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Escherichia coli DnaA protein: specific biochemical defects of mutant DnaAs reduce initiation frequency to suppress a temperature-sensitive *dnaX* mutation

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Abstract

The *Escherichia coli dnaA73*, *dnaA721*, and *dnaA71* alleles, which encode A213D, R432L, T435K substitutions, respectively, were originally isolated as extragenic suppressors of a temperature-sensitive *dnaX* mutant. As the A213D substitution resides in a domain that functions in ATP binding and the R432L and T435K substitutions affect residues that recognize the DnaA box motif, they might be expected to reduce ATP and specific DNA binding, respectively. Therefore, a major objective was to quantify the biochemical defects of the mutant DnaAs to understand how the altered proteins suppress the temperature-sensitive phenotype of a *dnaX* mutant. A second purpose was to address the paradox that mutant proteins with substitutions of amino acids essential for recognition of the DnaA box motifs within the *E. coli* replication origin (*oriC*) may well be inactive in initiation, yet chromosomal *dnaA* mutants expressing DnaA proteins with the R432L and T435K substitutions are viable at temperatures from 30 to 39 °C. We show biochemically that mutant DnaAs carrying R432L and T435K substitutions fail to bind to the DnaA box sequence. The A213D mutant is sevenfold reduced in its affinity for ATP compared to wild-type DnaA, and its affinity for the DnaA box sequence is also reduced. However, the reduced activity of the A213D mutant in *oriC* plasmid replication appears to arise from a defect in DnaA oligomerization. Although the T435K mutant fails to bind to the DnaA box sequence, other results suggest that DnaA oligomerization stabilizes the binding of the mutant DnaA to *oriC* to support its partial activity in initiation in vitro. These results support a model that suppression of *dnaX* occurs by reducing the frequency of initiation to a manageable level for the mutant DnaX so that viability is maintained.

Keywords: DnaA; DNA; Origin; Replication; Initiation; Chromosome

1. Introduction

Escherichia coli DnaA protein has several biochemical functions which are essential for the protein to initiate chromosomal DNA replication [1]. In summary, DnaA first binds to the individual DnaA boxes within the replication origin, *oriC* [2,3,41]. In a complex with ATP [4], DnaA then oligomerizes [5–7] to unwind a region of duplex DNA near the left border of *oriC* [8–10]. DnaA next recruits DnaB in the DnaB–DnaC complex [8,11,12]. After the release of DnaC [8], DnaB translocates on each parental DNA strand, occasionally interacting with primase, which synthesizes primers that are extended by DNA polymerase III holoenzyme during bidirectional replication fork movement [13,14]. These functions correlate with the four domains of DnaA protein [5,15]. Domain I near the N-terminus functions in oligomerization [5–7,16], and is linked by Domain II to a highly conserved region composed of Domains IIIa, IIIb and IV. Based on the crystal structure of the homologous region of DnaA

Abbreviations: Cs, cold-sensitive; IPTG, isopropyl-β-D-thiogalactopyranoside; Ts, temperature-sensitive.

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from the thermophilic *Aquifex aeolicus* [17], the structure of Domain IIIa, which binds ATP, is a five-stranded parallel β-sheet sandwiched between α-helices. As DnaA is a member of the AAA^+ class of ATPases [18], Domain IIIb contains sensor motifs that presumably function to distinguish whether ATP is bound or has been hydrolyzed to ADP [17]. Domain III residues also function in DnaA oligomerization [19], consistent with the role of the corresponding domain in other AAA^+ proteins that are oligomeric [17]. Domain IV [5,20–22] includes a helix-turn-helix motif involved in DNA binding [17,23], and a region around residues 372–381 that interacts with acidic phosopholipids to promote nucleotide exchange [24].

The *dnaX* gene encodes the τ and γ subunits of DNA polymerase III holoenzyme, whereby translational frame shifting gives rise to a truncated γ subunit containing the first 430 amino acids of τ and a unique, C-terminal 431st residue (reviewed in McHenry [25]). We previously described three conditional *dnaA* alleles that are able to suppress the temperature-sensitive phenotype of a *dnaX* mutant [26]. These mutants were isolated by selecting at semi-permissive temperatures of 39–40 °C, for spontaneous temperature-resistant revertants which were present in wild-type cultures at a frequency of about 1×10^{-7}, and screening isolates to identify those which concomitantly were cold-sensitive. The latter class corresponded to approximately 1% of the temperature-resistant isolates and about 10% of those which were cold-sensitive had extragenic suppressor mutations in *dnaA* [27]. Of the four mutants isolated, three were studied further. In otherwise wild-type strains, the *dnaA* mutants failed to grow at 20 °C or at 42–44 °C specifically because of failure to initiate replication. At temperatures from 30 to 39 °C, they grew with slightly increased generation times and initiations were asynchronous with a reduction of 30–40% in *oriC* content per cell. The *dnaA dnaX*(Ts) double mutants were also both cold- and temperature-sensitive. At 30–34 °C, their growth rates were similar to the respective *dnaA* mutant alone, but the origin content per cell was further reduced to about 50% the wild-type level with increased initiation asynchrony. At 39 °C, the double mutant also grew with approximately the same growth rate as the suppressor mutant and formed colonies with an efficiency of approximately one, relative to 34 °C, but did not complete the replication of all chromosomes in all cells, even after 8 h of incubation in the presence of rifampicin to inhibit new initiations [28,29].

Several observations support the model that the *dnaA* alleles compensate for the defect of the *dnaX* mutant at semi-permissive temperature (39–40 °C) by reducing initiation frequency and, therefore, chromosome content per cell to a level such that the *dnaX* mutant, even with reduced polymerase activity, replicates enough chromosomes to maintain viability. First, initiation was inefficient in all the mutants studied. Second, the *dnaX*(Ts) mutant could be suppressed also by introduction of *oriC* mutations that reduced initiation efficiency and chromosome content; the degree of suppression was proportional to the defectiveness of the mutant *oriC*. Third, temperature sensitivity of *dnaX*(Ts) was exacerbated by introduction of a *dnaA* mutation that caused over-initiation. Fourth, suppression of *dnaX*(Ts) by a suppressor *dnaA* mutation was not possible on a poor medium on which reduction of chromosome content to less than one per cell would be expected to be lethal [28,29]. Fifth, Skovgaard and Løbner-Olesen [30], also isolated suppressor mutants which were less active than $dnaA^+$ in initiation at temperatures from 30 to 39 °C, causing inefficient and asynchronous initiation.

The suppressing *dnaA* alleles (*dnaA73*, *dnaA721*, and *dnaA71*) encode A213D, R432L, and T435K substitutions, respectively. Because the A213D substitution resides in an α-helix in Domain IIIa, which interacts with ATP, nucleotide binding activity of the mutant DnaA may be impaired. Previous biochemical studies revealed that the R432L and T435K substitutions residing in a loop of Domain IV have greatly reduced affinities for individual DnaA boxes, including the consensus motif (TTATCCACA) [22], and that a T435M substitution was inactive in initiation because the mutant protein fails to recognize the DnaA box sequence [21]. In confirmation, structural studies showed that arginine 432 and threonine 435 directly interact with nucleotides of the DnaA box motif [23]. Although the *dnaA* mutants encoding the R432L and T435K substitutions might be inviable because of the failure to bind to *oriC*, they and the mutant encoding the A213D substitution are obviously healthy enough to support DNA replication in vivo. Faced with this paradox, we undertook a biochemical approach to quantify the biochemical defects of the mutant DnaAs. For simplicity, we refer hereafter to the mutant DnaAs by their amino acid substitutions. We show that A213D and T435K are partially active in initiation at *oriC* in vitro at a temperature where they can suppress the temperature-sensitive phenotype of the *dnaX* mutant. Apparently, T435K can recognize *oriC*, but direct assays failed to show that either T435K or R432L could bind to a DNA fragment containing an individual DnaA box. With R432L, we were unable to show that the mutant protein could support *oriC* plasmid replication in vitro. Other results suggest that, despite a lower affinity for ATP, the replication defect of A213D is in DnaA oligomer formation.

2. Materials and methods

2.1. Bacterial strains, plasmid, and growth conditions

E. coli C600 (*thr leu thi supE44*, *lacY*, *tonA*), from the Walker Lab Collection, was the host for routine plasmid maintenance and isolation. Strain JW27 is a derivative of strain C600 but carries *tnaA*::Tn10 closely linked to the *dnaA46*(Ts) allele. Strains SXC601, SXC602 and SXC603 carry the *dnaA73*, *dnaA721*, and *dnaA71* alleles, which encode the A213D, R432L and T435K substitutions, respectively, and are isogenic with strain C600 [28,29]. Strain BL21(DE3) (pLysS) (Novagen) was used to overproduce wild-type or mutant DnaA, each linked to an N-terminal polyhistidine