A Structural Basis for the Regulatory Inactivation of DnaA

Qingping Xu1†, Daniel McMullan2†, Polat Abdubek2†, Tamara Astakhova3†, Dennis Carlton4†, Connie Chen2†, Hsiu-Ju Chiu1†, Thomas Clayton4†, Debanu Das1†, Marc C. Deller4†, Lian Duan3†, Marc-Andre Elsliger4†, Julie Feuerhelm2†, Joanna Hale2†, Gye Won Han4†, Lukasz Jaroszewski3,5†, Kevin K. Jin1†, Hope A. Johnson4†, Heath E. Klock2†, Mark W. Knuth2†, Piotr Kozbial5†, S. Sri Krishna3,5†, Abhinav Kumar1†, David Marciano4†, Mitchell D. Miller1†, Andrew T. Morse3†, Edward Nigoghossian2†, Amanda Nopakun4†, Linda Okach2†, Silvya Oommachen1†, Jessica Paulsen2†, Christina Puckett2†, Ron Reyes1†, Christopher L. Rife1†, Natasha Sefcovic5†, Christine Trame1†, Henry van den Bedem1†, Dana Weekes5†, Keith O. Hodgson6†, John Wooley3†, Ashley M. Deacon1†, Adam Godzik3,5†, Scott A. Lesley2,4† and Ian A. Wilson4†* 

Regulatory inactivation of DnaA is dependent on Hda (homologous to DnaA), a protein homologous to the AAA+ (ATPases associated with diverse cellular activities) ATPase region of the replication initiator DnaA. When bound to the sliding clamp loaded onto duplex DNA, Hda can stimulate the transformation of active DnaA–ATP into inactive DnaA–ADP. The crystal structure of Hda from Shewanella amazonensis SB2B at 1.75 Å resolution reveals that Hda resembles typical AAA+ ATPases. The arrangement of the two subdomains in Hda (residues 1–174 and 175–241) differs dramatically from that of DnaA. A CDP molecule anchors the Hda domains in a conformation that promotes dimer formation. The Hda dimer adopts a novel oligomeric assembly for AAA+ proteins in which the arginine finger, crucial for ATP hydrolysis, is fully exposed and available to hydrolyze DnaA–ATP through a typical AAA+ type of mechanism. The sliding clamp binding motifs at the N-terminus of each Hda monomer are partially buried and combine to form an antiparallel β-sheet at the dimer interface. The inaccessibility of the clamp binding motifs in the CDP-bound structure of Hda suggests that conformational changes are required for Hda to form a functional complex with the clamp. Thus, the CDP-bound Hda dimer likely represents an inactive form of Hda.

© 2008 Elsevier Ltd. All rights reserved.

*Corresponding author. E-mail address: wilson@scripps.edu.
Abbreviations used: Hda, homologous to DnaA; AAA+, ATPases associated with diverse cellular activities; oriC, chromosomal replication origin; DUE, DNA unwinding element; RIDA, regulatory inactivation of DnaA; MAD, multiwavelength anomalous dispersion; CDP, cytidine-5′-diphosphate.
Introduction

In *Escherichia coli*, the initiation protein DnaA, when complexed with ATP (DnaA–ATP), binds to the 9-bp DnaA boxes within the chromosomal replication origin (oriC) to initiate DNA replication.1 At the beginning of initiation, 20–30 DnaA–ATP proteins bind to the origin and oligomerize into a large nucleoprotein complex, which facilitates melting of the adjacent AT-rich, 13-bp DNA unwinding element (DUE).2 The initiation of chromosomal DNA replication is tightly regulated to ensure only one replication per cell cycle.3 Three regulation mechanisms have been identified. First, an oriC sequestration mechanism inactivates the newly replicated oriC through binding of the SeqA protein. SeqA has a higher affinity for hemimethylated GATC sites within the oriC region. For example, a single copy of the dataA locus, located 470 kb downstream from oriC, could bind an unusually large number of DnaA molecules. DnaA boxes are also present in the promoter regions of many genes. Third, a mechanism known as RIDA (regulatory inactivation of DnaA) negatively regulates DnaA by converting active DnaA–ATP into inactive DnaA–ADP following the initiation.6–8 DnaA–ADP cannot induce melting of the origin and thus is ineffective for replication initiation.1

The two essential components of the RIDA system are the sliding clamp of DNA polymerase III (DnaN or δ-subunit) and Hda (homologous to DnaA, also referred to as Idab).9,10 When the sliding clamp is loaded onto double-stranded DNA, Hda can promote hydrolysis of DnaA–ATP to DnaA–ADP.11–13 As a result of RIDA, the level of DnaA–ATP in the cell, which peaks in *vivo* around the initiation of replication, decreases rapidly after the start of replication.8 The Hda-mediated RIDA process is an important mechanism for preventing overinitiation,14,15 and hda-deficient cells do in fact overinitiate.9,16 Hda is a dimer in solution and binds to the sliding clamp via its N-terminal sliding clamp binding motif.13,17 Besides being involved in RIDA, Hda may also have other cellular functions, since it also interacts with the plasmid RK2 replication initiation protein TrfA.10,18 Details of how and where RIDA occurs in the cell currently are not clear. In *vivo*, the RIDA interaction of DnaA–Hda is dependent on the sliding clamp loaded onto double-stranded DNA.12 In *vivo*, RIDA may occur on the pre-replication sliding clamps that remain on the lagging strands after synthesis of Okazaki fragments.13 It has also been suggested that Hda binds to the sliding clamp at the replication fork and that RIDA occurs at the fork.5 Another possibility is that Hda could interact with the DnaA–ATP origin complex after opening of the DUE.4,6,19

A typical Hda is about 250 residues in length. It is classified as an AAA+ (ATPases associated with diverse cellular activities) ATPase due to its sequence homology to the AAA+ ATPase region of DnaA (domains III and IIIb). A recent *in vitro* reconstitution of *E. coli* RIDA activity revealed that a conserved arginine in the box VII motif of Hda is required to stimulate ATP hydrolysis in DnaA and suggests that a conserved AAA+ type of interaction takes place between Hda and DnaA during RIDA.13

Members of the AAA+ superfamily of ATPases are found in all kingdoms of living organisms. These ATPases act as motors or switches and control a wide range of biological processes.20–24 Structural characterization of AAA+ modules has revealed that they range of biological processes.20–24 Structural characterization of AAA+ modules has revealed that they
consist of two domains: an N-terminal P-loop NTPase homologous domain (the base domain) and a smaller C-terminal helical bundle domain (the lid domain). The nucleotide binding site is located at the domain interface (see Nucleotide binding site). AAA+ proteins generally form ring-shaped oligomeric assemblies, where one AAA+ subunit inserts residues from a conserved motif (box VII) into the ATP interaction site of its adjacent subunit. ATP hydrolysis is enabled once the bipartite nucleotide binding pocket is formed, and contact is made between a conserved arginine (“arginine

![Image](https://example.com/image1)

Fig. 1 (legend on previous page)
finger”) from the box VII motif in one subunit and the γ-phosphate of the bound nucleotide in the adjacent monomer. ATP hydrolysis often results in intrasubunit and intersubunit conformational changes, which can be employed in many cellular events. The AAA+ ATPases are key components of DNA replication and repair complexes, such as the replication initiator DnaA. The opening of DUE by DnaA does not involve ATP hydrolysis, since a nonhydrolyzable ATP analog can functionally replace DnaA–ATP. Thus, it appears that ATP hydrolysis in DnaA is employed in its inactivation.

Table 1. Data collection and refinement statistics

<table>
<thead>
<tr>
<th>Statistic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength (Å)</td>
<td>0.9793</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>29.6–1.75</td>
</tr>
<tr>
<td>No. of observations</td>
<td>204,590</td>
</tr>
<tr>
<td>No. of reflections</td>
<td>55,436</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.7 (99.7)</td>
</tr>
<tr>
<td>Mean I/σ (I)</td>
<td>9.1 (1.8)</td>
</tr>
<tr>
<td>R cryst (%)</td>
<td>0.10 (0.64)</td>
</tr>
</tbody>
</table>

Model and refinement statistics

<table>
<thead>
<tr>
<th>Statistic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution range (Å)</td>
<td>29.6–1.75</td>
</tr>
<tr>
<td>No. of reflections (total)</td>
<td>55,402</td>
</tr>
<tr>
<td>No. of reflections (test)</td>
<td>2813</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.5</td>
</tr>
<tr>
<td>Bond angle (°)</td>
<td>1.47</td>
</tr>
<tr>
<td>Bond length (Å)</td>
<td>0.014</td>
</tr>
<tr>
<td>Average isotropic</td>
<td>24.1</td>
</tr>
<tr>
<td>B-value (Å²)</td>
<td>0.10</td>
</tr>
<tr>
<td>Protein residues/atoms</td>
<td>461/3802</td>
</tr>
<tr>
<td>Ligand and water molecules</td>
<td>575</td>
</tr>
</tbody>
</table>

ESU indicates estimated overall coordinate error. R cryst = ∑|F calc − |F obs|/ ∑|F obs|, where |F calc| and |F obs| are the calculated and observed structure factor amplitudes, respectively. R free = as with R cryst but for 5.1% of the total reflections chosen at random and omitted from refinement.

Results and Discussion

Overall structure

The structure of Hda from *S. amazonensis*, complexed with cytidine-5'-diphosphate (CDP) and magnesium (Fig. 1a and b), was determined to 1.75 Å resolution by multiv wavelength anomalous dispersion (MAD) with selenomethionine-labeled protein (Table 1). The structure was refined to R cryst and R free values of 16.6% and 20.1%, respectively. The model displays good geometry with 99.3% favorable mainchain torsion angles (no Ramachandran outliers) and 98.4% favorable side-chain rotamers according to MOLPROBITY. The final model contains a dimer (A12–241, B11–241) in which each monomer is complexed with a CDP and a magnesium ion. In addition, 571 solvent molecules (1 thiocyanate, 14 ethylene glycols, 1 sodium, 1 chloride, and 554 water molecules) were modeled. The main chain for residues A0–11, B0–10, and A32–33 and the side chains for residues A12, A36, A134, A150, B11–12, B81, B102–103, B136, B192, B221, and B229 were disordered and were not included in the final model.

The Hda monomer (Fig. 1b) displays a molecular architecture typical of AAA+ ATPases. The N-terminal domain I (residues 1–174, the base domain) has a RecA-like fold containing the Walker A (the P-loop), Walker B, and sensor I motifs. An extended N-terminal loop contains a short strand (β1) that has previously been shown to bind to the sliding clamp. The C-terminal domain Ib (residues 175–241, the lid domain) is a short four-helix bundle (α5–α8) that contains the sensor II motif. The two domains of Hda are similar to domain III (rmsd of 1.57 Å for 138 Cα) and domain IIb (rmsd of 1.56 Å for 48 Cα) of DnaA [Protein Data Bank (PDB); IDs 2hcb and l8q‡] when compared separately. The most significant difference between domain I of Hda and domain III of DnaA is the size of the insertion between β3 and β4 (the “steric wedge” in DnaA) (Fig. 1c). This region often varies among different AAA+ ATPases and is used to classify them. In Hda, this insertion is shorter than most others and contains three 310 helices (n1, n2, and n3) (Fig. 1a). Helices n2–n3 are equivalent to the second helix of a

‡ http://www.pdb.org/
The η1 of Hda is shorter than the equivalent helix of DnaA. The Hda insertion η1–η3 is structurally similar to the clamp loaders, such as the clamp loader delta subunit. However, Hda does not possess the corresponding long η3–β4 loop found in the clamp loader, which is critical in binding the sliding clamp. Since the β3–β4 insertion resides next to the clamp binding motif (β1), it could play a role in binding the sliding clamp.

The orientation between the base and lid domains varies significantly between DnaA and Hda. A superposition of the base domains of DnaA (PDB ID 2hcb) and Hda reveals that the lid domain of Hda is rotated a further 47° toward its base domain around the principal axis of the bound nucleotide (Fig. 1c).

**Hda dimer**

The crystallographic asymmetric unit contains a homodimer (Fig. 2). The two monomers are similar to each other with an rmsd of 0.99 Å for all Cα atoms, with the most significant differences in the orientation of the two C-terminal helices, α7 and α8 (Fig. 1c). The Hda dimer resembles a flat rhombic prism, with dimensions of 79 Å×62 Å×43 Å, in which the two N-terminal base domains are aligned along the short axis, while the two C-terminal lid domains are aligned along the long axis of the rhombus. The dimer buries a surface area of 2045 Å² from each monomer (Fig. 2). Molecular masses of 52,480 and 54,030 Da were determined by two independent runs of analytical size-exclusion chromatography in combination with static light scattering. Because an Hda monomer has a calculated molecular mass of 27,059.1 Da (mass determined by liquid chromatography/mass spectrometry was 27,060.8 Da), size-exclusion chromatography with static light scattering suggested that it forms a dimer in solution, consistent with its packing in the crystal. The bulk of the dimer interface is formed from interactions between the two base domains. These interactions are clustered around β1 and η1. The positions of the two N-terminal extended loops (11–21) are swapped, with the N-terminal loop from one subunit making extensive contacts with the β3, η1, and η2 from the second subunit. Together, these N-terminal loops form a short antiparallel β-sheet that contributes 25% of the buried surface area to the dimer interface. Additional dimer interactions are between the lid domain of one monomer and the base domain of the second monomer. Specifically, the N-terminal portion of α3 of the base domain is docked between α7 and α8 of the lid domain from the other subunit.

The Hda homodimer represents a novel mode of AAA+ oligomerization. AAA+ proteins frequently form hexameric rings in a “head-to-tail” manner such that the arginine finger from one subunit can interact with the nucleotide of the adjacent one. However, the two monomers in the Hda dimer are arranged in a “head-to-head” manner. As a result, the dimerization of Hda places the two nucleotide binding sites close to each other, while the two corresponding arginine fingers are located far apart on the perimeter (Fig. 2).

The transcription activator NtrC1 is an example of an AAA+ protein that forms a dimer in its inactive form through a swapped N-terminal linker when its receiver domain is unphosphorylated. However, the NtrC1 dimer differs significantly from the Hda dimer. The noncrystallographic 2-fold axis in the NtrC1 dimer is parallel with the linker region consisting of two long, antiparallel helices. The NtrC1 dimer does not involve the base domains of the AAA+ regions. In Hda, the noncrystallographic 2-fold axis is perpendicular to the antiparallel β-sheet in the N-terminal region. In contrast to the critical role of the NtrC1 linker in its dimerization, the N-terminus of Hda does not appear to be
essential for dimerization, since HdaΔN26 can still dimerize.\textsuperscript{13}

**Nucleotide binding site**

Well-defined electron density at the nucleotide binding site identified a bound CDP that presumably was obtained during protein expression in \textit{E. coli} (Fig. 3a). The possibility of ADP (or GDP) was excluded due to poor fit to the electron density, lack of favorable hydrogen bonds to stabilize the adenosine base, and steric clashes with the protein. Bound CDP is surprising since ADP or ATP was expected based on similarity to DnaA and other AAA+ proteins. However, CTP has previously been shown to bind to ATPases. For example, the \textit{E. coli}

---

**Fig. 3.** Nucleotide binding site of Hda. (a) CDP (shown in sticks) with the experimental density contoured at 2.5σ. The map was calculated after density modification improvement of the initial MAD phases. The conserved magnesium ion (pink), coordinated by water molecules (red), and Thr65 (not shown) stabilize the β-phosphate of the CDP. (b) Interaction between the protein and the cytidine base and sugar of CDP. (c) Comparison of motifs Walker A and sensor II of Hda (orange) and DnaA (gray). The corresponding residues for DnaA (PDB ID 2hcb) are shown in parentheses. (d) Sequence conservation pattern of the P-loop region of putative DnaA and Hda proteins. The DnaA group of sequences (785 sequences) were selected based on the presence of both a C-terminal DNA binding motif and an ATPase domain. The putative Hda sequences (94 sequences) were selected by full-length homology to \textit{E. coli} Hda and of length between 210 and 270 residues.
DnaA–CTP complex was effective in opening the DUE (but not effective in subsequent steps of the initiation process). The conformation of the CDP is very similar to the nucleotides ADP and AMP–PCP bound to DnaA. The cytidine base is stabilized through hydrophobic stacking interactions and three hydrogen bonds with the protein, along with two water-mediated hydrogen contacts (Fig. 3b). The binding site clearly favors CDP, and replacement of cytidine by uracil would result in the loss of two hydrogen bonds. These hydrogen-bond interactions involve the main chain of Tyr30 and the side chain of Arg185. Since Arg185 is the only side chain involved in hydrogen-bonding interaction with the base, it could play a role in specifying the substrate. Arg185 and the cytidine binding pocket are conserved in E. coli and highly conserved in other putative Hda proteins. Thus, it seems likely that other Hda proteins can also bind CDP.

The conserved motifs Walker A, Walker B, sensor I, and sensor II found in AAA+ proteins are all present in S. amazonensis Hda. The position of the sensor II motif of Hda is significantly different from that of DnaA due to the change in orientation of the lid domain Ib (Figs. 1c and 1d). The conserved arginine residue in the sensor II motif (e.g., Arg277 in Aquifex aeolicus DnaA) typically interacts with the γ-phosphate of ATP. However, the corresponding residue in Hda (Arg210) interacts with Asp24 of the base domain I, as well as the α-phosphate of CDP (Fig. 3c). Hda has a variant (58-GPVKSGRT-65) of the Walker A consensus sequence (GXXGXXGK[ST]). Lys61 physically occupies the same space as the DnaA box VIII (sensor II) Arg277. The Arg64 side chain interacts with the β-phosphate of CDP (Fig. 3c). The conformation of Arg64 is identical to the Lys64Arg mutant of RuvB from Thermotoga maritima.

**NTP hydrolysis and possible roles of nucleotides**

Hda shares many of the structural features found in DnaA and other members of the AAA+ ATPase superfamily, including a conserved nucleotide binding site. However, no experimental evidence is available to suggest that it has NTPase activity. It has been proposed that Hda could form a typical AAA+ head-to-tail dimer. Such a model is not consistent with the crystal structure determined here. The homodimer of Hda precludes a mechanism through a canonical arginine finger type of interaction between two Hda monomers. The CDP binding site is buried within the dimer interface (Fig. 2), and, despite a few solvent channels nearby, access to CDP is limited. No space is available to form a bipartite complex similar to other AAA+ ATPases. Significant structural changes, such as dimer dissociation, would be required to achieve NTP hydrolysis.

It seems likely that Hda has lost its ability to tightly bind or efficiently hydrolyze NTP. The presence of Arg64 in the Walker A motif appears to be detrimental to NTP binding and hydrolysis. All structurally known P-loops have lysines at this position. Mutational studies on other AAA+ ATPases have indicated that the conserved lysine in the Walker A motif is essential. For example, Lys-to-Arg mutants of E. coli RuvB and DnaC are defective in both ATP binding and hydrolysis. The requirement of a conserved lysine becomes more apparent when the Walker A motif is analyzed in all pertinent bacterial sequences currently in Pfam PF00308. The sequences fall into two groups: one with a C-terminal DNA binding domain (likely DnaA proteins) and one that has the prototypical DnaA AAA+ module only (likely Hda proteins). In the first group, for which ATP is required, the conserved lysine is invariant (Fig. 3d). In the second group, both the Walker A motif and the lysine position show greater sequence variability, as do the Walker B and sensor I motifs. The loss of the strictly conserved lysine suggests that the γ-phosphate of NTP is less important for Hda function. Structurally, an overlay of AMP–PCP of DnaA onto the CDP site of Hda indicates that the guanidinium group of Arg64 would clash with the γ-phosphate (Fig. 3c) and would therefore disrupt NTP binding.

Hda has retained its ability to bind NDP as the residues involved in binding ribose (His65) and α- or β-phosphate (Gly63, Arg210, Arg64) are invariant or highly conserved. A possible role of the CDP is to promote dimer formation and maintain its conformation. The smaller cytidine base may facilitate greater plasticity between the two domains compared to adenosine.

As ATP/ADP is found in all other AAA+ structures and the nucleotide binding pockets are highly conserved in AAA+ proteins, the possibility that Hda can also bind ADP cannot be excluded from this study. If Hda were to bind ADP like DnaA, it is likely that ADP may affect the conformation of Hda locally or globally. Due to the larger size of the adenosine base, the region between 28 and 34 of Hda must move outward to accommodate it (Fig. 4). Changes in this region could propagate to nearby regions, such as the linker region between domains I and Ib, the first helix of domain Ib (αE), and the clamp binding motif region. Thus, any association of ADP with Hda could disrupt interactions between domains I and Ib in Hda, and potentially alter the dimerization state or the conformation of the clamp binding region.

**The sliding clamp binding motif and the clamp interaction**

Hda can form a stable functional complex with the sliding clamp in vitro with a stoichiometry of Hda:clamp or, less likely, Hda:clamp. Previous studies have shown that a conserved hexapeptide motif, QL[S/P]LPL, at the N-terminus of E. coli Hda is essential for binding the sliding clamp. This motif is also widely conserved in other clamp binding proteins. Hda from S. amazonensis possesses a similar conserved sequence motif (13-QLSLPV-18) at its N-
terminus (Fig. 1a and b). In the Hda dimer, the clamp binding motifs of each subunit form a short, antiparallel \( \beta \)-sheet at the noncrystallographic 2-fold axis of the dimer. Mutational studies on this motif\(^{13,17} \) have suggested that Gln13, Leu16, and Val18 (equivalent to Gln21, Leu24, and Leu26 of \( E. \) coli) are critical for binding the sliding clamp. Interestingly, the side chains of these residues are buried in the dimer interface. The Gln13 from one subunit makes contact with the backbone of the adjacent subunit through two hydrogen bonds (Fig. 5a). Leu14, Ser15, and Pro17 in this motif are solvent exposed and, along with conserved residues Leu97, Leu101, and Phe104, located at the \( \beta3-\beta4 \) insertion (\( \eta2 \) and \( \eta3 \)), and Phe83 from \( \beta3 \), form a hydrophobic patch of dimensions 25 Å \( \times \) 22 Å, with a central ridge and a shallow cavity on either side (Fig. 5b).

Several characterized clamp binding motifs adopt extended conformations and bind to a conserved location between the second and third domains of the \( \beta \)-subunit (Fig. 6a).\(^{33,40-42} \) The clamp binding motif of Hda is also likely equivalent to other clamp binding motifs and interacts with the same binding site on the clamp.\(^{7,43} \) The clamp binding motif of Hda (QL[P]LP) is similar in sequence to the Pol IV clamp binding motif (QLVGL),\(^{41} \) thus, it is expected that the Hda clamp binding motif will adopt a similar conformation when bound to the clamp. However, the 2-fold symmetric clamp binding motifs of the Hda dimer are partially buried and have an unusual conformation (Fig. 5). Clearly, they cannot bind the clamp in a canonical manner in their current conformation without significant steric clashes. As a result, the CDP-bound dimer of Hda observed in the crystal structure is likely unable to bind the clamp and thus is likely inactive in RIDA, unless the hydrophobic patch of Hda binds the sliding clamp in an unconventional manner.

Hda must then adopt a different conformation in order to bind to the clamp in a canonical manner. This alternative structure could involve dissociation of the clamp binding motifs from the rest of the dimer to an extended conformation, or the Hda dimer itself may dissociate and interact with the clamp in a monomeric form (Fig. 6b). Dissociation of Hda clamp binding motifs (or the dimer) may occur spontaneously in solution. However, deuterium exchange mass spectroscopy experiments have indicated that the clamp binding motifs of the Hda–CDP dimer are not flexible in solution (data not shown), consistent with the crystal structure. Thus, it appears that additional factors are needed to
regulate the conformation of the clamp binding motifs of Hda, such as a conformational switch associated with binding of different nucleotides (e.g., ADP and CDP; see above) and a weak intrinsic NTPase activity. The physiological role of the inactive Hda dimer is currently unclear; it may offer additional means to regulate Hda activity so that it only becomes active when associated with the sliding clamp.

Since the clamp binding motif of Hda is located at its N-terminus rather than at the C-terminus as in Pol IV, the Hda molecule will likely be located close to the middle domain of the β-subunit (Fig. 6a). It is not clear whether the functional Hda–clamp complex for RIDA involves an Hda monomer or an Hda dimer at each site of the β-subunit (Fig. 6b). Although the conserved site on the clamp generally interacts with a monomer, Hda was previously shown to interact with the clamp as a dimer. However, a recent study suggested a possible monomeric interaction between Hda and the clamp. Further biochemical experiments and the complex structure of Hda and the sliding clamp will help resolve such questions.

Arginine finger and Hda–DnaA interaction

In order to utilize the conserved mode of ATP hydrolysis in AAA+ proteins, it is expected that DnaA and Hda form a heterodimeric complex during the RIDA reaction. The arginine finger, Arg168, of E. coli Hda is critical for the deactivation of ATP–DnaA, whereas the corresponding Arg161 of S. amazonensis Hda is located near the middle of a short helix α4 (box VII). The arginine fingers are fully exposed to solvent.

Residues in the immediate surrounding of Arg161 are also highly conserved among Hda, as well as DnaA (Figs. 1a and 7). Arg161 is preceded by an acidic (D/E) residue (Asp157) and a smaller polar (S/T) residue (Ser160), followed by a hydrophobic residue (Trp164). Additionally, two other residues,
Phe126 and Asn130, from the neighboring α3 (the central helix) are also highly conserved. Asn130 hydrogen bonds with Arg161 in one of the Hda subunits, and both are located on the same helical surface and are fully exposed to solvent. Arg161 and Trp164 are in different conformations in the two Hda monomers, indicating that these residues are flexible.

In contrast to DnaA, which is present in all bacteria, Hda is predominantly found in proteobacteria, which suggests that Hda originally evolved from DnaA to acquire its regulatory function. Due to the high level of similarity in both sequence and structure in the arginine finger regions (i.e., Phe126, Asn130, Glu134, Ser160, Arg161, Trp164, and Gly165) of both DnaA and Hda (Figs. 1a and 7), it has been proposed that Hda may interact with DnaA in a similar fashion to the DnaA self-assembly.4,19 Structural studies on A. aeolicus DnaA with ADP and an ATP analog have illustrated a rigid-body movement in the two AAA+ subdomains between the ATP and ADP states.19,26 Although there is no Hda homolog and likely no Hda-dependent RIDA in A. aeolicus, the structural changes of DnaA associated with different nucleotide states and the mode of DnaA self-assembly are likely to be generally applicable to DnaA in other bacteria due to the highly conserved AAA+ region (37% sequence identity between A. aeolicus and S. amazonensis).27 We therefore made use of these DnaA models in order to gain further insights into how the base domain and the arginine finger of Hda interact with DnaA.

The Hda dimer seems to be compatible with the mechanism suggested for the DnaA self-assembly structure.4,19 A geometrically plausible model can be built by replacing a DnaA molecule with an Hda dimer (or monomer) at the "ATP end" (the side where the γ-phosphate of the ATP bound to DnaA is accessible)19 of the DnaA self-assembly through superimposition of the base domains of DnaA and Hda. Extensive contacts are made through domain III of DnaA in the DnaA filament self-assembly.19 The corresponding Hda secondary structure elements that make contact with the DnaA−ATP site are α3 (120–135) and α4 (156–166). Helix α3 is close to a groove on the surface of domain III, and α4 makes contacts with domain IIIb (Fig. 8a).

This model is consistent with known AAA+ interactions for ATP hydrolysis. The γ-phosphate of ATP sits next to the strictly conserved Arg161 and Asp157 (Fig. 8b); therefore, both these residues likely play a catalytic role in ATP hydrolysis. An acidic residue, corresponding to Asp157, is conserved in many AAA+ modules, such as glutamate in Pol III delta subunit and aspartate in NSF-D1.20 Asp157 is close to the Walker B motif of DnaA and located at the gate of the binding pocket where the γ-phosphate of the ATP would contact the solvent. Thus, a possible role for Asp157 is to help the Walker B motif stabilize a nucleophilic water, which would interact with the γ-phosphate. Asp157 is not, however, conserved in DnaA (Figs. 1a and 4) and may explain why DnaA−ATP in the origin of replication assembly does not efficiently self-hydrolyze.

In the transition from DnaA−ATP to DnaA−ADP, the wedge opening, where Hda binds, will narrow through interdomain movement between the III and IIIb domains of DnaA.19,26 It is plausible that the conformational changes induced by ATP hydrolysis in DnaA could drive the dissociation of Hda and DnaA−ADP. The released Hda could then be recycled for the next reaction. The cellular concentration of Hda (~50 dimers/cell) is much less than that of DnaA (500–2000 molecules/cell);13 thus, reuse of the Hda is important in RIDA.

**Possible association of Hda with the membrane**

The replicatively active DnaA−ATP is regenerated from DnaA−ADP by acidic phospholipids in the presence of oriC and ATP.44 A region near the N-terminus of the bridging helix (residues 349–383) of E. coli DnaA, between the lid domain (IIIb) and the DNA binding domain (IV), is critical in membrane-mediated nucleotide release.4,44 The T7 epitope-tagged form of Hda was associated with the inner

---

**Fig. 8.** Hypothetical models for DnaA−Hda interaction. (a) Putative interaction between the base domain of Hda (shown in ribbon representation) and DnaA. Domains III, IIIb, and IV of DnaA are shown as surfaces and in gray, magenta, and cyan, respectively. (b) The bipartite active site for ATP hydrolysis is formed by the conserved residues in the DnaA−Hda (white/green) heterodimeric complex shown in (a).
membrane by immunoblot analysis. Interestingly, the lipid binding region of DnaA corresponds to a highly conserved region of Hda (residues 225–241; Fig. 1A). The α8 helix of Hda is equivalent to the bridging helix of DnaA, although shorter in length. Additionally, Arg328 of E. coli DnaA, whose mutation affects membrane binding in DnaA, is also highly conserved in Hda and located in the same region (adjacent to the side chain of Lys236 of Hda). Thus, it is possible that Hda could interact with the membrane in a similar fashion to DnaA.

Materials and Methods

Protein production

The gene encoding a putative Hda from S. amazonensis SB2B (GenBank YP 927791,119775051 gi: 119775051) was amplified by PCR (polymerase chain reaction) from genomic DNA using Phire DNA polymerase (Stratagene) and primers corresponding to the predicted 5’ and 3’ ends. The PCR product was cloned into plasmid pSpeedET, which encodes an expression and purification tag followed by a tobacco etch virus protease cleavage site (MGSDKIHHHHHHENLYFQG) at the amino terminus of the protein. The cloning junctions were confirmed by DNA sequencing. Protein expression was performed in E. coli strain GeneHogs (Invitrogen). At the end of fermentation, lysozyme was added to the culture to a final concentration of 250 μg/mL, and the cells were harvested. After one freeze–thaw cycle, the cells were homogenized in lysis buffer [50 mM Hepes, pH 8.0, 50 mM NaCl, 10 mM imidazole, and 1 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP)] and passed through a Microfluidizer (Microfluidics). The lysate was clarified by centrifugal ultrafiltration (Millipore). Hda was crystallized using the nanodroplet vapor-diffusion method 46 with standard JCSG crystallization protocols. 47 Initial screening for diffraction was carried out using the Stanford Automated Mounting system 48 at the Stanford Synchrotron Radiation Laboratory (SSRL, Menlo Park, CA). To determine its oligomeric state, we analyzed S. amazonensis Hda using a 0.8 × 30-cm Shodex PROTEIN KW-803 column coupled with miniDAWN static light scattering and Optilab differential refractive index detectors (Wyatt Technology). The mobile phase consisted of 20 mM Tris, pH 7.4, and 150 mM NaCl. The molecular mass was calculated using ASTRA 5.1.5 software (Wyatt Technology).

Crystallization and data collection

The crystallization reagent contained 0.2 M sodium thiocyanate and 20% (w/v) PEG (polyethylene glycol) 3350 at pH 6.9. Ethylene glycol was added as a cryo-protectant to a final concentration of 10% (v/v). MAD data were collected at SSRL on beamline 11-1 at wavelengths corresponding to the peak (λ1) and high-energy remote (λ2) of a selenium MAD experiment. The data sets were collected to 1.75 Å at 100 K using a Mar CCD 325 detector (MarUSA).

Structure determination and refinement

The crystals were indexed in orthorhombic space group P212121; with unit cell dimensions a = 55.04 Å, b = 64.03 Å, and c = 153.64 Å. Data processing and structure solution were carried using an automatic structure solution pipeline XSOLVE developed at the Joint Center for Structure Genomics. The MAD data were integrated and reduced using Mosflm 49 and then scaled with the program SCALA 50 of the CCP4 suite. 51 Phasing was performed with SHELXD 52 and autoSHARP. 53 The automated model building was performed with ARP/WARP. 54 Refinement was carried out using REFMACS 55 interspersed with manual building using COOT. 56 The model geometry was analyzed with MOLPROBITY 57 to assess the Ramachandran plot, side-chain rotamers, hydrogen bonds, steric clashes, and van der Waals contacts. Data and refinement statistics are summarized in Table 1.

Accession code

Coordinates and structure factors have been deposited in the PDB under accession code 3bos.

Acknowledgements

The project was sponsored by the National Institute of General Medical Sciences Protein Structure Initiative (P50 GM62411, U54 GM074898). Portions of this research were carried out at the SSRL. SSRL is a national user facility operated by Stanford University on behalf of the U.S. Department of Energy Office of Basic Energy Sciences. The SSRL Structural Molecular Biology Program is supported by the Department of Energy Office of Biological and Environmental Research and by the National Institutes of Health (National Center for Research Resources, Biomedical Technology Program, and the National Institute of General Medical Sciences). The contents of this work are solely the responsibility of the authors and do not necessarily represent the official views of the National Institute of General Medical Sciences or the National Institutes of Health. We greatly appreciate the very helpful comments of Dr. James Berger and his group.

References

Crystal Structure of Hda


