complex between the Pol III HE and primed DNA. We exploited a genetic selection using a dominant negative variant of the polymerase catalytic subunit that can effectively compete with wild-type Pol III α and form initiation complexes, but cannot elongate. Suppression of the dominant negative phenotype was achieved by secondary mutations that were ineffective in initiation complex formation. The corresponding proteins were purified and characterized. One class of mutant mapped to the PHP domain of Pol III α, abating interaction with the ε proofreading subunit and distorting the polymerase active site in the adjacent polymerase domain. Another class of mutation, found near the C terminus, interfered with τ binding. A third class mapped within the known β-binding domain, decreasing interaction with the β2 processivity factor. Surprisingly, mutations within the β binding domain also ablated interaction with τ, suggesting a larger τ binding site than previously recognized.

The *Escherichia coli* DNA polymerase III holoenzyme (Pol III HE) serves as a prototype for cellular replicases in all cellular systems (for a review, see Ref. 1). It encompasses the standard tripartite composition found in all branches of life: (i) a DNA polymerase that is non-processive and exhibits no special properties by itself (2, 3), (ii) a sliding clamp processivity factor, Pol III δ, that recircles DNA and contacts the polymerase and ε proofreading subunit locking the enzyme into a processive complex (4–8), and (iii) a “clamp loader” the DNAX complex (τ2γδβ′χψ) that expels the energy of ATP hydrolysis to assemble β2 onto a primer terminus and then chaperones the associated Pol III α subunit onto β2 (9, 10). Together, these three assemblies form a tight initiation complex on primed DNA in the absence of dNTPs (11). Upon addition of dNTPs, the Pol III HE advances rapidly and processively to synthesize at least 150 kb of DNA without dissociating and, perhaps, the entire *Escherichia coli* chromosome if a blocking lesion is not encountered (12, 13).

Components of the DNAX complex are also involved in processive elongation. The presence of the τ subunit is required to protect the elongating complex from dissociation by removal of β2 by exogenous DNAX complex (14). The presence of δ and δ′, primarily characterized for their role in initiation complex formation are also required for processive elongation (15). The χ subunit contributes to processive elongation by interaction with single-stranded DNA-binding protein, stabilizing Pol III HE on the replication fork (16–20).

Significant information is available regarding subunit interactions within the Pol III HE derived from quantification of physical interactions with subassemblies in solution and by determination of the structure of subassemblies (7–9, 21–33). More limited information is available regarding the dynamic interactions between Pol III HE subunits in the presence of all reaction components and the importance of these interactions at discrete reaction stages. To address this deficit in our understanding, we exploited a dominant negative Pol III α that contains a mutation in one of the critical acidic catalytic residues. Pol III α D403E can efficiently form initiation complexes but is unable to elongate (34). This results in sequestration of primer termini and blockage of competing wild-type Pol III HE (10).

This provided the basis for a genetic selection to isolate secondary mutations within *dnaE* D403E that caused loss of the dominant negative phenotype. We exploited this selection and identified several diverse mutations, spatially dispersed in three separate domains of Pol III α. Physical and enzymological characterization of these variant proteins provided additional insight regarding the positions of subunit interaction within Pol
III α and the importance of these interactions in the initiation complex formation stage of the replicative reaction.

Experimental Procedures

Bacterial Strains

Strains used in this study are listed in Table 1. BL21(DE3) was used for expression of T7 promoter-containing plasmids. Both TOP10 and JCL60 were used for the selection of suppressor mutants. JCL60 and BL21(DE3) contain the integrated lacI promoter (arabinose inducible) and both origins of replication) generating pJCL5. JCL60 was pre-
pared by integrating the lacI promoter into E. coli TOP10 using a commercial lacI lysogenization kit.

Plasmids

Plasmids used in this study are listed in Table 1. pJCL5 contains dnaE D403E (plus N-terminal His6 and biotinylation tag) under control of the pBAD promoter (arabinose inducible).

pJCL5-lacZα-T7 is identical to pJCL5 except that it contains a T7 promoter upstream of the pBAD promoter and lacZα downstream of the dnaE D403E (Fig. 1). Overlap PCR (35) was used to place the lacZα downstream of the dnaE D403E. Primers S7269 (5’-GTGTGACACCTCTTTATTAGTCAAACTCCAG-TTCCACC) were used to amplify a DNA fragment containing the 3’ end of dnaE D403E using pDEFER.2 as a template (1645 bp). Primers S9833 (5’-TAAGGAGCTGTACATATGAC-CATGATTACGCATTC) and A10047 (5’-GCGGCCGCTTA-TTAGGCCCATGCGATTTAGCCG) were used to amplify lacZα from E. coli MG1655 genomic DNA (210 bp). The overlap PCR fragment was generated using the outside primers S7269 and A10047 and the 1645- and 210-bp PCR fragments from above as templates. The resulting overlap PCR fragment (1842-bp fragment) was digested with BglII and NotI and ligated into the pJCL5 vector using pDEFER.2 as a template (1645 bp).
and A6276 using the 1110- and 339-bp PCR fragments from above as templates. The resulting overlap PCR fragment was digested with SphI and BamHI (1.25 kb) and ligated into pJCL5-lacZα-T7 digested with SphI and BamHI (8.8 kb) to generate pJCL5-lacZα-T7.

*sd*e8 Expression Vectors (Double Mutant; *dnaE* D403 and *sde*)—pJCL8 expresses *dnaE* D403E,A877E (*sde8*), from the T7 promoter. The *sde8* mutation in pJCL8 was originally isolated from a genetic selection using the pJCL5 plasmid as the parental vector. The plasmid also contained an additional mutation (D792G) that was shown not involved in the suppression phenotype as separation of the D792G from the A877E did not alter the suppressor phenotype of A877E (data not shown). Consequently no further characterization of D792G was performed. To move the *sde8* mutation into a clean T7 expression plasmid (away from D792G), overlap PCR was used to amplify *dnaE* D403E,A877E. Primers JCL42 (5′-TTTTCTTGAGCGATCAGC-GACC) and JCL44 (5′-AGACGTGGCGGT-TTCC) were used to amplify two PCR fragments from the pDFER.2 plasmid bearing the *dnaE* D403E mutation. The overlap PCR fragment was generated by using the outside primers JCL42 and JCL41 and using the 2171- and 815-bp fragments from above as templates. The resulting overlap PCR fragment (2966 bp) was digested with EcoRI and HindIII and ligated into pDFER.2 (EcoRI and HindIII sites) generating pJCL8. The pJCL21 plasmid expresses *dnaE* D403E,W1134C (*sde50*) from the T7 inducible promoter. The *sde50* mutation in pJCL21 was originally isolated from a genetic selection using the pJCL5 plasmid as parental vector. To construct pJCL21, the pJCL5 plasmid harboring the *sde50* mutation (*dnaE* D403E, W1134C) was digested with SphI/PstI plasmid to remove the *sde343* (*dnaE*) from the T7 promoter. The PCR fragment was cloned into pET11.N0.1 (397 bp) generating pJCL24. The pJCL25 plasmid expresses *dnaE* D403E,A877E, D403E,W1134C, sde343 from the T7 promoter. The *sde343* from the T7 promoter. The *sde343* from the T7 promoter. The *sde343* from the T7 promoter. The *sde343* from the T7 promoter. The *sde343* from the T7 promoter.

The pJCL24 and pJCL25 plasmids express *dnaE* D403E, L1157Q (*sde50*) and *dnaE* D403E Δ981–927 (sde388) from the T7 inducible promoter, respectively. The sde388 mutation was generated by using the outside primers JCL42 and JCL41 and using the 2171- and 815-bp fragments from above as templates. The resulting overlap PCR fragment (2966 bp) was digested with EcoRI and HindIII and ligated into pDFER.2 (EcoRI and HindIII sites) generating pJCL8. The pJCL21 plasmid expresses *dnaE* D403E,W1134C (*sde50*) from the T7 inducible promoter. The *sde50* mutation in pJCL21 was originally isolated from a genetic selection using the pJCL5 plasmid as parental vector. To construct pJCL21, the pJCL5 plasmid harboring the *sde50* mutation (*dnaE* D403E, W1134C) was digested with SphI/PstI plasmid to remove the *sde343* (*dnaE*) from the T7 promoter. The PCR fragment was cloned into pET11.N0.1 (397 bp) generating pJCL21.

The pJCL24 and pJCL25 plasmids express *dnaE* D403E, L1157Q (*sde50*) and *dnaE* D403E Δ981–927 (sde388) from the T7 inducible promoter, respectively. The sde388 mutation was generated by using the outside primers JCL42 and JCL41 and using the 2171- and 815-bp fragments from above as templates. The resulting overlap PCR fragment (2966 bp) was digested with EcoRI and HindIII and ligated into pDFER.2 (EcoRI and HindIII sites) generating pJCL8. The pJCL21 plasmid expresses *dnaE* D403E,W1134C (*sde50*) from the T7 inducible promoter. The *sde50* mutation in pJCL21 was originally isolated from a genetic selection using the pJCL5 plasmid as parental vector. To construct pJCL21, the pJCL5 plasmid harboring the *sde50* mutation (*dnaE* D403E, W1134C) was digested with SphI/PstI plasmid to remove the *sde343* (*dnaE*) from the T7 promoter. The PCR fragment was cloned into pET11.N0.1 (397 bp) generating pJCL21. The pJCL24 and pJCL25 plasmids express *dnaE* D403E, L1157Q (*sde387) and *dnaE* D403E Δ981–927 (sde388) from the T7 inducible promoter, respectively. The sde388 mutation was generated by using the outside primers JCL42 and JCL41 and using the 2171- and 815-bp fragments from above as templates. The resulting overlap PCR fragment (2966 bp) was digested with EcoRI and HindIII and ligated into pDFER.2 (EcoRI and HindIII sites) generating pJCL8.

The pJCL26 plasmid expresses *dnaE* D403E,Q238K mutation (*sde343*) from the T7 promoter. The *sde343* mutation in this plasmid was originally isolated from a genetic selection using the pJCL5-lacZα-T7 plasmid as the parental vector. In this case, the *sde343* mutation was moved into the pET11.N0.1 expression vector by overlap PCR. The 5′ region of *dnaE* bearing the Q238K mutation was amplified using oligos JCL45 (5′-GGCCTGCAAGAGCGAGCC) and JCL46 (5′-ACGCATATTTCTGCGGCCGA) using pDFER.2 as template. The region encoding the *dnaE* D403E mutation was amplified using oligos JCL47 (5′-TCTGCGCGCAAGATATGCGT) and JCL48 (5′-TACAGGCCTAAGCTTTCAGTCCC). The overlap PCR fragment was generated using the outside primers JCL45 and JCL48 and using the 728- and 2176-bp PCR fragments from above as templates. The resulting overlap PCR fragment at 2884 bp was digested with Stul and Pstl (2873 bp) and ligated into the Stul and Pstl sites of pDFER.2 (7187 bp) generating pJCL26.

*sd*e8 Expression Vectors (Single sde Mutation Only)—The pET11.N0.1 plasmid fuses a His6 tag and a biotinylation sequence to the N terminus of the Pol III α subunit under control of the T7 promoter (22, 24). Site-directed mutagenesis was performed by using the QuickChange Lightning Multi Site-directed Mutagenesis Kit (Agilent) using pET11.N0.1 as a template. For pET11.sde8, the WT sequence TCCGGGCGTTTGACCGT was changed to TCCGGGAGTTTGACCGT. For pET11.sde50, the WT sequence GCCAGCCTGCGTCGT was changed to GCCAGCCTGCGTCGT. For pET11.sde387, the WT sequence GTGGAACGTAGGTTTT was changed to GTGGAAACGTAGGTTT. For pET11.sde388, the WT sequence GTGCTGGAA[AACGTATACGT] ... TTCCGG-GTCGTC[G]CCGGAAGAG was changed to GTGCTGGAA[D]GCCGGAAGAG. All mutations were verified by sequencing.

**Mutant Isolation and Characterization**

The selections to isolate suppressors of *dnaE* D403E using pJCL5 and pJCL5-lacZα-T7 plasmids were identical except that pJCL5-lacZα-T7 contains a lacZα reporter (Fig. 1). The plasmids were transformed into TOP10 or JCL60, respectively (Table 1: strains JCL52 and JCL68). The cells were grown to late-log phase in L-broth + 25 μg/ml of kanamycin and then plated onto LB + 25 μg/ml of kanamycin + 0.2% arabinose plates to induce the pBAD promoter. Eight independent selections were performed with pJCL5-lacZα-T7 and 11 with pJCL5 to identify spontaneous suppressors. All resultant colonies were patched onto LB + 25 μg/ml of kanamycin + 0.2% arabinose ± 40 μg/ml of X-Gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside), to provide a visual indication of promoter integrity and polar effects caused by poor *dnaE* expression (Fig. 1). In suppressor selections harboring the pJCL5-lacZα-T7 plasmid: white colonies indicated mutated genes that were not expressed, whereas blue colonies indicated an intact promoter and normal levels of *dnaE* expression. The plates were incubated at 37 °C for 2 days. Spontaneous suppressors arose at an average frequency of 1.6 × 10⁶ for strain JCL52 and at an average frequency of 1 × 10⁵ for strain JCL68. To confirm the suppressor mutation was linked to the plasmid, the plasmid DNA was purified from each mutant using a Qiagen mini-prep kit, and the plasmid DNA was backcrossed into the parent strain (JCL60) and tested for its ability to suppress the dominant negative phenotype of *dnaE* D403E and expressed (blue when streaked on plates containing X-Gal) (Fig. 1).

Immunoblots were performed on all plasmid-linked mutants to check for expression of full-length *dnaE* D403E, Sde protein (Fig. 1), 2 ml of cells were grown to late-log phase *A*₆₀₀ = ~0.8, and induced with 1 mM isopropropyl 1-thio-β-D-galactopyranoside in the presence of 20 μM d-biotin. 1 ml of cells were harvested by centrifugation, and lysed with 5 mg/ml of lysozyme on
ice for 20 min, 1 min at 37 °C. A total of 40 μl of the soluble protein, isolated after centrifugation, was separated on a 4–20% SDS-PAGE gel and run next to full-length purified Pol III α as a marker. The SDS-PAGE gel was transferred to PVDF membrane, and incubated with a streptavidin-horseradish peroxidase-conjugate (GE Life Sciences) at a 1:1500 dilution in phosphate-buffered saline (PBS) to present visualization of the biotin tag. The immunoblot was developed using the enhanced chemiluminescence developer (GE Healthcare). All full-length intragenic suppressors were sequenced on both strands to identify mutations using the primers JCL37 (5′-CGAATCTTTCAAGG), JCL22 (5′-CTCAAAAGGATTACGG), JCL24 (5′-CTGGAGAGCTTCTG), JCL26 (5′-GGCCAAACATCGGTCT), JCL28 (5′-GGTTACGGATTTAC), and JCL30 (5′-CACCCATATCAACCG).

**Purification of Sde Pol III α Subunits**

**Purification of Sde Mutant α Proteins (Lacking dnaE D403E)—BL21(DE3) strains containing overexpression pET11. sde** plasmids were grown in 3.0 liters of L-broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl) + 100 μg/ml of ampicillin at 23 °C at 220 rpm on a rotary shaker. It was necessary to express all mutants at 23 °C to avoid degradation and to maximize the amount of soluble protein. Cells were grown to stationary phase and harvested by centrifugation 3 h after induction.

Cells were lysed as described (34) in the presence of 3 mg/ml of lysozyme, 5 mM benzamidine, 1 mM PMSF, and 5 mM EDTA (34). The Pol III α-fusion protein was precipitated in 50% (w/v) ammonium sulfate, followed by Ni²⁺-nitrotriacetic acid column chromatography as described (24). After Ni²⁺-nitrotetracetic acid chromatography, peak fractions were monitored using the gap-filling assay and/or by 4–20% SDS-PAGE gels to detect full-length protein. Fractions were assayed for protein concentration using the Coomassie Plus Bradford Assay Kit (Pierce). Purity of the protein was assessed on 4–20% SDS-PAGE gels.

Gap-filling assays for protein activity could not be used because the Pol III α D403E-sde proteins are catalytically inactive because of the dnaE D403E mutation. Pooled TALON column peak fractions (>50% peak concentration) were diluted in Buffer I (50 mM imidazole (pH 6.5), 25 mM NaCl, 1 mM EDTA, 20% glycerol, 5 mM DTT) to a conductivity equivalent to Buffer I and loaded onto a Bio-Rex 70 column, 1-ml bed volume, at a flow rate of 200 μl/min as previously described (36). The column was washed with 4 column volumes of Buffer I, and the protein was eluted with a 10-column volume 25–300 mM NaCl gradient. Again purified α was analyzed as described above.

**Biochemical Assays**

**Pol III HE Reconstitution Assay—This assay measures the processive function of Pol III on long single-stranded DNA templates. Assays were performed as described (34) with the following differences. Pol III HE replication assays (25 μl) were assembled on ice using the τ complex (150 fmo1; 6 mM τ₃δδ⁴χψ final), β₂ (150 fmo1; 6 mM dimer final), DnaG primase (1 pmol; 40 mM final), SSBₜ (single-stranded DNA-binding protein) (15 pmol; 600 mM final), mixed M13Gori DNA (58 fmol as ssDNA circle; 2.3 mM final), four nTPs (0.2 mM each final), four dNTPs (48 μM each dATP, dCTP, and dGTP final); 18 μM [³²P]dTTP (100 cpn/pmol), and 10 mM (final) magnesium acetate. The reaction was initiated by adding the WT or mutant Pol III α subunit as the last step and then immediately placing the reaction at 30 °C for 5 min.**

**Gap Filling Polymerase Assay—The gap filling assay measures the ability of Pol I to fill in the gaps of a nuclease-activated calf thymus DNA as a template. It does not require interaction with the other Pol III holoenzyme subunits. Gap filling assays were performed as described (34).**

**Pol III HE Competition Assay—This assay measures the ability of a mutant Pol III α to assemble at a primer terminus and thereby block catalysis by Pol III α. Pol III reaction mixtures were assembled on ice (34) using mutant Pol III α polymerase (25–150 fmo1; 1–6 mM final).** Reactions were initiated by adding the WT Pol III α (50 fmo1; 2 mM (final)) immediately placing the reaction at 30 °C for 5 min. Control experiments included heat denaturation of the competitors before addition to the assay. In these cases, no inhibition of the wild-type activity was observed.

**Surface Plasmon Resonance**

A BLACore 3000 instrument was used to quantify α-β₂β, α-ε, and α-τ interactions. Pol III α was immobilized on a streptavidin chip by a biotin-streptavidin interaction (26, 32, 37). A flow rate of 5 μl/min in HPKG buffer (50 mM Hepes (pH 7.4), 100 mM potassium glutamate, 10 mM magnesium acetate, 0.005% P-20 surfactant) at 25 °C was used for α-β₂ and α-ε interactions. A flow rate of 25 μl/min was used to measure the α-τ interaction. All buffers were filtered and degassed before use. The Sensor Chip SA (BLACore) was conditioned with three 1-min injections of 1 mM NaCl, 50 mM NaOH prior to attachment of wild-type Pol III α or Sde subunits. For α-β₂ and α-ε interactions, a total of 3500 response units of Pol III α was loaded per flowcell. For the α-τ interaction, a total of 500 response units of α was loaded per flowcell. Flowcell 1 was not derivatized and
was used as a control for background subtraction. Flowcells 2, 3, and 4 were used individually for different WT or mutant Pol III α subunits.

α-β₂ Binding—As observed previously (24), the on- and off-rates for β₂ binding were too fast to measure. Thus, chip regeneration was not necessary. The dissociation constant \( K_D \) for α-β₂ binding was determined by running a series of concentrations in quadruplicate of β₂ (40 μM each of 50, 100, 250, 500, 1000, 2500, 5000, and 10,000 nM) over the Sde mutant or WT α protein and determining the equilibrium level of β₂ bound. The \( R_{\text{max}} \) level of each response curve was plotted as a function of the β₂ concentration and fit to the 1:1 Langmuir model using BIAevaluation 4.1 software as described (24). The \( K_D \) values shown for the α-β₂ interaction are derived from the average of the quadruplicate determinations.

α-ε Binding—Binding conditions were similar to that described in Ref. 37. Three different concentrations of ε subunit (40 μL of 500, 1000, and 1500 nM) were run over a Pol III α-derivatized flowcell. The dissociation constant \( K_D \) was calculated by measuring the ratio of the determined rate constants for \( k_{\text{on}} \) and \( k_{\text{off}} \) at each concentration injected using nonlinear regression analysis using SigmaPlot software. The \( K_D \) was derived by the equation \( K_D = k_{\text{off}}/k_{\text{on}} \). ε was allowed to dissociate for 3 h, which is sufficient for complete dissociation of α-ε complexes. Thus, regeneration of the chip surface was not required. The dissociation constant \( K_D \) values shown were derived from the average of the \( K_D \) measured at each concentration for the individual α-ε interaction.

α-τ Binding—As observed previously (24), τ binds to wild-type Pol III α with high affinity. We could not find conditions that would dissociate the τ from α without denaturing Pol III α. A total of 200 μL of τ (50 nM) was injected over the surface of the α-derivatized chip at a flow rate of 25 μL/min (27) to prevent mass transport effects seen in the response curves at lower flow rates (5 μL/min) (data not shown). The concentration of τ was set at 50 nM so that a direct comparison of the dissociation constants could be made across the different WT- and mutant-α derivatized subunits (22, 24). Dissociation was measured for 2000 s. Dissociation constants were determined by globally fitting the resulting sensorgrams to a 1:1 Langmuir model using BIAevaluation 4.1 software.

Results

The availability of a dnaE D403E that encoded a protein that was inactive for elongation but competent for initiation complex formation provided a convenient genetic selection for secondary mutations that suppressed the dominant negative phenotype. These mutations would be expected to generate proteins that were defective in the initiation complex formation stage of the replicase reaction causing loss of their ability to compete with wild-type Pol III α.

We generated a plasmid that expressed dnaE D403E in trans under control of an arabinose-inducible promoter. Cells containing this plasmid were inviable in the presence of arabinose. Eleven independent selections were conducted to identify plasmid-borne spontaneous suppressor mutations. The most convenient selection protocol is diagramed in Fig. 1. Cells were grown in liquid culture in the absence of arabinose and then plated in the presence of arabinose to kill cells lacking suppressors of the dominant negative allele. Surviving cells were designated sde mutants (suppressor of dnaE). The presence of X-Gal on the plates and the placement of the lacZα fragment downstream of dnaE D403E permitted elimination of those trivial
Genetic Selection for Initiation Complex Formation Defects

mutations resulting from defects in the arabinose-inducible promoter, and polar mutations that caused decreased expression of dnaE. Blue colonies, indicating expression of lacZα, were grown, plasmid was isolated, and backcrossed into JCL60 to confirm that the sde mutation was linked to the plasmid. Viable cells were grown, lysed, and protein extracts were subjected to immunoblots to screen for those cells that expressed full-length Pol III α, eliminating mutants that caused truncation. Plasmids expressing normal levels of full-length Pol III α containing sde mutations were sequenced. Five containing diverse point mutations were subjected for further characterization. One deletion mutant that had lost the loop previously shown to be required for interaction of Pol III with DnaE was also characterized. These are shown mapped onto the homologous residues within the crystal structure of Taq Pol III α (38) (Fig. 2).

To provide a biochemical check for the predicted phenotype, each of the selected sde mutants were expressed and the corresponding proteins purified. As expected, all were reduced in their ability to compete with wild-type Pol III α in Pol III HE reconstitution assays (Fig. 3).

Pol III α expression plasmids were constructed that contained sde mutations in the absence of the D403E mutation. This allowed potentially catalytically active Sde proteins to be purified using polymerization assays and further biochemical and biophysical characterization. All proteins were purified (Fig. 4).

Biochemical analysis indicated variable levels of reduction of overall Pol III HE activity in reconstituted Pol III HE assays.
Genetic Selection for Initiation Complex Formation Defects

Sde8 (Q238K) is the only protein that showed a severe e-binding defect. The Q238K mutation resides in a domain that has previously been characterized as the PHP domain of Pol III α that binds e (37).

Sde50 and Sde387, both containing mutations (W1134C and L1157Q, respectively) within the C terminus, containing at least part of the τ binding site (24), exhibited severely decreased τ binding in solution (17- and >100-fold, respectively). Surprisingly, mutations in the β-binding domain (Sde8 and Sde388) also resulted in nearly complete ablation of τ binding (Table 3).

Discussion

Previous studies have characterized static interactions between Pol III HE subunits by biophysical measurements in solution and through structural studies. The goal of our study was to exploit a dominant negative variant of Pol III α to identify suppressor mutations that made the resulting proteins less fit to compete with wild-type Pol III α during the initiation complex formation stage of the replicase reaction. Several point mutations and a selected deletion of the β binding loop were selected for further study.

Sde343, containing a mutation in the PHP domain (Q238K) that binds e, was inert for e binding and was catalytically inactive. This mutation occurs adjacent to residue 237 of the Pol III α PHP domain, which has been shown in cross-linking studies to interact with the flexible C-terminal sequence of e (39). Previously, we observed defects in polymerase activity created by point mutations (34) and deletions (22) within the PHP domain. An e-β2 interaction makes significant contributions to the stability of initiation complexes (7, 8) and may contribute to the observed phenotype. An alternative explanation could be that the sde phenotype is caused by a deformation of the active site of the enzyme, diminishing its ability to interact with the primer terminus and form initiation complexes.

TABLE 3
Sde Pol III α affinity for β2, e, and τ

| Mutant protein | \( k_{\text{on}} \) \( \text{M}^{-1} \text{s}^{-1} \) | \( k_{\text{off}} \) \( \text{s}^{-1} \) | \( K_D \) \( \text{nM} \) | Affinity compared to WT | Maximum stoichiometry achieved*
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<td>β2 binding</td>
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<tr>
<td>DnaE WT</td>
<td>7.4 × 10^4 (±1.1)</td>
<td>5.3 × 10^{-4} (±0.8)</td>
<td>7.2 (±0.4) nM</td>
<td>100</td>
<td>0.27</td>
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<td>Sde387 (L1157Q)</td>
<td>6.6 × 10^4 (±0.4)</td>
<td>6.6 × 10^{-4} (±0.8)</td>
<td>11.7 (±3.9) nM</td>
<td>62</td>
<td>0.60</td>
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<tr>
<td>Sde387 Δ881-927</td>
<td>7.2 × 10^4 (±0.3)</td>
<td>5.3 × 10^{-4} (±0.7)</td>
<td>15.8 (±1.7) nM</td>
<td>46</td>
<td>0.57</td>
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<td>e binding</td>
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<tr>
<td>DnaE WT</td>
<td>7.3 × 10^4 (±1.4)</td>
<td>8.5 × 10^{-4} (±1.4)</td>
<td>10.0 (±0.7) nM</td>
<td>72</td>
<td>0.63</td>
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<tr>
<td>Sde387 (L1157Q)</td>
<td>6.6 × 10^4 (±0.4)</td>
<td>6.6 × 10^{-4} (±0.8)</td>
<td>11.7 (±3.9) nM</td>
<td>62</td>
<td>0.60</td>
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<tr>
<td>Sde387 Δ881-927</td>
<td>7.2 × 10^4 (±0.3)</td>
<td>5.3 × 10^{-4} (±0.7)</td>
<td>15.8 (±1.7) nM</td>
<td>46</td>
<td>0.57</td>
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<td>τ binding</td>
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<tr>
<td>DnaE WT</td>
<td>9.8 × 10^4</td>
<td>1.6 × 10^{-5}</td>
<td>0.16 nM</td>
<td>100</td>
<td>0.68</td>
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<tr>
<td>Sde387 (L1157Q)</td>
<td>6.6 × 10^4 (±0.4)</td>
<td>6.6 × 10^{-4} (±0.8)</td>
<td>11.7 (±3.9) nM</td>
<td>62</td>
<td>0.60</td>
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<td>Sde387 Δ881-927</td>
<td>7.2 × 10^4 (±0.3)</td>
<td>5.3 × 10^{-4} (±0.7)</td>
<td>15.8 (±1.7) nM</td>
<td>46</td>
<td>0.57</td>
</tr>
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</table>

* The values in this column represent the maximal stoichiometry achieved between β2, e, or τ relative to Pol III α bound to the chip. Note that for β2 and some of the other proteins with weak binding Sde variants that the level of analyte was not saturating.

* ND, not detectable.
A loop presented by the β-binding domain of Pol III α has been shown to be an important site of physical and functional interaction of the polymerase with β₂ to form a processive replication complex (6, 22–24, 32). Sde388, containing a deletion of this loop is unable to bind β₂ in solution and presumably is unable to form a stable initiation complex, consistent with the loss of the dominant negative phenotype of the Sde388 derivative of dnaE D403E. A mutation in a buried internal residue (Sde8; A877E) within the β binding domain leads to a significant, but lesser decrease in static β₂ binding in solution. Structural studies have demonstrated a significant conformational change in Pol III α upon initiation complex formation where the β₂ binding domain rotates by about 20°, bringing it in alignment with the path of exiting duplex DNA from the polymerase (6). We have demonstrated that in the absence of DNA that the N-terminal domains containing the PHP domain and the palm and thumb domains that form the polymerase active site hold the β-binding domain in a low affinity state (32). It is possible that the A877E mutation puts the β₂ binding domain in a low affinity state or hampers the conformational change in the presence of primed DNA during the Pol III HE initiation complex formation reaction. However, this notion is speculative and will require further experimentation to test this hypothesis.

We have demonstrated that the C terminus of Pol III α is involved in binding of the τ subunit of the clamp loader (22, 24). C-terminal mutations L1157A and F1159A reduce the affinity of Pol III α for τ 36- and 740-fold, respectively (24). Structures of Taq Pol III α, which has a different fold and is thought to be non-homologous to E. coli Pol III α in this region, also binds Taq τ through its C terminus (40). Thus, our finding sde mutations (L1157Q and W1134C) in the C terminus is consistent with a τ binding defect and extends our knowledge of the τ binding site on Pol III α. We note that the residues mutated in sde50 and 387 define the edges of a ridge that contain exposed hydrophobic residues that might also be involved in τ interaction. Interaction of Pol III α with τ is required for chaperoning Pol III α onto nascently loaded β₂ and a physiologically relevant rate of initiation complex formation (10, 41), is consistent with the observed phenotype.

Information is also available regarding the τ partner in the binding interaction. We had demonstrated that a deletion of the C-terminal domain V of τ ablated binding to α, identifying it as the sole α binding domain (26). A structure is available for the C-terminal domain of τ (42). The Pol III α binding sequences of τ have been localized to the extreme C terminus of τ in a region that is unstructured in solution in the absence of binding partners (42).

More surprising was the observation of a τ binding defect in the two sde mutants (A881–927 and A877E) that are located in the β₂ binding domain. The β₂ binding domain and the C-terminal domain that binds τ are independently folding domains, so we do not expect this result to be due to a folding defect. Deletion of the C terminus has a minimal effect on β₂ binding passed over the indicated attached Sde subunits. c, overlayed sensorgrams of 50 nM passed over the indicated attached Sde mutant α subunits. In a, b, and c, DnaE WT (black); Sde8 (A877E, red); Sde50 (W1134C, green); Sde343 (Q238K, gray); Sde387 (L1157Q, orange); Sde388 (A881–927, blue).
and other mutations within the β binding domain do not have a deleterious effect on τ binding (24). In deletion studies, we have observed that deletion of N-terminal sequences that include the polymerase portion of Pol III α (palm, thumb, and partial fingers deletion) interferes with τ binding (22). It is possible that additional τ interactions occur beyond the C-terminal domain. The observed decrease in τ binding is even more severe than that of β2 in the sde8 (A877E) mutation. We note that in the structure of Taq Pol III α bound to the Taq τ C-terminal domain that a contact was observed with the Taq Pol III α β2 binding domain (40). The authors of this study were uncertain if the interactions observed were due to crystal packing forces or a functional contact. Our findings suggest that the β binding domain is important for physical interaction of τ and functional importance in initiation complex formation. An alternative explanation would be that there are cooperative regulatory interactions between the β-binding domain and the C-terminal domain and that Sde 388 and Sde 8 induce a low affinity τ-binding conformation within the C terminus of these proteins. Together, these mutants increase our knowledge of the complex protein interactions within the DNA replicase required for replication initiation.

References