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Structure of the N-Terminal Oligomerization Domain of DnaD Reveals a Unique Tetramerization Motif and Provides Insights into Scaffold Formation

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Abstract

DnaD is a primosomal protein that remodels supercoiled plasmids. It binds to supercoiled forms and converts them to open forms without nicking. During this remodeling process, all the writhe is converted to twist and the plasmids are held around the periphery of large scaffolds made up of DnaD molecules. This DNA-remodeling function is the sum of a scaffold-forming activity on the N-terminal domain and a DNA-dependent oligomerization activity on the C-terminal domain. We have determined the crystal structure of the scaffold-forming N-terminal domain, which reveals a winged-helix architecture, with additional structural elements extending from both N- and C-termini. Four monomers form dimers that join into a tetramer. The N-terminal extension mediates dimerization and tetramerization, with extensive interactions and distinct interfaces. The wings and helices of the winged-helix domains remain exposed on the surface of the tetramer. Structure-guided mutagenesis and atomic force microscopy imaging indicate that these elements, together with the C-terminal extension, are involved in scaffold formation. Based upon our data, we propose a model for the DnaD-mediated scaffold formation.

Keywords

DnaD; DNA replication; *Bacillus subtilis*; primosomal; crystal structure

Introduction

Initiation of bacterial DNA replication is orchestrated by the master bacterial replicator DnaA and the staged assembly of a primosomal complex that remodels the DNA at the replication origin *oriC*, to load two replicative hexameric ring DNA helicases on the exposed single strands.^{1–4} Ring helicases are tethered around the single-stranded DNA, translocate the replication forks in opposite directions along the DNA, and unwind the double-stranded DNA in the process, fueled by chemical energy released from ATP binding/hydrolysis.⁵ Occasionally, replication forks may be arrested along the DNA, leading to replisome collapse and reinitiation at random sites distant to *oriC*. PriA coordinates the restart primosome and reassembly of the replisome at such sites.⁶ The DnaA and PriA proteins are highly conserved in all bacteria, but the rest of the primosomal proteins appear to be different, reflecting subtle mechanistic differences in primosome assembly and

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function in different species. For example, while both the Gram-positive *Bacillus subtilis* and the Gram-negative *Escherichia coli* have a helicase loader (DnaI and DnaC, respectively), no homologues of the *E. coli* PriB, PriC, and DnaT primosomal proteins are found in *B. subtilis*.² Instead, the DnaD and DnaB proteins, with no homologues in *E. coli*, have been implicated in primosomal assembly in *B. subtilis*.^{7,8}

DnaD is an essential protein^{9,10} that interacts with the master replication initiator protein DnaA¹¹ and the primosome-restart helicase PriA.¹² It has been implicated in the early steps of the primosomal cascade and is recruited at a membrane attachment point by DnaB^{13,14} to complete primosomal assembly and, together with DnaI, the loading of the replicative helicase DnaC.^{12,15–18} A genetic link between the PriA, DnaD, and DnaB proteins reinforces the notion of a coordinated cascade for the assembly of the restart primosome.¹⁹ In addition to its specialized role at *oriC*, DnaD has been implicated in DNA repair,¹⁰ recombination,⁸ initiation of plasmid replication,⁹ and initiation of sporulation in *B. subtilis*.²⁰ It also exhibits a novel global remodeling activity on supercoiled DNA; it opens up supercoiled plasmids and untwists the duplex, thus converting all the superhelical writhe into twist.^{21,22} DnaD molecules form a large macromolecular ‘scaffold,’ around the periphery of which the DNA is forced and held.^{22,23} This unique DNA-remodeling function is the sum of oligomerization and DNA binding activities of DnaD’s two distinct domains: an N-terminal domain (Nd; 16,056 Da) that mediates oligomerization and scaffold formation and a C-terminal domain (Cd; 13,730 Da) that binds DNA and exhibits a second DNA-induced oligomerization activity.²⁴ The two domains must be covalently linked into the same polypeptide chain in order to obtain the full wild-type DNA-remodeling effects. Interestingly, DnaB, which acts in the same primosomal cascade, exhibits a lateral DNA-compaction activity,²² and its molecular architecture resembles that of the *E. coli* γ -complex involved in the loading of the ring-shaped processivity factor onto the DNA.¹⁶ The significance of such activities for the initiation of DNA replication is not clear at present, but in addition to its helicase co-loading activity with DnaI,^{15,16,18} DnaB has been proposed to act as a ‘sensor,’ bridging large segments of the nucleoid at its membrane attachment site while at the same time sensing for the *oriC*.²² Once the *oriC* has been located onto the membrane attachment site, DnaD is recruited to remodel locally the nucleoid and assist the interaction of DnaA with the *oriC*. Other architectural proteins are also known to facilitate remodeling of the *oriC* by DnaA *in vitro*,^{25–28} and proteins such as HU β and H-NS suppress²⁹ or enhance³⁰ the thermosensitive phenotype of *dnaA46ts*, providing a genetic link between DNA-remodeling proteins and initiation of DNA replication.

In an effort to understand the mechanism of the DnaD-mediated DNA remodeling, we have solved the crystal structure of the scaffold-forming Nd and carried out structure-guided mutagenesis and atomic force microscopy (AFM) imaging to gain insights into the molecular details that underpin the formation of the DnaD scaffolds. Based upon our data, a structural model for scaffold formation is proposed and discussed.

Results and Discussion

The crystal structure of DnaD Nd reveals an extended winged-helix fold

The structure of the oligomerization domain of DnaD was refined against data to 2.0 Å spacing (see Supplementary Fig. S1 for an example of representative electron density), with a final *R*-factor of 18.1% and an *R*_{free} of 21.0% (see crystallographic and refinement statistics in Table 1). The overall structure reveals a winged-helix (WH) architecture with polypeptide extensions at both the N- and C-terminal ends of the classical WH fold (Fig. 1). DnaD Nd has no significant sequence homology with any other protein structurally characterized to date: sequence identity values in the range of 6–23% are obtained from structure-based sequence alignments with other WH proteins with which structural

similarity was detected using programs such as Dali³¹ and TOPS³² (Supplementary Table S1). Despite their unrelated sequences, the structures of DnaD Nd and WH proteins such as the BlaI repressor,³³ ADAR1,³⁴ genesis,³⁵ and the ESCRT II domains^{36,37} are superimposable with r.m.s.d. values in the 1.7–3.5 Å range, for selected C α atoms (Supplementary Table S1).

Two molecules of DnaD Nd are present in the asymmetric unit of the lattice (Fig. 1b and c). It is known from biochemical studies²⁴ that the domain is tetrameric in solution; a tetramer arrangement is observed (Fig. 1d) when crystallographic symmetry is applied. The dimer in Fig. 1b shows P2 symmetry about a central axis perpendicular to the plane of the page. Despite the overall symmetry in the arrangement of the monomers, detailed contacts in the dimer interface do not obey perfect symmetry and some equivalent side chains have nonidentical conformations. Dimers arrange themselves into the tetramer (Fig. 1d) also by P2 symmetry, about a vertical axis, again perpendicular to the plane of the page but orthogonal to the previously mentioned dimer axis (Fig. 2a).

The most striking feature of the WH fold in DnaD Nd is the presence of extensions at both termini (Fig. 1), which account for more than a third of the whole structure. The N-terminal extension includes a helix–strand–helix (H1'–S1'–H2') structural element, which appears to be unique to DnaD; on the other hand, the smaller C-terminal extension, made up of an alpha helix (H3'), is analogous to a similar extension found in the BlaI repressor (Supplementary Fig. S2). Inspection of the structure shows that both extensions, the N-terminal one in particular, are involved in the dimerization and tetramerization contacts.

Pairing of monomers and the dimer interface

The single β -strand present in the 24-amino-acid-long H1'–S1'–H2' motif forms a small β -sheet when two monomers pair up with each other with P2 symmetry, as mentioned before (Figs. 1a and b and 2a). Therefore, it appears that the resulting dimer is stabilized by the hydrogen bonding between the strands, in addition to a wealth of further contacts as outlined below. The dimerization interface is distinguished from the tetramerization interface in terms of surface area and interactions. The dimer contacts, between chains A and B (and chains A' and B', see Fig. 1b and d), cover an area of about 1524 Å², larger than the tetramerization interface, which is 1262 Å² in total and discontinuous, being made up of B–A' and A'–A' interactions. The dimer contacts include five strong (less than 3.0 Å) hydrogen bonds (Table 2) and several buried side chains (more than 90% of their area becomes solvent inaccessible; see Table 2). Among these buried groups, Ile17, Leu22, Leu35, and Ile36 are notable because they cluster into an extensive interaction hot spot of interlocking residues. Figure 2b shows an overall view of the interaction surface, with the more important side chains highlighted.

While both interfaces are significantly hydrated, water-mediated interactions occur almost exclusively at the periphery of the dimer interface, as opposed to the tetramer in which hydration is more widespread across the contact area (Fig. 3a). Accordingly, only 5 buried (or relatively buried, with less than 5 Å² accessible to the solvent) waters are observed in the dimer interface, while the tetramer interface has as many as 12 inaccessible water molecules. Thus, in terms of both the entropic cost of this hydration and the types of interactions observed, the dimer contacts result in a tighter association than the tetramerization interface, consistent with the native DnaD protein existing predominantly as a dimer in solution, with tetramers and higher oligomers forming at higher concentration as the scaffold is assembled.

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The tetramer interface and the tetramerization motif

Interactions that govern the formation of the tetramer appear generally weaker and less numerous than those involved in the dimer interface (Table 3). Only one strong electrostatic contact is present, and the shape of the interacting surfaces is such that there is no extensive hot spot as defined by burial of solvent-accessible area. The only remarkable feature is the presence of a highly symmetrical cluster of threonine residues at the center of the tetrameric assembly. These threonines (Thr14 and Thr16) are provided by the β -strands of the N-terminal extensions that pair up in the dimers; the face of each resulting double-stranded β -sheet then presents a threonine tetrad to another dimer so that the two sets come together into a tight core of eight threonine side chains, as shown in Fig. 3b. The interlocking is very effective because it is stabilized both by nonpolar hydrophobic contacts, contributed by the methyl groups, and by hydrogen-bonding interactions, from the hydroxyl functions of the threonine residues (Fig. 3b). This arrangement appears to be a unique feature of DnaD Nd, as is not observed in any other oligomeric protein with known structure. However, the tetramerization motif does not seem to be conserved across the sequences of homologous DnaD Nd proteins (Supplementary Fig. S2), although the preceding glycine (Gly13) and successive proline (Pro18) clearly show some degree of conservation. Even in the absence of strict conservation, it is possible that different residues pack together at the core of the tetramer; in particular, the space occupied by two water molecules, within each monomer and adjacent to the β -strands, means that some flexibility may allow the strands to rearrange their position and accommodate a range of side-chain groups in the tetramer core. In addition to the striking tetramerization motif, another region likely to provide a significant contribution to the interface is the crossover between helix H1' (residues 3–10) from one dimer and H3' (residues 103–111) from another dimer, where intervening nonpolar side chains yield a series of zip-like hydrophobic contacts. Notably, all groups involved in these interactions belong to the N- and C-terminal extensions, which appear to be a characteristic of the WH fold of DnaD Nd.

The role of the wing hairpin

Since DnaD Nd is involved predominantly in scaffold formation rather than DNA binding,²⁴ its extended WH domain participates in protein–protein interactions that underpin DnaD-mediated scaffold formation. In addition to the N- and C-terminal extensions, another striking feature of the WH fold in DnaD Nd is the wing-hairpin structure, which is pronounced and relatively long compared to a large set of nonhomologous (less than 20% sequence identity) WH proteins [Protein Data Bank (PDB) codes: 1Q1H, 1BJA, 1ILG, 1LEA, 1ZAR, 2A61, 2FXA, 1FT9, 1G6N, 1DPR, 1SAX, 1SEJ, and 1BIA] (Supplementary Fig. S2), as indicated by structural superpositions. Given the unique character of this structural feature, we speculate that it might be involved in protein–protein interactions key for scaffold formation. The role of the wing was examined through a protein engineering experiment in which the wing hairpin was truncated to form a much smaller structure. The EDQNGIK segment was replaced with the GS dipeptide sequence to produce the Nd_{GS} and DnaD_{GS} proteins (see Supplementary Fig. S3). With this protein engineering experiment, we removed the extended structure to generate a new hairpin where two short strands are still connected by a tight, type II β -turn. In our turn design, the sequence GS was chosen considering the conformation of the polypeptide chain and the propensities of amino acids to form and stabilize turn structures (see Plasmid constructs and mutagenesis). The hairpin truncated mutant proteins were expressed at high levels in the soluble fraction, purified to homogeneity, and confirmed to associate into tetramers for Nd, and dimers for DnaD in solution, by size-exclusion chromatography (data not shown). Circular dichroism (CD) analysis verified that this hairpin truncation did not induce gross structural changes either in the native DnaD or in the Nd (Supplementary Fig. S4).

AFM was used to compare the scaffolds formed by Nd and Nd_GS, which revealed striking differences (Fig. 4). At 6 nM, Nd_GS formed irregular aggregates that were small and elongated. Even at 30 nM, abnormal aggregates were apparent, growing elongated or very tall out of the mica surface. The Nd_GS aggregates are, therefore, distinctly different from the characteristic scaffolds formed by Nd, indicating that Nd_GS exhibits a defect in scaffold formation.

The role of the H3 helix

Given the strategic positioning of the H3 helix, which is prominently exposed at four corners of the tetrameric assembly (Fig. 1d), we envisaged that it might be involved in scaffold formation. Although the H3 helix is used in WH proteins to make contacts to the DNA in the major groove,³⁸ it also appears to be able to engage in protein–protein interactions as seen in the ESCRT-II complex.^{36,37} Examination of the three-dimensional coordinates, together with solvent-accessibility calculations, indicated that two residues on the H3 helix, Asn70 and Arg73, are very exposed to the solvent and project outward from the protein surface. It is tempting to postulate that these residues might be mediating scaffold formation. The role of the H3 helix was, therefore, investigated by mutating the N70 and R73 residues to alanines. AFM imaging of the resulting N70A and R73A mutant Nd proteins revealed abnormal aggregates (Fig. 4). Compared to the native Nd, irregular aggregates and smaller abnormal scaffolds were apparent at 6 and 30 nM, indicating that both mutations affect the ability of native Nd to form scaffolds. These data suggest that the wings and the H3 helices in the tetramer are involved in inter-tetramer interactions that are important in scaffold formation.

The role of the C-terminal extension

Given that the structure shows that the C-terminal (H3') helix is in a prominent, significantly exposed position, we devised two experiments to test its role in scaffold formation. With the first approach, residue Leu112, which points away from the molecule, was mutated to alanines in both the native DnaD and the Nd. In the second approach, three residues (Gln, Ser, and His) were added to the N-terminus of the protein, which is placed orthogonally, on the surface of the tetrameric assembly and proximal to H3', so that any lengthening of the N-terminal end would project out of the surface of the tetramer and sterically hinder other tetramers interacting with H3'. The GSH tripeptide was added to the native DnaD and used to engineer the GSH-N70A, GSH-R73A, and GSH-DnaD_GS mutant proteins. All GSH-tagged proteins were expressed in the soluble fraction purified to homogeneity and confirmed to associate into tetramers for Nd and dimers for DnaD in solution by size-exclusion chromatography (data not shown). CD analysis also verified that the GSH addition did not induce gross structural changes in the native DnaD or the Nd (Supplementary Fig. S4).

The GSH-DnaD proteins were examined by AFM, in the presence of pBSK, and found to form aggregates that were unable to open up supercoiled plasmids, indicating no regular scaffold formation (data not shown). The inability of GSH-DnaD to form scaffolds was verified further by its inability to shift intact supercoiled plasmids in agarose gels (Fig. 5a). Such shifts are dependent upon the formation of Nd-mediated scaffolds.^{22–24} GSH-DnaD protein appeared to shift only the open circular R-form of the plasmid, and upon treatment of the samples with proteinase K, the R-plasmid was released (compare lanes 2–5 and 7–10 in Fig. 5a). This is not because GSH-DnaD exhibits some DNA-binding defect or fails to bind to supercoiled DNA. In fact, GSH-DnaD binds to a synthetic single-stranded oligonucleotide and produces shifts comparable to the native DnaD²³ (Fig. 5b). GSH-DnaD also binds to supercoiled plasmid, as shown by the characteristic stimulation of topoisomerase I activity in the presence of increasing concentrations of GSH-DnaD²¹ (Fig.

5c). We conclude that GSH-DnaD binds to supercoiled and relaxed DNA but fails to produce the characteristic shifts of supercoiled plasmids in agarose gels because of its failure to produce native scaffolds. By comparison, the DnaD Cd, which is also unable to produce scaffolds, fails to shift supercoiled plasmids despite the fact that it binds to supercoiled DNA and forms DNA-induced oligomers.^{21,24}

In order to verify further that the GSH insertion at the N-terminus affects scaffold formation, we constructed the GSH-Nd protein and examined scaffold formation by AFM. Small aggregates were observed at 6 nM, which became larger at 30 nM (Fig. 6a). These aggregates were very different from the characteristic scaffolds observed with the native Nd (compare Fig. 5 and Fig. 6a). By comparison, Nd-L112A even at 30 nM also formed abnormal scaffolds compared to native Nd (compare Fig. 4 and Fig. 6b), while DnaD-L112A retained its ability to bind to DNA (Fig. 7). However, the scaffolds formed by Nd-L112A are more regular compared to other mutant scaffolds and appear to grow high up from the surface of the mica (see enlarged images in Fig. 6b). In that respect, they are also different from the wtDnaD scaffolds. This difference could be the result of subtle changes in tetramer–tetramer association.

The combined data show that the GSH insertion and the L112A mutation cause defects in scaffold formation without affecting binding to DNA. This is consistent with previous studies showing that the major DNA-binding activity resides on Cd and binding to DNA results in Cd oligomerization and duplex untwisting.^{21,24} The Nd mediates scaffold formation that appears to enhance duplex untwisting by Cd.²¹ It forms tetramers in solution,²⁴ and the crystal structure suggests that the H1'–S1'–H2' insertion at the N-terminus is intimately involved in dimerization and tetramerization. Indeed, deletion of the H1'–S1'–H2' insertion results in insoluble protein, which is likely to be the result of dimer and tetramer destabilization and exposure of the surface hydrophobic patches involved in dimerization and tetramerization (Supplementary Fig. S5). In conclusion, these experiments suggest that the H3' helices in the tetramer are involved in inter-tetramer interactions that are important in scaffold formation.

The effects of mutations in the H3 and H3' helices and the wing on the DNA-remodeling function of native DnaD

While scaffolds formed by the Nd appear similar to those formed by the native DnaD,^{23,24} the DNA-binding activity resides on the Cd,²⁴ and the two domains are likely to cooperate for DNA remodeling. In fact, the formation of scaffolds opens up supercoiled plasmids and is manifested by shifts of the plasmids in agarose gels.²⁴ Also, binding of Cd to DNA is sufficient to induce helix untwisting but the native DnaD appears to be more efficient at untwisting the DNA helix than Cd.²¹ Therefore, the presence of the scaffold appears to enhance the DNA untwisting activity of Cd. We argued that defective scaffold formation may be manifested indirectly by effects on the shifts of supercoiled plasmids in agarose gels, which will be different from that caused by native DnaD. The N70A, R73A (H3 helix), L112A (H3' helix), and DnaD_{GS} (wing truncation) mutants in the native DnaD were used to study the effects on gel shift assays.

Agarose shift assays revealed that all mutants formed nucleoprotein complexes that shifted higher up the gel, but subtle differences were apparent (Fig. 7). Higher concentrations of the N70A and DnaD_{GS} proteins were required to produce complete shifts compared to native DnaD (compare lanes 1–3, 4–6, and 13–15 in Fig. 7). Also, the DnaD_{GS} shifts appeared more diffuse relative to the rest. The R73A mutant did not produce intermediate shifts, exhibiting very big nucleoprotein complexes that stayed in the well (lanes 7–9), while the L112A mutant produced slightly bigger nucleoprotein complexes at 105 μM compared to native DnaD (compare lanes 3 and 12). These data are consistent with irregular scaffold

formation and intact DNA-binding activities, hence resulting in subtle differences in the positions of the shifted nucleoprotein species.

A model for scaffold formation

Canonical WH proteins are usually versatile DNA-binding proteins utilizing contacts between the so-called recognition helix H3, the wing, and the DNA. The H3 and the wing interact either with the major grooves or with the minor grooves of DNA, depending on whether these elements are located on the most basic electrostatic surface of a protein.³⁸ However, some WH proteins do not bind DNA, and their H3 and wing elements are involved in protein–protein interactions instead.^{37,38} Although DnaD is a nonspecific DNA-binding protein, the main function of Nd is to mediate scaffold formation rather than to bind DNA. Instead, a major DNA-binding activity resides on a separate Cd.²⁴ The Nd forms a tetramer, with three key secondary structural elements exposed on the surface: H3 recognition helices, wings, and C-terminal H3' extensions. The defects observed in scaffold formation by the DnaD_{G5}, N70A, and R73A mutants suggest that the exposed H3 helices and wings participate in the formation of scaffolds. In addition, both the L112A mutation and the GSH addition at the N-terminus affected scaffold formation. The N-terminal H1'–S1'–H2' insertion participates in dimerization and tetramerization at the core of the structure and is unlikely to be involved in scaffold formation directly. However, the GSH insertion at the N-terminus is expected to project out of the structure in the vicinity of the orthogonal C-terminal H3' helix, thus sterically preventing the H3' helix from participating in inter-tetramer interactions.

Given that Nd forms concentration-dependent scaffolds in a manner similar to the native DnaD protein, we propose a possible structural model for scaffold formation (Fig. 8a) based on the arrangement of the molecules as seen in the crystal lattice. It is reasonable to assume that some of the interactions that govern scaffold formation are also present in the lattice. Indeed, contacts between molecules across the crystal are made almost exclusively by groups from the H3 helix and the wings (Fig. 8b). The H3' terminal extension is not involved in crystal contacts. Presumably, this can be explained because in the lattice, a particular set of scaffold interactions dominate, thus giving the crystal its order and directionality of molecules that are lacking from the seemingly irregular scaffolds observed by AFM. Also, since the wing is associated with a high level of flexibility (Supplementary Fig. S6), it is likely that a particular or more common conformation of the β -hairpin is selected by the crystallization conditions, while different wing conformations may be present in molecules across the scaffold, affecting the direction and orientation of the molecular packing. Regardless of possible differences in the exact orientation of molecules in the lattice *versus* the scaffold, it is striking to see that both Asn70 and Arg73 of H3 are involved in a number of inter-molecular crystal contacts. Interestingly, in the Nd lattice, there is sufficient space between Nd tetramers to accommodate four Cds, which would be required in the scaffold of the native DnaD. It is, therefore, attractive to propose that the lattice packing of Nd molecules provides a valid snapshot of some of the key interactions that are likely to govern the association of molecules into the unique structural scaffolds that underlie the DNA-remodeling function of DnaD.

Native DnaD is predominantly dimeric with tetramers and higher oligomer species forming at high concentrations.²³ We propose that the dimer exists in two conformations, D2 and D2*, depending on the orientation of the Cds relative to the Nds (Fig. 9). Only D2* can form tetramers D4 that will be required for the formation of the scaffold. In the absence of DNA, the D2 species is the dominant one and in equilibrium with small amounts of D2* and D4 as shown in Fig. 9. Binding of D2 to DNA via the Cds stabilizes the D2* conformation and shifts the equilibrium toward the D4 tetramers and the formation of the scaffold. The D4–DNA species would act as a seed for the formation of the scaffold. This explains the

strange behavior of native DnaD in DNA-binding gel shift assays. In such assays, it forms an initial complex with DNA, which is consistent with a dimer, but as the concentration of DnaD increases, the complex starts to shift higher up the gel with free DNA remaining intact at the bottom of the gel.²³ It appears that the DnaD–DNA complex acts a seed that attracts binding of more DnaD to it, rather than the free DNA, to form the scaffold.

Experimental Procedures

Plasmid constructs and mutagenesis

The native DnaD and Nd proteins were produced from the pET22b-DnaD²³ and pET22b-Nd²⁴ constructs, respectively. The plasmid constructs for the GSH-DnaD and GSH-Nd proteins were engineered by cloning the NdeI–HindIII or the NdeI–XhoI fragments, containing the *dnaD* and Nd genes, respectively, into the same sites of pET28a to produce the pET28a-DnaD and pET28a-Nd constructs, respectively. The pET28a-DnaD construct produced a protein with a removable N-terminal His₆ tag, while the pET28a-Nd construct produced a protein with removable N-terminal and nonremovable C-terminal His₆ tags. The removable tag was cleaved by thrombin (Novagen) cleavage, according to the manufacturer's instructions. After thrombin treatment, a GSH tag remained attached at the N-terminus, producing the GSH-DnaD or GSH-Nd proteins.

The N70A and R73A mutants were constructed in the native DnaD, GSH-DnaD, and Nd proteins, while the L112A mutant was constructed in the native DnaD and Nd proteins. All single point mutants were engineered using the pET22b-DnaD,²³ pET22b-Nd,²⁴ or pET28a-DnaD constructs, as appropriate, and the QuikChange II-E site-directed mutagenesis kit (Stratagene), according to the manufacturer's instructions.

The wing structure typical of the WH fold has a type II β -hairpin as assigned using the program PROMOTIF.³⁹ In order to shorten the wing, we replaced residues 87–93 with a Gly-Ser dipeptide sequence (Supplementary Fig. S3), which is commonly used in type II β -hairpin structures⁴⁰ and generally accommodates amino acid substitutions in type II β -hairpins.⁴¹ The new, shorter designed β -hairpin was modeled in COOT⁴² and analyzed with PROMOTIF.³⁹ Supplementary Figure S1 shows the predicted model of the shortened hairpin in comparison with the wild type. The wing truncations were constructed in the native DnaD (DnaD_{GS}), GSH-DnaD (GSH-DnaD_{GS}), and Nd (GSH-Nd_{GS}) proteins using a two-step PCR splice by overlap mutagenesis procedure.⁴³

The constructs coding for the N-terminal H1'–S1'–H2' truncated DnaD and Nd proteins were engineered using suitable primers (Supplementary Table S2) and PCR with the pET22b-DnaD and pET22b-Nd substrates, respectively, followed by cloning into the NdeI–HindIII and NdeI–XhoI sites of pET22b, respectively.

All the oligonucleotides and proteins used in this study are shown in Supplementary Tables S2 and S3, respectively.

Protein purifications

Native DnaD and its mutants (N70A, R73A, L112A, and DnaD_{GS}) were purified as described before.^{23,24} The only difference is that DnaD_{GS} was overexpressed at 20 °C.

For the purifications of the GSH-DnaD and its mutants (N70A, R73A, and DnaD_{GS}), overexpressions were carried out at 37 °C (N70A and R73A) and 20 °C (DnaD_{GS}). Cells were harvested in buffer A (50 mM sodium phosphate, pH 7.5, 500 mM NaCl, and 20 mM imidazole) and 10% w/v sucrose, sonicated, and then incubated for 30 min at 37 °C with benzonase (Novagen). The cell extract was clarified by centrifugation at 17,500 rpm for 30

min. Total protein was precipitated with ammonium sulfate (2.9 g per 10 mL) and centrifuged at 17,500 rpm for 30 min. The protein pellet was resuspended in buffer A and loaded onto His-trap-Ni²⁺ chelating column pre-equilibrated with buffer A. The protein was eluted with a 20- to 500-mM imidazole gradient in buffer A. Relevant fractions were pooled, and N-terminal His tag was removed by thrombin (Novagen) treatment at 20 °C overnight. The protein solution was then reloaded onto His-trap-Ni²⁺ chelating column pre-equilibrated with buffer A. The flow-through of the His-trap column containing the protein was collected, precipitated with ammonium sulfate, and centrifuged at 17,500 rpm for 30 min. The protein pellet was resuspended in TED (50 mM Tris pH 7.5, 2 mM ethylenediaminetetraacetic acid, and 1 mM DTT) with 600 mM NaCl and loaded onto a Superdex S-75 gel filtration column pre-equilibrated with TED and 350 mM NaCl. Final samples were made up to 10% v/v glycerol before snap-freezing in liquid nitrogen for storage at -80 °C.

The Nd and GSH-Nd proteins and their mutants were purified as described elsewhere,²⁴ with the following differences: the overexpressions of Nd_{G5} and GSH-Nd_{G5} were carried out at 20 °C, and, for the GSH-Nd proteins, treatment with thrombin to remove the N-terminal Histag preceded the final gel filtration step.

The purity of all protein preparations was checked by SDS-PAGE analysis, and their correct oligomerization states were verified by comparative analytical gel filtration (data not shown).

Crystallization and preparation of protein for phase determination

Crystallization of the native DnaD Nd was carried out as described before.⁴⁴ For obtaining phase information, samples of the DnaD Nd were prepared in the presence of selenomethionine (SeMet) by transforming the expression plasmid in methionine auxotroph *E. coli* B834 (DE3) cells. An overnight culture (2 mL) of B834 (DE3) cells, grown at 310 K in Luria-Bertani medium supplemented with 50 µg mL⁻¹ ampicillin, was transferred to 1 L of LeMaster medium⁴⁵ with 50 mg L⁻¹ SeMet (Calbiochem) and 50 µg mL⁻¹ carbenicillin (Melford) in a baffled 2-L flask. Cells were grown at 310 K and shaken at 200 rpm until an optical density (*A*₆₀₀) of 0.7 was reached. Protein expression was then induced by adding isopropyl-β-D-thioga-lactopyranoside to a final concentration of 1 mM, and cultures were further grown overnight. Purification and crystallization of SeMet Nd were carried out as described for the native protein but at the lower protein concentration of 10 mg mL⁻¹, in contrast to 20 mg mL⁻¹ for the unlabeled protein.⁴⁴

Data collection and phasing

Phase information was obtained by means of the multiwavelength anomalous dispersion (MAD) method using a highly redundant two-wavelength SeMet-MAD data set collected from a single SeMet derivative crystal at the ID23-1 beamline of the European Synchrotron Radiation Source (Grenoble, France). The selenium absorption edge was determined by an X-ray fluorescence scan, and the peak (0.9798 Å) and the remote (0.976 Å) data sets were collected with 1° oscillation, an exposure of 1 s with a 98% attenuated beam. The SeMet derivative crystals diffracted to 2.2 Å (peak) and 2.1 Å (remote) spacing. Data on the native DnaD Nd crystals were collected in-house as previously described.⁴⁴ All data sets were processed with the programs MOSFLM⁴⁶ and SCALA⁴⁷ of the CCP4 suite.⁴⁸

Heavy atom location and phase calculation on the SeMet-MAD data set was carried out with SHELXD⁴⁹ and SHARP/autoSHARP,^{50,51} using all 10 possible SeMet sides of the two molecules in the asymmetric unit cell and showing excellent phasing statistics and a high-quality initial map.

Model building and refinement of the atomic coordinates

Manual building of the model was carried out using COOT⁴² and refined against the native data using TLS refinement⁵² in REFMAC5.⁵³ The optimal number of TLS groups was determined using the TLSMD server.⁵⁴ The model consists of chains A and B, with 1–115 and 1–117 residues, respectively. The residues 116–128/118–128 (chains A/B) are not included in the model, because only weak, broken peaks of electron density were obtained for these regions. The sequence of C-terminal tail is mainly made up of almost exclusively small, polar, charged amino acids, consistent with this region forming a random-coil, disordered structure connecting the Nd and Cd. Also, relatively weak density was obtained for the loop region 88–92 of chain A, which is consistent with the high level of flexibility of this region (see *B*-factor plot in Supplementary Fig. S6). In chain B, this loop region is engaged in crystal contacts and, therefore, less flexible. Data processing and model refinement statistics are summarized in Table 1. The model was evaluated using the MOLPROBITY server^{55,56} and shows excellent geometry and no Ramachandran outliers. Surface area calculations were carried out with the program AREAIMOL,⁴⁸ and interactions between the molecules were analyzed with LIGPLOT⁵⁷ and visually examined in COOT. All structural figures were prepared using PyMOL.⁵⁸

Electrophoretic mobility shift assays (EMSAs)

Agarose EMSA was carried out as described before,²² using 4.2 nM supercoiled pBSK and various concentrations (15, 45, and 105 μ M) of proteins, as appropriate. Polyacrylamide EMSA was carried out in 50 mM Tris, pH 7.5, 0.1 mM ethylenediaminetetraacetic acid, 200 mM NaCl, 4 mM MgCl₂, 1 mM DTT, and 2.5 nM 5'-³²P-labeled 34mer oligonucleotide (5'-TGCATGCCTGCAGGTCGACTCTAGAGGATCCCCC-3') in the presence of 700 or 800 nM DnaD or GSH-DnaD. Binding reactions were incubated at room temperature for 15 min and resolved by nondenaturing polyacrylamide gel electrophoresis. Electrophoresis was carried out in 0.5 $\times$ TBE. The single-stranded oligonucleotide was radiolabeled using γ -³²P-ATP and T4 polynucleotide (NEB) and separated from unincorporated γ -³²P-ATP using a MicrospinTM S-200 HR spin column (Amersham Biosciences), according to the manufacturer's instructions.

Topoisomerase-I-based untwisting assays

Topoisomerase-I-based untwisting assays were carried out as described before,²¹ using 21 nM supercoiled pBSK and various concentrations (0.5, 1, 8, 16, and 32 μ M) of native DnaD and GSH-DnaD proteins, as appropriate.

AFM

Nd-DnaD and its mutants (N70A, R73A, L112A, Nd_{GS}, and GSH-Nd) were diluted to 6–30 nM in TED and 100 mM NaCl. For imaging in air, 10- μ L samples were incubated with freshly cleaved mica (Agar Scientific) for 1 min, rinsed gently with 300 μ L of dH₂O, and allowed to dry under a gentle flow of nitrogen gas. All AFM imaging was carried out with a Digital Instruments Nanoscope IIIa Multimode AFM with a type E scanner (Veeco, Santa Barbara, CA) in tapping mode. The cantilevers used were silicon-tapping probes with a spring constant of 34.4–74.2 N/m (Olympus, OMCL-AC160TS). The tapping set point was adjusted to minimize probe-sample interactions. Images were recorded in both topography and phase modes with a pixel size of 512 $\times$ 512, flattened, and analyzed with the Nanoscope software.

PDB accession numbers

The atomic coordinates (PDB code 2V79) have been deposited in the PDB, European Bioinformatics Institute—Macromolecular Structure Database, Cambridge, United Kingdom[‡].

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Abbreviations used

Nd	N-terminal domain
Cd	C-terminal domain
AFM	atomic force microscopy
WH	winged helix
PDB	Protein Data Bank
CD	circular dichroism
MAD	multiwavelength anomalous dispersion
EMSA	electrophoretic mobility shift assay

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Fig. 1.

Ribbon representations depicting the structure of the DnaD Nd. (a) Schematic representation of the monomer with its WH folding topology. The typical structural elements of the WH architecture are shown in a semitransparent mode. The N- and C-terminal extensions unique to the WH fold of DnaD, termed H1', S1', H2', and then H3', respectively, are highlighted. (b) Two monomers of DnaD Nd form a symmetrical dimer through the pairing of their S1' strands (for a diagram of the dimer and tetramer symmetry, see Fig. 2a). (c) Side view of the dimer rotated 90° relative to the previous image. (d) Figure showing the tetrameric assembly, which is the predominant oligomeric state of the DnaD Nd in solution.

Fig. 3.

Key features at the interfaces. (a) Hydration at the dimer and tetramer interfaces: surface representations of the dimer interface, on the left-hand side, and the tetramer interface, on the right-hand side. The diagrams show how water molecules (shown in blue) and ions (in green) mediate interactions exclusively at the periphery of the contact area in the case of the dimer interface. On the other hand, several waters are found interspersed across the tetramer contacts, resulting in over twice as many water molecules becoming buried in the latter case compared to the dimer interface. (b) The tetramerization motif: the tetramer appears to be stabilized by a striking structural feature: one threonine tetrad, with two Thr14/Thr16 pairs (shown in a space-filling model) provided by the N-terminal extension β -strands hydrogen-bonded in the dimers, associates with another one to pack onto a tight core of eight threonine side chains. The interlocking involves both nonpolar hydrophobic contacts, contributed by the methyl groups (highlighted in gray), and hydrogen-bonding interactions, from the hydroxyl functions of the threonine residues.

Fig. 4.

AFM images of scaffolds formed by Nd and its mutants. Scaffolds formed by native Nd and the Nd_{GS}, N70A, and R73A mutants are shown at 6 and 30 nM, as indicated. At 30 nM, native Nd forms a scaffold that covers the entire surface (bottom left), while mutants can only form irregular aggregates and smaller abnormal scaffolds. Scale bars represent 400 nm with a Z range of 3 nm.

Fig. 5.

The interaction of GSH-DnaD and its mutants with DNA. (a) Agarose gel shifts of supercoiled pBSK plasmid (4.2 nM) treated with GSH-DnaD and its mutant proteins (105 μ M), as indicated. Control reactions with native DnaD (lanes 1 and 6) and pBSK plasmid only (lane 11) are shown. Samples from binding reactions were treated or not with proteinase K (PK), as indicated. Only the relaxed R-form of pBSK was shifted by the GSH-DnaD and its mutants compared to all of the plasmids with the native DnaD protein. (b) Comparative gel shift assays in a nondenaturing polyacrylamide gel using a radioactively labeled synthetic oligonucleotide (2.5 nM) and different concentrations of native DnaD (lane 1, 700 nM; lane 2, 800 nM) and GSH-DnaD (lane 3, 700 nM; lane 4, 800 nM). Lane C shows a control with radioactive oligonucleotide but no proteins. The characteristic initial shift followed by a supershift at higher concentration²³ is apparent for both proteins. The observed supershifts are due to scaffold formation by native DnaD and irregular oligomerization by GSH-DnaD. (c) A comparison of the untwisting activities of native DnaD and GSH-DnaD proteins. Native DnaD binds to supercoiled plasmids and untwists duplex DNA, thus stimulating the activity of topoisomerase I.²¹ The topoisomerase I activity was assayed in the presence of increasing concentrations (0.5, 1, 8, 16, and 32 μ M) of native DnaD and GSH-DnaD proteins, as indicated. Control reactions with untreated pBSK in the presence and absence of PK (lanes 1 and 2, respectively) and topoisomerase-I-treated pBSK (lane 3) are also shown. The positions of relaxed (R), linear (L), and supercoiled (SC) pBSK in the gel are indicated for clarity. Both proteins stimulate the topoisomerase I activity, indicating untwisting of the duplex. The inhibition of topoisomerase I at higher concentrations of the proteins is the result of steric block that prevents topoisomerase I from accessing the DNA.

Fig. 6. The GSH-Nd and Nd-L112A proteins form defective scaffolds. (a) AFM images of GSH-DnaD scaffolds at 6 and 30 nM, as indicated. Scale bars represent 200 nm (top) and 1 μ m (bottom). (b) AFM images of L112 scaffolds at 30 nM. Zoomed (top) and distant (bottom) images are shown. The scale bars represent 1 μ m (top) and 2 μ m (bottom) for the zoomed and distant image, respectively, with a Z range of 5 nm.

Fig. 7.

The interaction of native DnaD and its mutants with DNA. Comparative agarose gel shift assays of supercoiled pBSK (4.2 nM) in the presence of increasing concentrations (15, 45, and 105 μ M) of native DnaD and its mutants, as indicated. Lane C shows control plasmid DNA in the absence of protein.

Fig. 8.

A lattice-based model for some key interactions governing scaffold formation. (a) Cartoon representation of DnaD Nd molecules packing in the crystal lattice. Molecules along a plane or layer are shown, and overlapping molecules have been removed from the figure to make the association between molecules easier to view. (b) A selection of three molecules from the lattice is displayed, highlighting the inter-tetramer interactions involving the H3 helix and the wing hairpin.

Fig. 9.

A model for DNA-induced scaffold formation by DnaD. Native DnaD is a dimer in solution that exists in two conformations, D2 and D2*. The two forms are defined by the relative orientations of the Nd and Cd. Only D2* can form the tetramer D4, and only D4 can form the scaffold. In the absence of DNA, there is an equilibrium between D2, D2*, D4, and higher oligomers. This equilibrium is shifted predominantly toward D2. In the presence of DNA, the dimer interacts with the DNA and the D2* conformation is stabilized. The D2*–DNA complex acts as a ‘magnet’ to draw another D2* molecule to form a stable D4–DNA complex. This complex acts as the seed for scaffold formation. The crystal structure reveals how the dimer is formed (N2) and how two dimers form the tetramer (N4). The green balls represent the C-termini from where the Cds would be attached in the native protein. In the N4 tetramer, one dimer is represented in orange space-filling mode while the second dimer is in green ribbons for clarity.

Table 1

Data collection, processing, phasing, and refinement statistics

	DnaD Nd		
	Native	SeMet peak	SeMet remote
<i>Data collection</i>			
Space group	$P3_121$	$P3_121$	$P3_121$
Unit cell parameters			
a, b, c (Å)	78.7, 78.7, 124.6		79.1, 79.1, 124.4
α, β, γ (°)	90.0, 90.0, 120.0		90.0, 90.0, 120.0
Wavelength (Å)	1.5418	0.9798	0.976
Resolution range (Å)	28.0–2.0 (2.1–2.0)	30–2.2 (2.3–2.2)	30.0–2.1 (2.2–2.1)
R_{sym} (%)	4.8 (37.6)	8.9 (41.8)	6.4 (36.6)
Mean $I/\sigma I$	24.4 (4.0)	19.0 (5.1)	24.4 (6.0)
No. of observations	177,843 (25,869)	310,519 (50,593)	360,835 (58,215)
No. of unique reflections	30,662 (4429)	23,412 (3354)	26,920 (3875)
Completeness (%)	99.8 (100.0)	100.0 (100.0)	99.9 (100.0)
Anomalous completeness (%)	–	97.4 (100.0)	98.1 (100.0)
Multiplicity	5.8 (5.8)	13.3 (15.1)	13.4 (15.0)
Anomalous multiplicity (%)	–	7.0 (7.8)	7.1 (7.8)
<i>Phasing statistics</i>			
Number of Se sites			10
Figure of merit (centric/acentric)			0.84/0.93
Phasing power			1.53
<i>Refinement</i>			
Resolution (Å)	28.0–2.0 (2.05–2.0)		
No. of reflections	29,103 (2119)		
$R_{\text{work}}/R_{\text{free}}$ (%)	18.1–21.0 (25.3–30.6)		
No. of atoms			
Protein (chain A/B)	1944 (958/986)		
Waters/Ions	231/14		
<i>B</i> -factors			

DnaD Nd			
	Native	ScMet peak	ScMet remote
Protein (chain A/B)	37.8/34.4		
Waters/Ions	43.1/39.1		
r.m.s.d.			
Bond length (Å)	0.012		
Bond angles (°)	1.126		

Numbers in parentheses are for the high-resolution shell.

^dFive percent of randomly selected reflections were excluded from the refinement for R_{free} calculation.

Table 2

Interactions identified in the dimer and tetramer interfaces

Dimer contacts				Tetramer contacts			
Interface area		1524 Å ²		1026 Å ²		236 Å ²	
Chain ID	Chain A	Chain B	Distance (Å)	Chain B	Chain A'	Chain A	Distance (Å)
Polar/ Electrostatic	M1 (mc)	Q111 (sc)	2.8	K3 (sc)	E108 (sc)	M1 (mc)*	2.9
	Q12 (mc)*	N19 (sc)*	2.9	Y107 (sc)*	M1 (mc)*		3.1
	G13 (mc)	N19 (mc)	3.1				
	S15 (mc)*	I17 (mc)*	2.8				
	S15 (mc)*	N19 (sc)*	2.5				
van der Waals	E32 (sc)*	S62(sc/mc)*	2.6				
	M61 (mc)	K26 (sc)	3.1				
	M1 (sc)*	I110 (sc)*	3.7	K3 (sc)*	G104 (mc)*	M1 (sc)*	3.8
	M9 (sc)*	L20 (sc)*	3.7	K3 (sc)	E108 (sc)	F6 (sc)*	3.8
	T16 (sc)	T14 (sc)	3.6	F6 (sc)*	Y107 (sc)*	M9 (sc)*	3.9
	N19 (sc)*	M43 (sc)*	3.8	F6 (sc)*	I110 (sc)*		
	L22 (sc)	M43 (sc)	3.7	I7 (sc)*	Q100 (sc)*		
	T23 (sc)*	M43 (sc)*	3.7	I7 (sc)*	G104 (mc)*		
	K26 (sc)*	G60 (mc)*	3.7	Q10 (mc/sc)*	W103 (sc)*		
	E32 (sc)	I36 (sc)	3.8	E11 (sc)*	Q100 (sc)*		
	E32 (sc)	T33 (mc)	3.6	T16 (sc)	T16 (sc)		
	T33 (mc)	E32 (sc)	3.8				
	L35 (sc)*	I36 (sc)*	3.7				
	I36 (sc)	I36 (sc)	3.4				

Interface area refers to the solvent-accessible surface area buried by each chain upon association. A' is the symmetry-related molecule to A (refer to the color scheme in Fig. 1: chain A=red, chain B=green, chain A'=blue, chain B'=orange). The asterisk symbol refers to contacts mirrored by symmetry, although within the dimer, symmetry is not strictly obeyed at the level of individual side chains. The abbreviations sc and mc refer to side chain and main chain, respectively. Interactions were examined and identified using the programs AREAIMOL and DISTANG of the CCP4 suite and LIGPLOT (see Experimental Procedures).

Table 3

List of amino acids which become significantly buried upon dimer formation

Residue	SIS (%), chain A/B
Ser15	95/93
Ile17	95/100
Asn19	92/92
Leu20	73/77
Leu22	99/100
Thr23	61/71
Glu32	98/95
Leu35	95/100
Ile36	100/100
Met43	78/85
Met61	92/100
Ile110	68/75
Glu114	61/42

Those side chains that are completely buried, or virtually so, are highlighted in boldface and are likely to make the strongest contribution to the stability of the interface.

SIS=Surface Inaccessible Surface.