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Research paper

Quantitative analysis of the mechanism of DNA binding by *Bacillus* DnaA protein☆

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ABSTRACT

DnaA protein has the sole responsibility of initiating a new round of DNA replication in prokaryotic organisms. It recognizes the origin of DNA replication, and initiates chromosomal DNA replication in the bacterial genome. In Gram-negative *Escherichia coli*, a large number of DnaA molecules bind to specific DNA sequences (known as DnaA boxes) in the origin of DNA replication, *ori*C, leading to the activation of the origin. We have cloned, expressed, and purified full-length DnaA protein in large quantity from Gram-positive pathogen *Bacillus anthracis* ($DnaA_{BA}$). $DnaA_{BA}$ was a highly soluble monomeric protein making it amenable to quantitative analysis of its origin recognition mechanisms. $DnaA_{BA}$ bound DnaA boxes with widely divergent affinities in sequence and ATP-dependent manner. In the presence of ATP, the K_D ranged from 3.8×10^{-8} M for a specific DnaA box sequence to 4.1×10^{-7} M for a non-specific DNA sequence and decreased significantly in the presence of ADP. Thermodynamic analyses of temperature and salt dependence of DNA binding indicated that hydrophobic (entropic) and ionic bonds contributed to the $DnaA_{BA}$·DNA complex formation. $DnaA_{BA}$ had a DNA-dependent ATPase activity. DNA sequences acted as positive effectors and modulated the rate (V_{max}) of ATP hydrolysis without any significant change in ATP binding affinity.

1. Introduction

DnaA protein functions as the initiator protein for chromosomal DNA replication in all prokaryotes studied to date [1–6]. Initiation of DNA replication appears to interface with a number of other cellular processes such as sporulation in *Bacilli* and lower eukaryotes such as fungi, and differentiation in mammalian cells. Unlike other proteins involved in DNA replication, initiator proteins appear to act as the central hub of regulation of initiation of a new round of DNA replication, a process that appears to be conserved from prokaryotes to mammals [7–19]. Thus, understanding *Bacillus* DnaA protein is essential in order to dissect the complex interrelationships between DNA replication and other cellular processes in the genus *Bacillus*.

Studies by Francois Jacob and colleagues made the major initial contributions leading to the unraveling of the genetic apparatus, which carries out DNA replication in *Escherichia coli* [4–6]. These studies identified many of the *E. coli* DNA replication genes, such as *dnaA*, *dnaB*, *dnaC*, etc. and delineated their temporal order of function. Using temperature-sensitive mutants, the *dnaA* gene-product was identified as functioning in the initiation of chromosomal DNA replication. These studies established that the *dnaA* gene-product or DnaA protein as the first protein involved in chromosomal DNA replication, hence dnaA gene designation. *In vitro* studies by Fuller and Kornberg demonstrated that *E. coli* DnaA is an oligomeric protein that binds ATP with high affinity and initiates replication of plasmid DNA containing *ori*C [1]. DnaA binds to origin DNA as a large complex comprising of 20–40 molecules forcing *ori*C DNA to form a loop [20,21]. This is an essential step and precedes the opening of the AT-rich DNA unwinding elements (DUE) in *ori*C [20–23]. Following melting of the DUE, DnaA recruits the DnaB·DnaC protein complex to further unwind the parental duplex DNA [24,25]. Once this open complex is formed, SSB binds to the ssDNA and prevents the strands from reannealing. The synthesis of RNA primers is then initiated by DnaG primase followed by the synthesis of the daughter strands by DNA polymerase III holoenzyme [13,26–28].

DnaA protein is a member of the AAA + family of ATPases [29]. Although most AAA + ATPases form closed-ring assemblies, DnaA

Abbreviations: EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol; TBE, tris–borate–EDTA buffer; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; rNTP, ribonucleotide triphosphate; $DnaA_{BA}$, DnaA protein of *Bacillus*; $DnaB_{BA}$, DnaB helicase of *Bacillus*; $DnaG_{EC}$, DNA primase of *E. coli*; $DnaB_{EC}$, DnaB helicase of *E. coli*; ssDNA, single-stranded DNA; MRSA, multidrug resistant *Staphylococcus aureus*; SSB, single-stranded DNA binding protein of *E. coli*.

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proteins tend to form long oligomeric chains. DnaA has four functional domains. The N-terminal domains (I and II) are involved in the DnaA–DnaB interaction, DnaA oligomerization, as well as regulation by other factors like DiaA protein. Domain III is the AAA + ATPase domain, involved in ATP binding/hydrolysis, ssDNA binding, and multimer formation [30,31]. The C-terminal domain IV is the DnaA box binding domain. There are two AT-rich 13-mer sequences as well as five 9-mer DnaA box sequences (consensus: $TT^{A}/_{T}TNCACA$) in *E. coli oriC* that bind to DnaA proteins [21]. ATP and ADP bind tightly to DnaA, however, ATP–DnaA and ADP–DnaA forms have differential binding affinities towards various DnaA boxes [13,32]. In *E. coli*, ATP–DnaA and ADP–DnaA bind with similar affinity to two DnaA boxes, R1 and R4, at the edges of the DnaA-binding region in *ori*C, whereas, ATP–DnaA binds with higher affinity to other DnaA boxes, R2, R3, and R4 [13]. In the *Streptomyces* origin of DNA replication, there are 19 DnaA boxes and DnaA-binding affinities towards these DnaA boxes are generally lower than that observed in *E. coli* [33]. Messer and coworkers concluded that DnaA proteins from different organisms use different strategies for DnaA box binding [33]. Some organisms use high affinity DnaA proteins, whereas, others use low affinity DnaA, particularly, those with large numbers of DnaA boxes and/or high GC content. Unlike domain IV, domain III of DnaA binds single-stranded DNA. Berger and coworkers studied binding of ssDNA to domain III of DnaA from *Aquifex aeolicus* by X-ray crystallography and demonstrated that DnaA forms a right-handed spiral filament that wraps around DnaA boxes and causes melting of the adjacent AT-rich DUEs with possible involvement of additional DnaA monomers [34]. In addition, ssDNA binding to domain III of DnaA likely advances DUE melting. Recent studies indicate that ATP-dependent assembly of *A. aeolicus* DnaA into a spiral oligomer creates a continuous surface that allows successive AAA + domains to bind and extend or stretch single-stranded DNA segments in the origin [35,36].

Replication mechanisms of chromosomal DNA of highly pathogenic bacteria remains poorly understood primarily due to difficulties in studying these organisms [37–39]. An important difference between Gram-positive *Bacilli* and *E. coli* is that under stressful environmental conditions, many of these organisms have the ability to survive indefinitely by forming endospores [16]. Once the conditions become favorable, they can then rapidly replicate resuming their pathogenesis cycles. This suggests that even though the basic DNA replication mechanism is similar, its regulation is likely different between these organisms. Krause et al. further defined the interaction of *Bacillus subtilis* DnaA with its origin of DNA replication and mapped the DnaA boxes as well as the DUE in the origin that unwinds during initiation [27].

DnaA proteins have a general tendency of multimer formation [40]. However, this property of DnaA proteins makes it difficult to purify and analyze *in vitro*. Purification requires either harsh physicochemical and enzymatic treatment or deletion of domain I to make DnaA monomeric [1,40]. We have chosen to study DnaA protein from *Bacillus* ($DnaA_{BA}$) because the full-length recombinant $DnaA_{BA}$ is highly soluble and monomeric without sequence modification. Consequently, it can be purified in large quantity in its native form that makes it ideal for a variety of studies *in vitro*. *Bacillus anthracis* has an origin of DNA replication similar to other prokaryotes with 13 identifiable DnaA boxes.

In general, most of the origin binding studies of DnaA protein have been carried out by electrophoretic mobility shift or gel binding assay (EMSA), foot printing, and biosensor analysis [1,21,22,33,40]. Although, each of these studies has unique features and provides specific information regarding DnaA, neither of these approaches provides true quantitative analysis under physiological conditions. In this study, we have carried out a quantitative analysis of DnaA from B. anthracis with its cognate DnaA boxes using fluorescence anisotropy titrations. Fluorescence anisotropy permits acquisition of data under a variety of physiological and equilibrium conditions and it neither requires separation of bound and free ligand nor immobilization of the ligand as in biosensors [41–44]. This analytical methodology has been useful in studying protein–DNA interactions involving a number of DNA binding proteins including, estrogen receptor interaction with estrogen responsive elements, tryptophan repressor binding to the tryptophan operator, and *E. coli* DNA primase binding to DNA [42,45–47]. This methodology is highly suitable for quantitative analysis of $DnaA_{BA}$ binding to DNA sequences. We have analyzed the unique properties of $DnaA_{BA}$ and its interaction with various DNA elements in the *Bacillus* origin of DNA replication quantitatively.

2. Materials and methods

2.1. Materials

All chemicals used to prepare buffers and solutions were ACS reagent grade and were purchased from Fisher Chemical Company (Pittsburgh, PA). Synthetic and modified oligonucleotides (with fluorescein moiety) were from Sigma Genosys Inc. (Woodland, TX). Nucleotides, ion exchange, and other chromatographic resins were from GE Biosciences Inc. (Piscataway, NJ).

2.2. Buffers

Buffer A contained 25 mM Tris–HCl (pH 7.5), 10% (w/v) glycerol, 0.01% (w/v) NP40, 1 mM EDTA (pH 8.0), and 5 mM $MgCl_2$. Buffer A_x indicates buffer A containing *X* mM NaCl. Buffer B was 5% glycerol, 1 mM EDTA (pH 8), 20 mM Hepes (pH 8), 5 mM $MgCl_2$, and 5 mM DTT. Buffer C was 25 mM Tris–HCl (pH 7.5), 10% (w/v) glycerol, 1 M NaCl and 0.005% (w/v) NP40. Buffer D contained 25 mM Tris–HCl (pH 7.5), 10% (v/v) glycerol, 0.1 mg/ml BSA, and 5 mM DTT. Buffer E consisted of 25 mM Tris–HCl (pH 7.9), 10% (w/v) sucrose, 250 mM NaCl and 0.001% NP40.

2.3. Cloning, expression and purification of $DnaA_{BA}$

The DnaA gene was amplified from genomic *B. anthracis (Stern strain) DNA*. $DnaA_{BA}$ was cloned into a T7 expression vector, pET30a (EMD Millipore, Billerica, MA), using the restriction enzymes *Nde*I and *Bam*HI. The pET30a–$DnaA_{BA}$ plasmid was used to transform *E. coli* BL21 (DE3) RIL strain (Agilent Technologies Inc., Santa Clara, CA). The protein was overexpressed in the *E. coli* cells by growing in 8 L batches of 2×YT medium containing 50 μg/ml kanamaycin and 12 μg/ml chloramphenicol at 37 °C to an OD600 of 0.4–0.6. Once proper OD was reached, 1-isoproply 1-thio-B-D-galactopyranoside (IPTG) was added to a final concentration of 0.5 mM. The cells were then grown for 12 h at 16 °C and the harvested by centrifugation at 7500 × g for 5 min at 4 °C. The cell pellet was resuspended in Buffer E and frozen at −80 °C until further use. *E. coli* cells were extracted with lysozyme following standard protocol. The extract was precipitated overnight using 0.35 g/ml ammonium sulfate on ice, followed by centrifugation at 43,000 × *g* for 30 min at 4 °C.

The protein pellet was resuspended in Buffer A_0 and diluted until the conductivity matched that of buffer A_{50} (fraction III). The diluted protein was loaded on a 5 ml Q-sepharose column connected in tandem to a 5 ml S-sepharose column, both were equilibrated with buffer A_{50}. The columns were washed with 100 ml A_{100}, disconnected, and the S-sepharose was eluted using a 100 ml gradient from A_{200} to A_{500}. The $DnaA_{BA}$ fractions were identified by carrying out SDS-PAGE and measuring the absorbance of the protein using a Nanodrop 2000C spectrophotometer.