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Keywords
novel, zinc, binding, fold, helicase, interaction, domain, Bacillus, subtilis, Dnal, helicase, loader, CMMB

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A novel zinc-binding fold in the helicase interaction domain of the Bacillus subtilis DnaI helicase loader

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ABSTRACT

The helicase loader protein DnaI (the Bacillus subtilis homologue of Escherichia coli DnaC) is required to load the hexameric helicase DnaC (the B. subtilis homologue of E. coli DnaB) onto DNA at the start of replication. While the C-terminal domain of DnaI belongs to the structurally well-characterized AAA+ family of ATPases, the structure of the N-terminal domain, DnaI-N, has no homology to a known structure. Three-dimensional structure determination by nuclear magnetic resonance (NMR) spectroscopy shows that DnaI presents a novel fold containing a structurally important zinc ion. Surface plasmon resonance experiments indicate that DnaI-N is largely responsible for binding of DnaI to the hexameric helicase from B. stearothermophilus, which is a close homologue of the corresponding much less stable B. subtilis helicase.

INTRODUCTION

DNA replication in all organisms is carried out by replisomes, multiprotein machines that contain a ring-shaped DNA helicase for separation of the strands of the DNA duplex (1). In bacteria, the helicase forms a hexameric structure that encircles single-stranded (ss) DNA. Several other replication proteins bind to the helicase and its loading onto DNA is one of the earliest steps in replisome assembly (2). In Escherichia coli, loading of the DnaB helicase hexamer onto ssDNA is assisted by DnaC, a loader protein that associates with it to form a ring of six DnaC molecules on one face of the helicase ring (3). In Bacillus subtilis and some other Gram positive bacteria such as Staphylococcus aureus, the corresponding hexameric helicase is named DnaJ and the helicase loader protein is DnaI. In contrast to E. coli DnaB, which is loaded onto ssDNA by ring opening (4,5), the B. subtilis helicase dissociates more readily into monomers and the role of DnaI is to assist its assembly on the ssDNA (6). This process is assisted in B. subtilis by a pair of DNA-remodelling co-loader proteins (called DnaB and DnaD), which guide the DnaC/DnaI complex to specific sites in the DNA (7–13). However, in vitro and in the presence of ATP, DnaI alone is sufficient for loading of the helicase onto ssDNA (6,14).

The B. subtilis helicase and its loader form a complex of six helicase and six DnaI molecules in analogy to the E. coli DnaB/DnaC complex (6). The amino-acid sequences of the helicase loaders from the two species indicate the presence of nucleotide-binding sites, in agreement with the requirement of ATP for helicase loading (1,15–17). In addition, experiments with DnaI (14) showed that it consists of two structured domains. The larger C-terminal AAA+ domain (1,2,16) contains the nucleotide-binding site and a cryptic site for ssDNA binding, whereas the N-terminal domain (here termed DnaI-N) seems to be primarily responsible for helicase binding and acts as a molecular switch that regulates the accessibility of the ssDNA-binding site in the C-terminal domain (14). DnaI is a larger protein than E. coli DnaC (36 versus 28 kDa) and DnaI-N is unrelated in sequence to the DnaC helicase from E. coli (15). As the B. subtilis DnaC helicase is a less stable protein that can exist as a mixture of oligomeric forms, its interactions with DnaI have mostly been inferred using the well-behaved stable hexameric helicase from B. stearothermophilus (in the following referred to as Bst DnaB), which shares 82% sequence identity and 92% similarity with the B. subtilis helicase.
The interaction between DnaI and Bst DnaB depends on the ability of DnaI to bind zinc (14). The zinc-binding site is located in DnaI-N and is lost in the mutants C67A, C70A and H84A. Remarkably, single mutations of the other two cysteine residues, C76 and C101, did not completely abolish zinc binding or helicase interaction, suggesting that side chains of these two residues might substitute for each other as zinc ligands.

To date, very little is known of the structural basis of interactions of helicase loaders with their helicase partners, and this has limited understanding of the mechanisms of helicase loading. Although no complete structure of any helicase loader has been determined to date at atomic resolution, the structure of the AAA+ domain of Aquifex aeolicus DnaC, missing the N-terminal helicase-binding domain, has recently been reported (2). Its structure shows association of domains as a helical filament that suggests a mechanism for association of the loader proteins with the DnaA replication initiator filament that suggests a mechanism for association of the loader proteins with the DnaA replication initiator (2).

The structures of several related hexameric DnaB-family helicases reported recently (18-21) all reveal unusual domain arrangements where the N-terminal primase-interacting domains form a trimer of dimers stacked on top of a hexamer of C-terminal RecA-like helicase domains. The only reported structural information on a helicase/loader protein complex comes from low-resolution single particle analysis of negative stain (3) and cryo-electron (22) microscopic images of the E. coli DnaB/DnaC complex, which revealed a ring of DnaC molecules stacked on the C-terminal face of DnaB, which in the presence of ATP results in closing of the central ssDNA-binding channel (22).

Here we present the first report of the 3D structure of the helicase-binding domain of a replicative helicase loader, the N-terminal domain of B. subtilis DnaI, and identify its zinc-coordinating residues. In addition, experiments have been conducted to delineate the boundary between the N- and C-terminal domains of DnaI to define the minimal domain required for binding to the Bst helicase.

MATERIALS AND METHODS
Expression and purification of DnaI and Bst DnaB

The production of full-length DnaI has been described earlier (14). The procedure used in the present work is provided in the Supplementary Data. The DnaI-N106 mutant protein (14) and the B. steatorthermophilus DnaB helicase were prepared as described (18), using an expression strain (15) provided by Dr Panos Soultanas (University of Nottingham, UK). Concentrations of all proteins were determined spectrophotometrically at 280 nm, using calculated values of ε280 (23).

Limited proteolysis and characterization of proteolytic fragments

Limited proteolysis experiments were done for 30 min at 30°C with solutions of 126 μg of full-length DnaI in 60 μl of storage buffer (50 mM Tris–HCl, pH 7.6, 10% (w/v) glycerol, 2 mM dithiothreitol, 1 mM EDTA) using a DnaI:trypsin ratio of 125:1. Additional experiments were conducted in the presence of 3 mM ATP, 12 mM MgCl2, or both. The products were analyzed by 12% SDS-PAGE. The gels were washed in water before selected bands were excised. N-terminal sequences of fragments were determined by Edman degradation (Biomolecular Resource Facility, Australian National University).

Construction of the expression system for DnaI-N106

Plasmid pND787, which contains the dnaI gene from pET-15b-dnaI (7) under transcriptional control of strong bacteriophage λ promoters in plasmid vector pND76 (24), was used as template for the PCR amplification of the dnaI deletion mutant with primer 5’-GAGATATA CATATGGAACCAATC-3’ incorporating an ATG initiation codon (italicized) as part of an NdeI site (underlined). Primer 5’-AAAAAGCGGTATACTCCGTTTGA CTGGACATTG-3’ was complementary to the 3’ end of the dnaI fragment (Mut1 sites underlined; complement of stop codon italicized). The PCR product was digested with NdeI and MluI and inserted into vector pND76 between the same restriction sites to yield pKL1272. The gene encoding the dnaI mutant [coding for the N-terminal 106 residues of DnaI, DnaI-N106 (Figure 1)] is under strict control of tandem heat-inducible phage λ promoters pR and pL. The plasmid pKL1272 was transformed into the E. coli strains AN1459 (25) and BL21(DE3)recA (26) for protein expression in LB or 15N-enriched media, respectively.

Expression and purification of DnaI-N106

Cells from the E. coli strain AN1459/pKL1272 were grown in 4 l of LB medium containing thymine (25 mg/l) and ampicillin (50 mg/l) at 30°C to A595 = 0.61. A rapid temperature shift to 42°C induced the overproduction of DnaI-N106, and growth was continued for a further 3 h (final A595 = 1.18). The cells (5.05 g) were harvested at 11 000 × g for 6 min, frozen in liquid N2 and stored at −70°C. All purification steps following cell growth were carried out at 0–4°C. Thawed cells were resuspended in 76 ml of lysis buffer (50 mM Tris–HCl, pH 7.6, 10% w/v sucrose, 100 mM NaCl, 2 mM dithiothreitol and 10 mM spermidine) and lysed using a French press at 12 000 psi. Proteins in the soluble fraction after centrifugation (39 000 × g, 1 h) were precipitated by addition of ammonium sulfate (0.4 g/ml). After stirring for 1 h, the suspension was centrifuged (39 000 × g, 45 min); the pellet was dissolved in 30 ml of buffer A (50 mM Tris–HCl, pH 7.6, 10% w/v glycerol, 2 mM dithiothreitol, 40 mM NaCl) and dialysed against two changes (11 each) of buffer A. The protein was applied to a Toyopearl DEAE-TSK650M column (2.5 × 14 cm) pre-equilibrated in buffer A. The column was washed with 100 ml of buffer A at a flow rate of 1 ml/min. DnaI-N106 eluted in the flow-through and solid ammonium sulfate (8.1 g) was added to the combined fractions (18 ml). The suspension was stirred for 1 h, and the pellet after centrifugation (39 000 × g, 45 min)
was dissolved in 4 ml of buffer B (50 mM Tris–HCl, pH 7.6, 10% w/v glycerol, 2 mM dithiothreitol, 100 mM NaCl). The solution was applied to a column (2.5 × 62 cm) of Sephadex G50 gel filtration resin pre-equilibrated in buffer B. The protein was eluted at a flow rate of 0.5 ml/min and the pooled fractions (21 ml) yielded 15 mg of purified DnaI-N106. The purification was monitored by 15% SDS–PAGE. The identity of the protein was confirmed by ESI-MS (VG Quattro II triple quadrupole mass spectrometer) using a 0.1% formic acid solution as above following growth of cells in 15N/13C-enriched medium (Silantes).

15N-labeled DnaI-N106 was produced using the E. coli strain BL21(DE3)recA/pKL1272 in 15N-enriched medium (Silantes, Munich, Germany), yielding 2.74 g of cells from a 11 culture. The purification process was as described above, except that the gel filtration step was replaced by chromatography over an 8 ml Mono-Q column (GE Healthcare; flow rate 1.5 ml/min) after dialysis against buffer C (50 mM Tris–HCl, pH 7.6, 2 mM dithiothreitol) containing 70 mM NaCl. 15N-labeled DnaI-N106 eluted at about 110 mM NaCl in a linear gradient (240 ml) of 70–150 mM NaCl in buffer C. The yield of purified 15N-labeled protein was 13 mg l−1 of culture.

Expression and purification of DnaI-N123 and DnaI-N132

C-terminally His6-tagged DnaI-N132 was purified as described previously (14). The DnaI-N123 construct was made using the QuickChange site-directed mutagenesis kit (Stratagene) by introduction of a TAA stop codon after that encoding Gln123 in the pET22b-dnaI plasmid described by Soultanas (14). The primers used were 5′-GC ATGTATATCCAGTAAGTCTTGTGAG-3′ and its complement (stop codon italicized). This strategy resulted in an untagged protein that was purified in the same way as DnaI-N106.

NMR sample preparation

NMR samples were prepared by dialysis of DnaI-N proteins against NMR buffer (10 mM sodium phosphate pH 7.0, 0.1% (w/v) sodium azide, 100 mM NaCl and 1 mM dithiothreitol). Following concentration to 0.5 ml by ultrafiltration (Amicon Ultrafree-4 centrifugal filter device with a 5000 molecular weight cutoff), D2O was added to 10% (v/v). The final NMR samples contained 1.1 mM unlabeled and 0.5 mM 15N-labeled DnaI-N106.

NMR measurements

All NMR experiments were carried out at 25°C using Bruker Avance 600 and 800 MHz NMR spectrometers equipped with TCI cryoprobe. The backbone resonance assignment was obtained from the analysis of a 3D NOESY-15N-HSQC spectrum [60 ms mixing time, 1.1max(1H) = 20 ms, 2max(15N) = 13 ms, 3max = 106 ms] together with 3D HNHA and 2D NOESY spectra. The side-chain resonances were assigned using NOEY, TOCSY and DQF-COSY spectra. A 15N-HSQC spectrum with the INEPT delays set to 20 ms yielded correlations via 2JHN couplings of the side chain of His84. NOE restraints were collected from the 3D NOESY-15N-HSQC spectrum, 2D NOESY spectra of the 15N-labeled and unlabeled samples in 90% H2O/10% D2O solution (60 and 40 ms mixing time, respectively) and a 2D NOESY spectrum in D2O solution (40 ms mixing time).

Resonance assignments of DnaI-N123 were obtained from 3D HNCA, HN(CO)CA and CBCA(CO)NH spectra using 15N/13C doubly labeled protein, prepared as above following growth of cells in 15N/13C-enriched medium (Silantes).

Structure calculations

The cross-peaks in the NOE spectra of DnaI were assigned and integrated using the program XEASY (27). Stereospecific resonance assignments were obtained for 21 C6H2 groups, 10 C5H2 groups, 1 C6H2 group, 5 isopropyl groups and 14 side-chain amides. The stereo-specific resonance assignments were determined by using the HABAS and GLOMSA routines implemented in the program CYANA (28), including J-coupling information from HNHA, COSY and TOCSY spectra to support the stereospecific assignment of C6H2 groups. The NMR structure calculations using CYANA (28) involved eight iterations of automatic NOE assignment using the routine CANDID (29) followed by a simulated annealing procedure starting from 100 random conformers that were annealed in 20 000 steps using torsion-angle dynamics. The 20 conformers with the lowest residual restraint violations were energy minimized in a shell of water using the protocol re_h2o.inp (30) and the program CNS (31) with standard parameters.

Table 1 shows an overview of the restraints used and structural statistics. Secondary structure elements and root mean square deviation (r.m.s.d.) values were calculated using the program Molmol (32). The chemical shifts and coordinates of the structure have been deposited in the BioMagResBank (accession code 15926) and PDB (accession code 2K7R).

Thiol titrations with DTNB

Samples of full-length DnaI, DnaI-C76A and DnaI-N123 were dialyzed extensively under N2 at 4°C against 50 mM Tris–HCl pH 8.0, 0.1 M NaCl. Reactions with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Sigma) were followed in matched quartz cuvettes in a UV-1700 UV-visible spectrophotometer (Shimadzu) at 23°C. The concentration of protein (~5 μM, in 580 μl of buffer) was first determined from a UV spectrum before a 10 mM solution of DTNB (15–20 μl) was added to initiate the reaction. The release of 5-thio-2-nitrobenzoate anion was monitored spectrophotometrically at 412 nm [ε412 = 14 150 M−1 cm−1; (33)] until no further change occurred (30–40 min).

DnaI-N/Bst DnaB interaction analysis

Surface plasmon resonance (SPR) experiments were conducted in a BIACore T100 instrument operating at 20°C at a flow rate of 5 μl/min, using streptavidin.
**Table 1. Structural statistics for the NMR conformers of DnaI-N106**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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</thead>
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<tr>
<td>Number of uniquely assigned NOE cross-peaks</td>
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</tr>
<tr>
<td>Number of non-redundant NOE upper-distance limits</td>
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</tr>
<tr>
<td>Number of scalar coupling constants (H-H)</td>
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</tr>
<tr>
<td>Number of dihedral-angle restraints</td>
<td>524</td>
</tr>
<tr>
<td>Number of distance restraints for zinc ion</td>
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</tr>
<tr>
<td>Intra-protein energy (kcal/mol)</td>
<td>d</td>
</tr>
<tr>
<td>Number of dihedral-angle restraint violations (°)</td>
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</tr>
<tr>
<td>Maximum NOE-restraint violations (Å)</td>
<td>0.30 ± 0.08</td>
</tr>
<tr>
<td>Maximum dihedral-angle restraint violations (°)</td>
<td>3.50 ± 1.01</td>
</tr>
<tr>
<td>r.m.s.d. to the mean for N, C α and C (Å)</td>
<td>0.78 ± 0.16</td>
</tr>
<tr>
<td>r.m.s.d. to the mean for all heavy atoms (Å)</td>
<td>1.27 ± 0.15</td>
</tr>
<tr>
<td>Ramachandran plot appearance</td>
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<tr>
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<td>Additionally allowed regions (%)</td>
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<td>Generously allowed regions (%)</td>
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</tr>
<tr>
<td>Disallowed regions (%)</td>
<td>0.0</td>
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</tbody>
</table>

**RESULTS**

**Protein constructs**

Limited proteolysis of DnaI with trypsin under native conditions showed a small number of distinct fragments corresponding to cleavage after Arg105, Lys111, Lys112 and Lys118 (Supplementary Figures S1 and S2). In the presence of ATP and MgCl₂, the preferred cleavage site shifted from Lys118 to Lys111, confirming that ATP binds to DnaI and may cause conformational changes. To probe the C-terminal boundary of the N-terminal domain of DnaI, the constructs comprising residues 1–106, 1–123 and 1–132 (referred to as DnaI-N106, DnaI-N123 and DnaI-N132, respectively) were prepared, with a His₆ tag at the C-terminal end of the DnaI-N132 domain. [Limited proteolysis with subtilisin indicated a cleavage site after residue 132 (14).] TOCSY spectra showed the best spectral quality for DnaI-N106, whereas the longer domains gave broader resonances indicative of aggregation. Subsequently, ¹⁵N-labeled samples were prepared of DnaI-N106 and DnaI-N123, and a ¹³C/¹⁵N-labeled sample of DnaI-N132.

**NMR resonance assignments**

The ¹⁵N-HSQC spectrum of DnaI-N106 presented well-dispersed resonances characteristic of a monomeric folded protein (Figure 2). Virtually complete resonance assignments were obtained, except for the backbone amide of Glu2, the C≡H group of Met1, the γ-, δ-, and ε-protons of Lys65, the C≡H group of Lys106 and H' of Leu87. In the course of the 3D structure determination, stereospecific resonance assignments were obtained for 21 pairs of CαH₂, five pairs of C-H₂ and one pair of CαH₂ protons as well as five pairs of γ- and δ-methyl groups of Leu and Val, five pairs of γ₁ methylene protons of Ile, and all side-chain NH₂ protons of Gln and Asn.

A TOCSY spectrum of DnaI-N132 indicated narrow resonances for about ten of the C-terminal residues in addition to the His₆ tag, indicating increased mobility and, therefore, no participation in the structured part of the N-terminal domain (data not shown). We therefore constructed the DnaI-N123 domain, expecting it to be more fully structured.

However, resonance assignments of DnaI-N123 were much more difficult than for DnaI-N106 because the signals were broad. Only backbone resonance assignments were attempted using a 0.9 mM ¹³C/¹⁵N-labeled sample.
Three-fold dilution did not improve the line widths of the NMR resonances, indicating that complete dissociation of the aggregates was not possible at the concentrations required for NMR. Most of the amide chemical shifts observed for this sample were the same as for DnaI-N106. For the segment with residues 107–123, tentative resonance assignments were obtained for only nine amino-acid residues.

Zinc-binding site

NMR spectroscopy cannot be used to determine directly the location of the zinc ion, so we confirmed the identity of zinc ligands by independent methods. DnaI-N106 contains one histidine, four cysteines (Figure 1) and one zinc ion (14), and all of the cysteine residues of full-length DnaI are in the DnaI-N106 domain. The $^{15}$N chemical shift of the $\text{Ne}^2$ resonance of His84 (221 ppm; Supplementary Figure S3) was characteristic of a zinc-bound imidazole nitrogen (34), confirming that the side chain of His84 is one of the zinc ligands (14). In addition, there was no sign of structural heterogeneity, indicating that the zinc ion was present in all protein molecules and in the same coordination environment. The affinity of DnaI-N for zinc was high, as treatment of a sample with 4 mM EDTA did not alter the appearance of the $^{15}$N-HSQC spectra. Thiol titrations of full length DnaI in triplicate with DTNB (0.32 mM, at pH 8.0) gave biphasic pseudo first-order kinetics, with one exposed thiol group titrating quickly ($k_{\text{obs}} = 0.09 - 0.16 \text{ s}^{-1}$), and three others about 50-fold more slowly; $k_{\text{obs}} = (1.9 - 2.7) \times 10^{-3} \text{ s}^{-1}$. Near identical stoichiometries and rate constants were observed for titration of DnaI-N123. The slowly reacting cysteines are presumed to be protected from reaction by being coordinated to zinc, and to titrate simultaneously as the metal-binding site is destroyed. In contrast, titration of the DnaI-C76A mutant protein with 0.25 mM DTNB under similar conditions showed only single-phase kinetics, with three thiols titrating slowly; $k_{\text{obs}} = (0.5 - 0.7) \times 10^{-3} \text{ s}^{-1}$. These data thus clearly identify His84 as a zinc ligand and Cys76 as the exposed faster-reacting cysteine residue, and suggest that the other three cysteine thiols are coordinated to the zinc ion. These conclusions were confirmed by 3D structure determination, as described below.

Structure of DnaI-N106

The structure of DnaI-N106 comprises four $\alpha$ helices and two $\beta$ strands. In addition, a short $3_10$ helix near the C-terminus was found in most of the NMR conformers (Figure 3A). The structure presents a novel fold, as a search of the protein data bank using the program Dali (35) failed to reveal a domain of similar structure. The zinc ion is coordinated by Cys67, Cys76, His84 and Cys101 (Figure 3A), whereas the side chain of Cys76 is located in the disordered loop between helix 4 and the first $\beta$ strand (Figure 3 and Supplementary Figure S4). The zinc ion probably plays an important structural role, as it ties together three sequentially distant segments of the polypeptide chain in a region where the structure has few hydrophobic residues (Figure 3D). In agreement with the zinc-coordinating residues found in the present NMR study, Cys76 is not conserved in B. amyloliquefaciens and B. pumilus, while all the other zinc-coordinating residues are (Figure 1). Nonetheless, some of the DnaI homologues from other bacteria show no evidence of a zinc-binding site. In those proteins,
the polypeptide segment between helix 4 and the first β strand is shorter which probably stabilizes the structure. Where this segment is longer, as in the homologue from *S. aureus*, it cannot be excluded that zinc binds at a similar site using a different set of cysteine and histidine residues (Figure 1).

The N-terminal 14 residues were disordered. As expected for a mobile polypeptide segment, the NMR signals of the N-terminal residues were also significantly narrower than those of the structurally defined part of the protein. Similarly, the 1H NMR signals of residues 63–82 in the loop between helix 4 and the first β strand showed a reduced line width.

Conceivably, the loop residues following helix 4 could assume a single, rigid conformation in the presence of the C-terminal ATP-binding domain. Although a detailed analysis of the longer construct DnaI-N123 was hampered by broad line widths, significant chemical shift changes observed for the loop residues suggest an interaction with the segment following the C-terminus of DnaI-N106 (Figure 3B). It cannot be excluded, however, that the chemical shift change is non-specific, reflecting spatial proximity of two or several DnaI-N domains induced by self-association of the C-terminal segment of DnaI-N123. This interpretation would be consistent with the observation that the NMR signals of residues 107–123 were particularly broad yet were at chemical shifts indicative of random coil structures.

As expected for a conserved fold, the side chains of many of the conserved hydrophobic residues have low solvent accessibility because they contribute to the hydrophobic core of the protein (Figure 4). Conversely, many of the residues with low side-chain solvent accessibility are hydrophobic. For example, the completely conserved residues Leu57, Gly82 and Pro85 are buried and the side chains of Phe37 and Tyr98 have low solvent accessibilities (Figure 4). Remarkably, the positions of Ile4, Leu8 and Val11 in the unstructured N-terminus of *B. subtilis* DnaI-N are also occupied by hydrophobic residues in all DnaI-N domains shown in Figure 1, although those residues are highly solvent accessible (Figure 4). Phe17 is also highly conserved despite high solvent accessibility of its side chain (>75%). Their conservation suggests a functional role of these flexible residues, presumably in protein–protein interactions.

**Interactions with *Bst* DnaB**

The interaction of *Bst* DnaB with all three DnaI-N constructs was probed by SPR, using a biotinylated oligonucleotide (dT35) immobilized on a streptavidin-coated surface. *Bst* DnaB was first injected over the oligonucleotide to a consistent binding level. In the presence of ATP under the conditions used, *Bst* DnaB binds to dT35 and dissociates very slowly; less than 9% dissociation was observed 30 min after injection. Subsequently, increasing concentrations of DnaI-N domains were injected. This experimental design allows for the real-time observation of the interaction of ssDNA-bound *Bst* DnaB with each of the DnaI-N constructs and direct comparison of the results.
Figure 3. Solution structure of *B. subtilis* DnaI-N106. Only residues 15–106 are shown since the initial 14 residues were disordered. The zinc atom is shown as a sphere (magenta) and the side chains of coordinating residues Cys67, Cys70, His84 and Cys101 are shown in yellow (cysteine residues) and blue (histidine). (A) Ribbon representation of DnaI-N106. The secondary structure elements are labeled as in Figure 1. Helix 5 is a 3_10 helix found in most but not all of the NMR conformers. (B) Same as (A), but color coded to reflect the changes in backbone amide chemical shifts observed in DnaI-N106 versus those in DnaI-N123 (blue: very small chemical shift changes >0.015 ppm (1H) or >0.15 ppm (15N); red: significant chemical shift changes >0.05 ppm (1H) or >0.5 ppm (15N). (C) Stereo view of a superposition of the backbone atoms of 20 NMR conformers. (D) Stereo view of a heavy-atom representation of the conformer closest to the mean structure of DnaI-N (only residues 15–106 are shown). The side-chains are color coded in blue (Lys, Arg, His), red (Asp, Glu), yellow (Ala, Cys, Ile, Leu, Met, Phe, Pro, Trp, Val) and gray (Asn, Gln, Ser, Thr, Tyr). The figure was prepared using the program Molmol (48).
All three DnaI-N domains (DnaI-N106, 123 and 132) interacted with Bst DnaB with association and dissociation rates that were fast, in contrast to the slower rates that full-length DnaI exhibited under similar conditions (14). Full-length DnaI is presumed to form a much more stable complex with the helicase because it oligomerizes cooperatively on the helicase surface via interactions among its C-terminal domains (2,14). This cooperativity in association and multiphasic dissociation made it impossible to obtain reliable thermodynamic data with full-length DnaI.

Provided that the sole contact between Bst DnaB and DnaI is in the zinc-binding region of DnaI-N106 as suggested in previous work (14), all three DnaI-N domains were anticipated to interact with similar strength. However, the experimentally determined $K_D$ values (DnaI-N106, 18.5 ± 0.9 µM; DnaI-N123, 7.3 ± 0.1 µM; DnaI-N132, 0.65 ± 0.02 µM; Figure 5) revealed significant differences, with the $K_D$ values decreasing with each longer construct, especially with DnaI-N123 in comparison with DnaI-N132. This indicates that the interaction of DnaI with Bst DnaB may extend beyond the folded core of the DnaI-N domain to the neighboring segments. Although the NMR experiments showed that this region is most likely unstructured in DnaI-N132, the function of the conserved, but unstructured, N-terminal segment of DnaI thus remains uncertain, though we cannot rule out that it might contribute modestly to helicase interaction.

DISCUSSION

3D structure determination of DnaI-N106, the folded core of the N-terminal domain of B. subtilis DnaI, revealed a zinc-binding module with a novel fold. High sequence conservation of DnaI-N was found only among proteins with putative primosomal functions. The zinc-binding motif of DnaI differs from conventional zinc fingers, in particular as the zinc-coordinating residues are separated as the zinc-coordinating residues are separated in the amino-acid sequence by two relatively long poly-peptide segments. The zinc-binding motif may play a purely structural role. It has been shown not to be involved in DNA binding, and study of alanine mutants of zinc-coordinating residues showed that impairment of zinc binding correlated with impaired binding to Bst DnaB (14). Although this is consistent with the zinc-binding region being involved in interaction with the...
helicase, it would also be readily explained if the primary role of the metal ion is to prevent unfolding of the DnaI-N domain.

By study of the binding of three different DnaI-N domain constructs to ssDNA-bound Bst DnaB helicase, we showed that the structured core domain DnaI-N106 bears most of the interacting residues, and that progressive C-terminal extension of this domain to 123 and 132 residues results in somewhat stronger binding (Figure 5). With reliable binding assays in hand, we are now in a good position to explore this interaction further by mutagenesis. Having defined the structure of DnaI-N may also facilitate structural study of the helicase/helicase loader complex by X-ray crystallography. The structural basis of this interaction in any organism is still poorly defined (2).

The stability of the DnaI-N fold clearly does not depend on conservation of hydrophobic side chains at positions 4, 8 and 11 in the mobile N-terminal segment of DnaI-N106. We have also observed these residues to be flexible in full length DnaI, indicating that they do not fold back to contact the C-terminal AAA+ domain (data not shown). Therefore we speculate that these residues, together with the highly conserved, solvent exposed hydrophobic residue at position 17, are required for binding to another protein. Specific protein–protein interactions, where a mobile terminal polypeptide segment of one protein binds to a well-structured domain of its binding partner, is a recurrent motif in bacterial replisomes, governing for example the interactions between the \( \psi \) and \( \gamma \) (36), \( \tau \) and \( z \) (37,38), and \( \varepsilon \) and \( z \) (39) subunits of the \( E. \ coli \) polymerase III complex, as well as the interactions of polymerases and many other proteins with the \( \beta \) sliding clamp (40), and of ssDNA-binding protein with its many binding partners (41). We were unable, however, to detect a strong interaction with \( Bst \) DnaB of a peptide corresponding to the N-terminal 18 residues of DnaI, and note that a version of DnaI lacking the N-terminal seven residues still bound its natural binding partner, the \( B. \ subtilis \) DnaC helicase, very strongly as detected by yeast two hybrid experiments (7).

The N-terminal domain of DnaI acts as a molecular switch that regulates the accessibility of the ssDNA-binding site of the C-terminal domain (14). In agreement with this notion, the fragmentation pattern observed after limited tryptic digestion of DnaI varied depending on the presence of ATP and \( \text{Mg}^{2+} \), which presumably bind to the Walker A and B motifs in the C-terminal domain (Supplementary Data). The change in fragmentation pattern may reflect a conformational change affecting the accessibility of the linker segment between the N- and C-terminal domains, although it cannot be excluded that the ATP-Mg\(^{2+}\) complex alone is sufficient to hinder and redirect the approach of trypsin.

\( B. \ subtilis \) DnaI and \( E. \ coli \) DnaC share significant sequence homology in their C-terminal domains, but there is no similarity between DnaI-N and the N-terminal segment of \( E. \ coli \) DnaC (15). \( E. \ coli \) DnaC cannot assume the DnaI fold determined here because it does not have enough residues in the N-terminal polypeptide segment, though this segment has also been shown to comprise at least part of the helicase binding domain of DnaC (42).

In view of the homology between the DnaI-N domains of \( B. \ subtilis \) and \( S. \ aureus \) (Figure 1), breaking the interaction of DnaI-N with the helicase would be of pharmaceutical interest. The 3D structure of DnaI-N determined in this work sets the stage for directed mutagenesis experiments and further structural studies to identify the interaction surface between the two proteins.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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