The roles of Deinococcus radiodurans Dps-1 and Dps-2 in nucleoid organization and in survival during oxidative stress

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THE ROLES OF DeINOCoccus RadiodURANS DPS-1 AND DPS-2 IN NUCLEOID ORGANIZATION AND IN SURVIVAL DURING OXIDATIVE STRESS

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

In

The Department of Biological Sciences

by

Khoa Huynh Nguyen
B.S. (Magna Cum Laude), Louisiana State University, 2008
May 2013
I would like to dedicate this dissertation to the most important people in my life: my family.

To my dad, Ut Van Nguyen and to my mom, Mui Huynh, both of you are one of the main reasons for my success today. Your unconditional love and encouragement have help through all the difficult times of my life. I greatly appreciate all the things that you have done for me and I am very grateful to have you as my parents.

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ABSTRACT

DNA protection during starvation (Dps) proteins are important for bacterial oxidative stress responses. This study aims to understand the roles and characteristics of the two Dps homologs in *Deinococcus radiodurans*: Dps-1 and Dps-2. Dps-2 contains a predicted signal peptide and *in vivo* localization of Dps-2 reveals that its location is non-cytoplasmic. β-galactosidase assays show that the *Dps*-2 promoter is upregulated in the presence of H$_2$O$_2$ and the results from the DNA protection assay show that Dps-2 is able to protect DNA efficiently against reactive oxygen species (ROS). Dps-2 has a C-terminal extension that is needed for assembly into a dodecamer but not for DNA binding.

Dps-1 is assembled from six dimers and stoichiometric experiments reveal that the stoichiometry of Dps-1 binding to 22 bp DNA substrate is 1:6, meaning that Dps-1 has six DNA binding sites. However, for the 26 bp DNA substrate, the stoichiometry is 1:4, suggesting that the protein can interact with both faces of a DNA duplex provided there are two consecutive major grooves on each face. Furthermore, mutation of the surface arginine (Arg132) causes a decrease in DNA binding, which indicates that this residue is involved in the path of DNA binding of Dps-1 after the initial contact is made with the N-terminus. A model for the mode of Dps-1 binding to genomic DNA is proposed based on these observations.

Dps-1 has a unique metal site at the end of the N-terminal extension and mutations of this site cause the protein to exist as a hexamer and this lead to a significant reduction in DNA binding. The mutant protein (Dps-HE) breaks down into dimers and loses its ability to bind DNA upon removal of divalent metals, whereas removal of metals from full-length Dps-1 has no effect on oligomeric state. These findings suggest that the N-terminal metal site is needed for
proper assembly, but once the protein oligomerizes to a dodecamer, metals are no longer required to maintain the dodecameric state.

These results suggest that the role of Dps-1 might be to organize genomic DNA while the role of Dps-2 might be to provide protection against incoming ROS.
CHAPTER 1: INTRODUCTION

Reactive Oxygen Species

Reactive oxygen species (ROS) are produced naturally by many organisms in multiple cellular processes. As electrons are passed down the electron transport chain, there is a chance that the electrons will leak from the chain. If those electrons interact with oxygen, ROS will form. In some cases, ROS can be beneficial for organisms and is required for the survival of the organisms. For example, phagocytes cells (such as macrophages and neutrophils) release ROS to kill invading pathogens (Hoppe & Swanson 2003; Cathcart 2004). However, when the concentration of ROS is higher than the cell defense capability, it can put the organism in a condition called oxidative stress. This condition usually occurs as a consequence of external stresses such as radiation, which can transfer energy to electrons of macromolecules to excite those electrons. The excited electrons can react with O$_2$ to form ROS (De Iuliis et al. 2009; Cabiscol et al. 2000). Some common ROS include hydrogen peroxide (H$_2$O$_2$), superoxide anion (O$^2-$) and hydroxyl radical (•OH) (Imlay 2003).

Due to its negative charge, O$^2-$ repels the negative charge on the phosphate backbone of DNA. Instead, it targets mainly the positively charged iron sulfur clusters of certain enzymes. For example, aconitase (an enzyme that participates in the cell respiration pathway) has an [4Fe-4S]$^{2+}$ cluster in its active form. O$^2-$ can oxidize that cluster to [4Fe-4S]$^{3+}$ which will get converted to [3Fe-4S]$^{1+}$. The loss of the iron will inactivate the enzyme and that will interfere with the respiration pathway. The released iron can in turn react with H$_2$O$_2$ to generate additional ROS (Imlay 2003).

H$_2$O$_2$ generally doesn’t cause significant damage to the cells but it can be converted into a more toxic form. In the presence of a transition metal such as Fe(II), it can be converted into
• OH via the fenton reaction. • OH is an extremely unstable species due to an incomplete valence shell. It can create lesions in DNA by taking electrons from the sugars and bases. Multiple lesions within the DNA can inactivate essential genes and kill the organisms. • OH can abstract electrons from proteins, causing the proteins to become a radical. The radical protein can then abstract electrons from other proteins, creating a series of protein crosslinking and inactivation. • OH can also interact with lipid to cause lipid peroxidation. This can change the membrane structure, alter movement of molecules across the membrane and negatively affect membrane bound proteins (Cabiscol et al. 2000; Imlay 2003).

**Survival Mechanisms of Microorganisms under Oxidative Stress Conditions**

The presence of excess ROS can damage the cells but it can also activate a number of regulons in various microorganisms that can help them survive. Some of these regulons include SoxRS, OxyR and PerR. The SoxR protein contains a [2Fe-2S]$^{+}$ cluster in its inactive form. In the presence of O$_{2}^{-}$, that cluster gets oxidized to an active [2Fe-2S]$^{2+}$ form. In this oxidation state, SoxR can activate SoxS, which will then activate the genes in its regulon. Some of the genes in the SoxRS regulon encode proteins that can help defend against ROS. These proteins include superoxide dismutase which can convert O$_{2}^{-}$ to H$_{2}$O$_{2}$ and O$_{2}$ (Imlay 2008; Gu & Imlay 2011) and endonuclease IV, which participates in DNA repair (Ramotar & Demple 1996; Imlay 2008). While superoxide anion was originally thought to bring about SoxR oxidation, more recent evidence points to redox-cycling drugs as the agents that are sensed by SoxR (Gu & Imlay 2011).

OxyR has a cysteine residue that can be oxidized by H$_{2}$O$_{2}$. Upon oxidation of this cysteine residue, OxyR undergoes a conformational change that allows it to bind to the -35 region of the genes in its regulon and enhance their transcription. Catalase, an important enzyme
that can break down H$_2$O$_2$ into H$_2$O and O$_2$, is upregulated by OxyR in *E. coli*. As the level of H$_2$O$_2$ declines, glutaredoxin (a protein that is upregulated by OxyR) can reduce the cysteine and inactivate OxyR. This negative feedback helps turn off the regulon when it is no longer needed (Imlay 2008).

In other bacteria such as *Bacillus subtilis*, H$_2$O$_2$ can inactivate PerR. The PerR protein is a repressor of genes in its regulon and many of those genes are analogs of those in the OxyR regulon (Imlay 2008). PerR has two metal binding sites. The first site binds zinc (Zn$^{2+}$) and is coordinated by four cysteine residues while the second site is coordinated by two histidine residues that can bind either manganese (Mn$^{2+}$) or iron (Fe$^{2+}$). When Mn$^{2+}$ is present at the second site, the protein is insensitive to H$_2$O$_2$. On the other hand, if Fe$^{2+}$ is bound at the site, the histidine residues will get oxidized by H$_2$O$_2$. This will lead to a conformational change in PerR, which will cause it to lose its DNA binding abilities. As a result, PerR can no longer repress the transcription of the genes in its regulon which will lead to derepression and activation of those genes (Lee 2006 & Helmann 2006). In addition to ROS-mediated regulation of gene activity as a means to combat oxidative stress, ROS may also be inactivated by reacting with antioxidants. And an important contributor to managing oxidative stress in eubacteria is Dps, as discussed below.

**Deinococcus radiodurans**

During the 1950s, the bacterium *Deinococcus radiodurans* (*D. radiodurans*) was discovered from a can of meat that had been sterilized with a high amount of radiation (Anderson et al.). It was first called *Micrococcus radiodurans* because of the similarity in the morphology with the genus *Micrococcus*. However, later analysis of the ribosomal RNA sequences revealed that *D. radiodurans* is more similar to the genus *Thermus* than *Micrococcus*. *Deinococcus* is
still classified as a separate genus because it is considered a mesophile (grows best at 30° C) and not a thermophile like *Thermus* species (Battista 1997; Makarova et al. 2001; Cox & Battista 2005).

*D. radiodurans* is a red-pigmented, non-motile, round-shaped, sporeless bacterium that often appears as clusters of cocci. It can be found in various environments ranging from animal feces and soil to desiccated, nutrient poor environments. Although *D. radiodurans* is a Gram positive bacterium, it shares similarities with Gram negative bacteria. In addition to the inner membrane and thick peptidoglycan layer, *D. radiodurans* also has an outer membrane. However, the outer membrane lacks the lipid A component and *D. radiodurans* does not have the genes that are needed for the synthesis of the lipopolysaccharide layer that is found in a typical gram negative bacterium (Raez & Whitfield 2002; Bos et al. 2004).

The genome of *D. radiodurans* is divided into four parts: chromosome I, chromosome II, a megaplasmid and a plasmid. Chromosome I contains the most genetic information with more than 2.60Mbp. There are more than 3,000 genes that are found in this bacterium and the genome is rich in GC content (>60%) (Cox & Battista 2005).

*D. radiodurans* is well-known for its extreme resistance to numerous stress conditions such as desiccation, starvation and notably high level of radiation. *D. radiodurans* can endure up to around 5000 grays of radiation. This type of exposure can introduce thousands of double strand breaks, which would kill most organisms. For example, a human cannot tolerate more than 5 grays of radiation and *Escherichia coli* will die upon exposure to less than 20 grays of radiation. Second, it can live in an arid environment for over 5 years with 10% viability (Daly & Minton 1995; Cox & Battista 2005).
Potential Contributors of *Deinococcus radiodurans* Extreme Resistance

The exact mechanism for the resistance abilities of *D. radiodurans* is still unclear; however, there are some potential contributors to this extreme resistance. First, *D. radiodurans* has multiple copies of its genome. In a nutrient-rich environment, *D. radiodurans* possesses up to 10 copies of its genome. During stationary phase, that number is reduced to around 4 copies (Hanson 1978; Harsojo et al. 1981). This number is somewhat higher compared to other bacteria such as *E. coli*, which has only 4 to 5 copies of its genome during ideal growth. Having multiple genome copies may be beneficial because it can serve as backup genetic information for the genes. The chance that a damaging agent can inactivate the same gene on all copies of the genome is relatively slim. Secondly, the extra genome copies can serve as templates during the recombination repair process. It has been reported that genome multiplicity is required to fix double-stranded breaks in *E. coli* (Krasin & Hutchison 1977; Battista 1997; Makarova et al. 2001).

Next, *D. radiodurans* has a toroidal nucleoid structure that might contribute to the extreme resistance. It has been proposed that the condensed ring-like nucleoid structure of *D. radiodurans* might help protect its DNA by making certain parts of the DNA inaccessible to the damaging agents. Furthermore, by having the nucleoid in a condensed form, it may help in blocking the diffusion of DNA break ends from the fragmented genome; therefore, enhancing the repair process (Levin-Zaidman et al. 2003; Englander et al. 2004). However, later findings have suggested that the toroidal structure does not contribute to radioresistance (Zimmerman & Battista 2005)

*D. radiodurans* has an unusually high ratio of Mn/Fe. In *D. radiodurans*, manganese is localized throughout the cells while iron is concentrated near the area between dividing cells.
Unlike Fe, Mn does not participate in the Fenton reaction to produce the toxic hydroxyl radicals. Manganese can protect the cells against reactive oxygen species through different mechanisms. Mn(II) can oxidize O$_2^-$ to produce Mn(III) and H$_2$O$_2$ while Mn(III) can react with H$_2$O$_2$ to produce oxygen, H$_2$ and Mn(II); therefore, scavenging the ROS. The positive charge on Mn can neutralize the negatively charged phosphate backbone, which may aid in nucleoid condensation. Mn is needed for the activity of various enzymes that defend against ROS such as superoxide dismutase. Finally, Mn can form complexes with other small metabolites such as orthophosphate that may prevent protein oxidation by ROS (Daly et al. 2004; Zimmerman & Battista 2005; Daly et al. 2007; Daly et al. 2010).

There are several compounds within _D. radiodurans_ that can serve as antioxidant. For example, _D. radiodurans_ has carotenoid, which is a compound that is responsible for giving this bacterium its red color. Carotenoids can sequester or react with peroxyl radicals to prevent the ROS from causing damage. When the genes that are responsible for the synthesis of carotenoid are mutated, _D. radiodurans_ becomes more sensitive to ROS (Woodall et al. 1997; Paiva & Russell 1999; Tian et al. 2007). Lipoic acid is also suggested to be an antioxidant in this bacterium due to its ability to traps ROS and sequester transition metals to block formation of toxic ROS (Manda et al. 2007; Zhang et al. 2009; Slade & Radman 2011).

_D. radiodurans_ has several mechanisms to repair damaged DNA. Some prominent repair systems include base excision repair (BER), nucleotide excision repair (NER) and recombinational repair (RR). In BER, the DNA glycosylase will identify the incorrect base and remove it, leaving a baseless site. AP endonuclease will then break the phosphodiester bond at the 5’ end of the AP site which will leave a 5’-deoxyribose phosphate and generate a nick. DNA polymerase I will then bind to the nick, resynthesize the correct base and leave a nick. DNA
ligases will then seal the nick (Krwawicz et al. 2007; Zharkov 2008). NER follows a similar pattern except a whole region of bases is removed as opposed to a single base in BER. This repair system involves the uvrA endonuclease. In RR, the RecA protein is a major component in carrying out homologous recombination process (Battista 1997; Liu et al. 2007). These repair mechanisms, however, are not unique to *D. radiodurans* (Eisen & Hanawalt 1999).

There are multiple enzymes in *D. radiodurans* that may provide protection against ROS. *D. radiodurans* has catalase, superoxide dismutase and peroxidase to break down ROS (White et al. 1999; Makarova et al. 2001). Furthermore, it also has nucleoid associated proteins that can organize genomic DNA and regulate gene expression which may help the bacterium survives during stress conditions. One example of these proteins includes the Dps (DNA protection during starvation) proteins.

**Dps Proteins: General Properties**

Dps was first discovered in *E. coli* where it was shown to be extremely abundant during stationary phase and capable of providing efficient protection against DNase. Strains in which the *dps* gene is inactivated were shown to be sensitive to H$_2$O$_2$ (Almiron et al. 1992). Dps proteins can protect bacteria against ROS through different mechanisms. First, it can physically protect the DNA against the damaging agents via binding and condensing the DNA. Interaction with DNA has been suggested to be due to the positively charged N- or C-terminal extension since the surface of Dps is dominated by negatively charged residues and lacks a typical DNA binding motif. In *E. coli*, Dps fails to bind DNA when the three N-terminal lysine residues are mutated (Calhoun & Kwon 2010) while in *Mycobacterium smegmatis* Dps1, DNA binding is lost when the last 16 residues of the C-terminal extension is truncated. DNA binding is not the only mechanism in which Dps protects the cells because *Agrobacterium tumefaciens* Dps is unable to
bind DNA due to its N-terminal extension being disabled on the protein surface (Ceci et al. 2003). Several Dps proteins also have ferroxidase activity so they can oxidize Fe(II) to Fe(III) to block the Fenton reaction in forming \( \cdot \text{OH} \). Not only that, they can also store the Fe(III) and eventually form a mineralized core within the protein shell. To some extent, Dps can contribute to the scavenging of the ROS by sequestering Fe(II) and by using \( \text{H}_2\text{O}_2 \) as the preferred oxidant (Chiancome & Ceci 2010).

Dps is often referred to as a “ferritin-like” protein because it can serve as an iron storage protein with ferroxidase activity and form a spherical shell. However, looking at the structures of the two proteins, there are some notable differences. First, Dps can only oligomerize to a dodecamer while ferritin exists as a 24-mer. Each monomer of Dps is composed of a four helix bundle with a short helix between the second and third helix (Figure 1.1). On the other hand, ferritin has an additional fifth helix at the C-terminus but lacks the short helix. This might be the reason why Dps cannot go beyond a dodecamer. In ferritin, each subunit contains its own ferroxidase center while in Dps, the ferroxidase center is localized between the interface of two subunits. Although both proteins are capable of forming a mineralized core, Dps can only hold up to around 500 Fe while ferritin can store over 4000 Fe atoms (Dörner et al. 1985; Yang et al. 2000; Barnes et al. 2002; Olsen et al. 2005; Pulliainen et al. 2005).

**Regulation of Dps Proteins**

In *E. coli*, Dps has been shown to be upregulated by OxyR during exponential phase and Integration Host Factor (IHF) and \( \sigma^\varphi \) during stationary phase. In the presence of \( \text{H}_2\text{O}_2 \), OxyR is activated and it can bind upstream of the Dps promoter to induce the expression of Dps. When the cells are starved, the IHF consensus sequence is required by \( \sigma^\varphi \) to upregulate Dps (Altuvia et al. 1994). When the level of ROS has decreased, Dps is downregulated by FIS (factor for
Figure 1.1: **Dps-1 structure.** Structure of dodecameric Dps-1. Each monomer is represented by a different color. The bottom panel is a detail view of an individual Dps-1 monomer with the four helix bundle (Protein Bank Database 2F7N).
inversion structuring protein) and H-NS (histone-like nucleoid proteins). During exponential phase, FIS is present at very high concentration and can bind at the dps promoter. FIS does not prevent binding of \( \sigma^{70} \) to the promoter, but it causes \( \sigma^{70} \) to be trapped in a closed complex formation. In this scenario, \( \sigma^{70} \) and FIS are serving as a co-repressor to prevent \( \sigma^8 \) from binding to the promoter. H-NS, which is present at high level throughout exponential and stationary phase, inhibits dps transcription by binding to the dps promoter and directly blocking \( \sigma^{70} \) from accessing the site. It should be noted that H-NS has no effect on the binding of \( \sigma^8 \) to the promoter. It is still unknown how OxyR can override the effect of FIS during the induction of Dps, but there are a couple of possibilities. First, binding of OxyR might cause a conformational change in the DNA that lead to the release of FIS from the complex and a reduction in the affinity of FIS for the promoter. Alternatively, binding of OxyR might cause structural change in the FIS- \( \sigma^{70} \) complex which can trigger an open complex formation to initiate transcription of Dps (Grainger et al. 2008; Schnetz et al. 2008). Dps has also been shown to be regulated at the translational level. For example, the amount of Dps is reduced in E. coli by ClpXP and ClpAP during exponential phase. ClpXP and ClpAP are both proteases that can bind and degrade Dps when there is no oxidative stress present (Schmidt et al. 2009; Calhoun & Kwon 2010).

**Dps in Deinococcus radiodurans**

There are two Dps homologs in *D. radiodurans*: Dps-1 and Dps-2. Dps-1 is different from other Dps homologs due to its inability to protect DNA against against hydroxyl radical-mediated degradation, a characteristic due to the continuous release of iron from the protein core (Kim et al. 2006). Looking at the structure of Dps-1 (Figure 1.1, top panel), it contains twelve four helix bundle monomers (Figure 1.1, bottom panel) that are found in a typical Dps protein. The dodecameric form of Dps-1 is a very stable and preferred species that does not dissociate
once it is formed. It does not break down unless it is treated with harsh chemical agents such as HCl for an extended amount of time (Grove & Wilkinson 2005). Dps-1 also has an N-terminal extension that is much longer than other Dps homologs such as *E. coli* and *Listeria innocua* Dps. Removal of the first half of the extension causes the protein to lose its DNA binding ability while truncation of the entire extension prevents dodecamer formation (Bhattacharyya & Grove 2007).

There are four metal binding sites within Dps-1. The first metal site is localized at the end of the N-terminus and beginning of the first helix. This site is coordinated by two histidines, one aspartate and one glutamate. Retention of the region of the N-terminal extension containing this metal site allows the protein to exist as a dodecamer, which suggest that this site might play a role in the oligomerization of Dps-1. The second metal site is found at the ferroxidase center and is coordinated by residues from two monomers (one histidine from one subunit and an aspartate and a glutamate from another subunit) (Figure 1.2a) (Kim et al. 2006). Most Dps homologs have this conserved site with the exception of a few homologs such as *Lactococcus lactis* DpsB in which there is no ferroxidase activity (Stillman et al. 2005). The last two metal sites are considered 3-fold symmetrical channels because they are coordinated by residues from three subunits. The iron entry channel is composed of negatively charged residues such as aspartate (Figure 1.2b) that create an increase in the negative electrostatic potential gradient toward the inner region of the protein. The iron exit channel has a cap with a sulfate ion between the two arginine residues. Beyond the cap is a hydrophobic tube that is created by three phenylalanines and within the protein shell are three glutamate (Fig. 1.2c) that can form a salt bridge with the nearby arginine. When this metal site was mutated, the protein was able to protect the DNA against hydroxyl radical as evidenced by little or no DNA degradation in the presence of H$_2$O$_2$ and Fe(II). However, this mutation did not affect dodecameric assembly or
Figure 1.2: Metal sites of Dps-1. (a) Ferroxidase center (His83, Asp110, Glu114) (b) Iron Entry Channel (Asp 181) (c) Iron Exit Channel (Arg205). Colored spheres represent the metals at each site and residues coordinating the metal sites are represented as stick figures (Protein Bank Database 2F7N)
ferroxidase activity which suggests that this site is responsible for the release of Fe from the protein shell that can react with ROS to damage DNA (Grove & Wilkinson 2005; Kim et al. 2006).

Although Dps-1 cannot protect DNA against ROS, it is capable of binding DNA. The exact mechanism for DNA binding is not completely understood but there have been some significant findings. First, it has been shown that Dps-1 interacts with DNA via the major groove and that DNA binding requires metals. When the protein was treated with bipyridyl, a metal ion chelator, DNA binding is reduced dramatically. When the metal is added back to the bipyridyl treated Dps-1, DNA binding is restored to normal. Furthermore, DNA binding requires DNA substrate of at least 22 bp leading to the inference that Dps-1 needs at least two consecutive major grooves to stably interact with the DNA. When the 18 bp DNA substrate was used, no complex was formed. Dps-1 is incapable of bending DNA. The structure of Dps-1 shows that the dimers are positioned in such a way that prevents the DNA to wrap around the protein (Bhattacharyya & Grove 2007).

Interestingly, Dps-1 is the first Dps homolog shown to exist as a dimer (Grove & Wilkinson 2005). The only other Dps homolog that can exist in an oligomeric state beside a dodecamer is Mycobacterium smegmatis (M. smegmatis) Dps in which a trimer species was observed at low temperature (Gupta & Chatterji 2003). The dimeric state of Dps-1 forms at low salt concentration or when the entire N-terminus is removed. Dimeric Dps-1 has ferroxidase activity and this is consistent with the ferroxidase center being localized between two subunits. It should be noted that the dimer does not form a mineralized core like the dodecamer. The dimeric Dps-1 binds DNA at a much lower affinity compared to the dodecameric state, which can be only partly explained by there being only two N-terminal extensions to interact with the
DNA as opposed to twelve in the dodecamer (Grove & Wilkinson 2005; Bhattacharyya & Grove 2007). Unlike the dodecameric form, dimeric Dps-1 is capable of protecting DNA against hydroxyl radical. This is most likely due to the lack of the three fold symmetry iron exit channel in the dimer form (Grove 2005; Bhattacharyya 2007; Kim 2006).

The second Dps homolog in D. radiodurans, Dps-2, is also composed of a four-helix bundle that can oligomerize to a dodecamer. In addition to an N-terminal extension, Dps-2 also has a C-terminal extension. This C-terminal extension might participate in DNA binding due to the presence of multiple positively charged residues (Cuypers et al. 2007). It has been reported that the removal of the C-terminal extension leads to the loss of DNA binding in M. smegmatis Dps (Roy 2007; Chiancone & Ceci 2010). Notably, Dps-2 is the first Dps homolog that has been reported to contain a signal peptide at the N-terminus. The presence of the signal peptide suggests that its location is noncytoplasmic. The crystal structure of Dps-2 also reveals five metal binding sites in the presence of iron, but the function of the metal sites in Dps-2 is not well studied. The metal sites are localized at the ferroxidase center (one at the center and one near the center), N-terminus and C-terminus (one surface exposed and one internal) (Cuypers et al. 2007). Notably, Dps-2 has a conserved tryptophan at position 71 that has been reported to play a role in trapping free electrons to block radical byproducts of the ferroxidase center from releasing (Bellapadrona et al. 2010).

My findings show that the Dps-1 N-terminal metal site contributes significantly to DNA binding and is required for dodecameric assembly. The path of DNA binding of Dps-1 involves the interaction with the surface exposed Arg132 and there are six DNA binding sites per dodecamer; these findings lead to a predicted path for DNA on the surface of Dps-1. A
comparison between the two Dps homologs shows that the role of Dps-1 might be to organize genomic DNA while the role of Dps-2 might be to provide protection against incoming ROS.
CHAPTER 2: THE UNIQUE LOCATION OF DPS-2 IMPLIES DIFFERENT FUNCTIONS FOR THE TWO DPS HOMOLOGS IN DEINOCOCCUS RADIODURANS*

Introduction

*D. radiodurans* belongs to the family *Deinococcaceae*, which is closely related to the genus *Thermus*. The environment in which this bacterium is found varies significantly, ranging from nutrient-rich environments to unfavorable environments where they may experience long periods of desiccation (Bauermeister et al. 2011; Dartnell et al. 2010). It is an obligate aerobe that is well known for its extreme resistance to ionizing radiation and desiccation. Both conditions can lead to massive DNA damage that can be detrimental to the cells. The exact reason for how *D. radiodurans* can survive exposure to thousand grays of radiation is not clear; nevertheless, this bacterium has several notable characteristics that may contribute to this resistance, including a ring-like nucleoid that can aid in recombination repair processes, an efficient DNA repair mechanism or a high level of Mn(II) that has been proposed to protect cellular proteins (Mattimore & Battista 1996; Daly et al. 1996; Makarova et al. 2001; Liu et al. 2003; Levin-Zaidman et al. 2003; Tanaka et al. 2004; Zimmerman & Battista 2005; Daly et al. 2004; Daly et al. 2007).

During starvation, desiccation, or other stress-related conditions such as radiation exposure, the amount of reactive oxygen species (ROS) that are produced can exceed the cell tolerance level. In the presence of transition metals such as Fe^{2+}, the ROS H_{2}O_{2} can be converted to hydroxyl radicals via Fenton chemistry. Hydroxyl radicals are very unstable

because they are missing the final electron to have a complete valence shell; therefore, they can react with and damage various macromolecules (Yu & Anderson 1997; Cornelis et al. 2011; Daly 2006). Prokaryotes have several different proteins that can defend against ROS and one of those proteins is the non-specific DNA binding protein Dps (DNA protection during starvation).

In many bacteria, Dps proteins have been reported to protect DNA through two different mechanisms: by physically binding the DNA and providing a barrier against ROS and by oxidizing Fe$^{2+}$ to prevent hydroxyl radical formation. Dps proteins are often considered ferritin-like proteins due to the sphere-like structure and ability to store iron. However, Dps is smaller than ferritin in that it can only oligomerize to a dodecamer while ferritin exists as a 24-mer. The monomer in Dps consists of a four helix bundle, and some Dps homologs have an N- or C-terminal extension that has been reported to participate in oligomerization and DNA binding (Martinez & Kolter 1997; Grant et al. 1998; Zhao et al. 2002; Gupta & Chatterji 2003; Ceci et al. 2004; Stillman et al. 2005; Su et al. 2005; Bhattacharyya & Grove 2007; Ceci et al. 2007; Roy et al. 2007). Most Dps proteins contain a conserved ferroxidase center localized between the interfaces of two subunits that can oxidize Fe$^{2+}$ to Fe$^{3+}$. That Dps proteins have a strong preference for H$_2$O$_2$ over molecular oxygen as the oxidant is also important, as this preference allows Dps to detoxify Fe(II) and H$_2$O$_2$ simultaneously (Zhao et al. 2002; Su et al. 2005; Ilari et al. 2005; Schwartz et al. 2010).

Dps was first discovered in *Escherichia coli* and it has been studied in a number of bacteria after that. Having more than one Dps homolog is uncommon and is only seen in some bacteria such as *Lactococcus lactis*, and *D. radiodurans* (Ceci et al. 2004; Stillman et al. 2005; Almirón et al. 1992; White et al. 1999). There are two Dps proteins in *D. radiodurans*: Dps-1 (DR2263) and Dps-2 (DRB0092) (Grove & Wilkinson 2005). Both Dps homologs contain the
four helix bundle monomer found in a typical Dps. For Dps-1, there is a novel metal site toward the end of the N-terminus that has been shown to play an important role in oligomerization and DNA binding. Dps-2 has both N and C-terminal extensions with the C-terminus containing a surface exposed metal site that is made up by three subunits (Su et al. 2005; Kim et al. 2006; Cuypers et al. 2007; Romão et al. 2007; Nguyen et al. 2012). Here we show the role of the Dps-2 C-terminus in oligomeric assembly and the ability of Dps-2 to protect against ROS. Our results suggest that Dps-1 and Dps-2 have different functions in vivo. Dps-1 is unable to provide protection against ROS; therefore, its job might be to participate in organization of the nucleoid and iron homeostasis. Dps-2 is localized noncytoplasmically and protects the DNA efficiently against ROS, suggesting a role in protection against exogenous ROS.

Materials and Methods

Cloning, overexpression and purification of Dps-2 and CLess

Wild type Dps-2 and CLess were purified and characterized as previously described (Reon et al. 2012)

Determination of oligomeric state

Sedimentation equilibrium was used to determine oligomeric state. CLess Dps-2 was dialyzed overnight at 4°C against AU buffer (20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM MgCl$_2$). The reference and solution sectors of an analytical cell with a double-sector centerpiece were loaded with AU buffer on one side and CLess Dps-2 on the other side. The cell was spun at 20°C at 10,000 rpm using a Beckman Optima XL-A analytical ultracentrifuge equipped with an An-60 Ti rotor. The cell was scanned at 5-hr intervals at 294 nm with a step size of 0.004 cm until the system reached equilibrium. Equilibrium sedimentation data were analyzed using SEDFIT software and fit to an equation describing a single non-interacting protein species.
Oligomeric assembly was also confirmed by crosslinking with glutaraldehyde and by native gel electrophoresis, as described (Bhattacharyya & Grove 2007).

**Thermal stability**

Dps-2 and CLess Dps-2 Dps-1 were diluted to 10 µM in a buffer containing 50 mM Tris pH 8.0, 100 mM NaCl and 5X SYPRO Orange (Invitrogen). Fluorescence emission resulting from dye binding to unfolded protein was measured over a temperature range of 1°C to 90°C in 1 degree increments for 45 s using an Applied Biosystems 7500 Real-Time PCR System using the SYBR green filter. The correction for the total fluorescence yield was made using reactions without protein. The resulting data were exported to Sigma Plot 9 and the sigmoidal part of the curve was fit to a four parameter sigmoidal equation.

**β-galactosidase assay**

For analysis of Dps-1 and Dps-2 promoter activity in *D. radiodurans*, the promoters were PCR amplified from *D. radiodurans* genomic DNA and digested with BglII. The PCR products were cloned into pRADZ1 containing a promoter-less lacZ gene. Both promoters were cloned in both their forward and reverse directions. For Dps-1, the cloned promoter fragment spanned positions -87 to -10 relative to the start codon; for Dps-2, a 130 bp DNA fragment spanning positions -131 to -2 relative to the start codon was used. The HU promoter (DRA0065; 187 bp spanning positions -191 to -5 relative to the start codon) was similarly amplified and cloned into the BglIII site of pRADZ1. Integrity of all constructs was confirmed by sequencing.

*D. radiodurans* cells were transformed with reporter constructs using the previously described protocol except chloramphenicol was used in place of mitomycin (Earl et al. 2002). The transformed cells were grown until they reach exponential phase, and cultures were then treated with either 10 mM H₂O₂ or 10 mM Fe(NH₄)₂(SO₄)₂ for 60 min. Cells were harvested by
centrifuging 500 µl culture while measuring OD\textsubscript{600}. The cell pellets were resuspended in 500 µl Z-buffer (60 mM Na\textsubscript{2}HPO\textsubscript{4}, 10 mM KCl, 1 mM MgSO\textsubscript{4}, 50 mM β-mercaptoethanol) supplemented with lysozyme (25 µg/ml) and DNaseI (50 ng/ml). After incubation for 30 min at 37°C, 20 µl of toluene was added. After another 60 minutes incubation at 37°C, 300 µl of 4 mg/ml o-nitrophenyl β-D-galactopyranoside (ONPG) was added to each aliquot. The reaction was stopped by the addition of 500 µl of 1 M Na\textsubscript{2}CO\textsubscript{3}. Absorbances were measured at 420 and 550 nm and the Miller unit activity was measured using the equation: 
\[
[A_{420}-(1.75* A_{550})/(\text{rxn time (min)}* \text{volume} * \text{OD}_{600})]*1000.
\]

**Electrophoretic Mobility Shift Assay (EMSA)**

To remove divalent cations, proteins were incubated with 50 mM bipyridyl for 20 min at 4°C. The bipyridyl or metalbipyridyl complex was removed by dialysis against a high salt buffer (10 mM Tris-HCl, pH 8.0, 500 mM KCl, 5% (v/v) glycerol, 0.5 mM β-mercaptoethanol, and 0.2 mM PMSF) at 4°C for 2 h. The bipyridyl-treated proteins were incubated with 800 nM of MnCl\textsubscript{2} for 6 h. DNA binding was assessed in 10 µl reaction mixtures where 100 ng pGEM5 was incubated with 10 pmol protein in binding buffer (20 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.05% (w/v) Brij58, 10 µg/ml BSA, 5% glycerol) at room temperature for 1 h. Reactions were resolved on 1% agarose gels with 0.5X TBE buffer (45 mM Tris-borate, pH 8.3, 1mM EDTA) and stained with ethidium bromide. EMSA was also performed with 26 bp DNA as described, except that the binding buffer contained 50 mM NaCl as the monovalent salt (Grove & Wilkinson 2005).

**DNA protection**

To analyze the ability of Dps-1 and Dps-2 to protect DNA against ROS, 100 ng pGEM5 was incubated with 10 pmol protein in the presence of 20 mM Tris-HCl pH 7.5 and 400 mM KCl.
for half an hour at room temperature. After that, 10 mM H$_2$O$_2$ and 150 μM Fe(NH$_4$)$_2$(SO$_4$)$_2$ were added followed by a 10 minute incubation at room temperature. DNase I protection assay was carried out by incubating 100 ng pGEM5 10 with 10 pmol Dps-2 or CLess Dps-2 in 20 mM Tris-HCl and 400 mM KCl at room temperature for half an hour. One U DNase I, 0.5 mM MgCl$_2$, and 0.5 mM CaCl$_2$ were added to the reaction mixture and incubated for 5 min at room temperature. Reactions were terminated with 2 μl of stop solution (5% SDS and 15% glycerol) and plasmids were run on a 1% agarose gel in a 0.5X TBE buffer and stained with ethidium bromide. Reactions were performed at least three times.

**Compaction of the nucleoid**

*E. coli* BL21 cells containing a ΔHU were transformed with pET5a-dps1 or pET5A-DrHU. Transformants were grown in 10 ml of LB broth containing 50 μg/ml ampicillin at room temperature until they reach an O.D. of 0.2. In order to overexpress the proteins, 1 mM IPTG was added to the cells for one hour. Two μl of each induce cell culture were mixed with 2 μl of 50 mg/ml 4',6-diamidino-2-phenylindole (DAPI) and incubated for 5 minutes. The cells were visualized using a Leica DM IRE2 at 100X NA 1.4 objective. The Leica A4 filter cube was used to view the nucleoid.

**Dps-2 localization in D. radiodurans**

To determine cellular localization of Dps-2, *D. radiodurans* transformed with plasmid pRADpsspEGFP was grown in 10 ml TGY with 3 μg/ml chloramphenicol at 25°C until cultures reached an OD600 of 0.8. The pRADpsspEGFP construct was created as previously described (Reon et al. 2012). Cells were viewed using a Leica DM IRE2 under 100X NA 1.4 objective.
Results

The C-terminal extension is required for dodecameric assembly

The crystal structure of Dps-2 shows that this protein has a lengthy C-terminal extension and that the surface exposed metal site at this region is coordinated by residues from three subunits (Figure 2.1). To study the role of this C-terminus, a mutant protein (CLess) was created in which the entire C-terminus is removed.

The Dps-2 interfaces were analyzed using the Protein Interfaces, Surfaces, and Assemblies (PISA) server (Krissinel & Henrick 2007). The PISA algorithm uses a given structure to predict the most thermodynamically stable assemblies. The PISA algorithm predicts two protein-protein interfaces that contribute significantly to stability of the Dps-2 dodecamer, one of which is the dimer interface with a total of 24 hydrogen bonds and salt bridges (Table 2.1). Interaction between the C-terminal loop and an adjacent subunit at the three-fold Dps-like axis is also predicted to be very significant for stability of dodecameric Dps-2, while interactions between subunits at the N-terminal (ferritin-like) axes are predicted to contribute less. Metal binding at various sites is also predicted to be significant for stability of the assembly, including metals coordinated at the ferroxidase center, at the C-terminal loop, and at the three-fold N-terminal ferritin-like iron entry channels. The PISA analysis predicts that dodecameric assembly of CLess Dps-2 would be compromised, and that dodecameric Dps-2 is significantly more stable than isolated subunits.

Dps-1 exists almost exclusively as a dodecamer in solution and the dodecamer fails to migrate from the wells of a native gel (Grove & Wilkinson 2005). Similarly, oligomeric state of Dps-2 was assessed by native gel electrophoresis, in which Dps-2 fails to migrate from the well with no faster migrating species detected (Figure 2.2A). The migration of CLess Dps-2 (Lane 4)
Figure 2.1: **Dps-2 structure.** Structure of dodecameric Dps-2. Each monomer is represented by a different color. The bottom panel is a detail view of the iron exit channel coordinated by residues from the C-terminal extensions (black shaded region) of three monomers. Colored spheres represent the metals.
was compared with that of Dps-dn, a truncated derivative of Dps-1 previously shown to exist exclusively as a dimer (lane 1) (Bhattacharyya & Grove 2007). Considering the shared four-helix bundle folds and the lower Mw of Dps-dn (Mw 17,364 Da) as well as its lower pI of 4.7 compared to the pI of 5.3 for CLess Dps-2, dimeric Dps-dn should migrate significantly faster than dimeric CLess Dps-2; the faster migration of CLess Dps-2 is therefore consistent with its existence as a monomer. As this result was unexpected based on reports that other Dps proteins

**Table 2.1.** PISA analysis predicts several interfaces that contribute to assembly of Dps-2

HB + SB represents number of hydrogen bonds and salt bridges. CSS; Complex Formation Significance Score, an indicator of interface relevance to complex formation, with CSS = 1.000 implying an interface that is essential to stability of the assembly. In addition to the listed interfaces, contacts to a metal near Fe$^{2+}$ at the ferroxidase center is also predicted to contribute to complex stability. Metals at the ferroxidase center are coordinated by two subunits, generating two distinct interfaces. Metal coordinated at the C-terminal loop contacts an adjacent subunit, likewise generating two distinct interfaces. *Not a significant contribution. Calculations done using the PISA server at www.ebi.ac.uk/msd-srv/prot_int/pistart.html and PDB 2C6R, treating all ligands as free particles.

<table>
<thead>
<tr>
<th>Type of interaction</th>
<th>HB+SB</th>
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<tbody>
<tr>
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<td>1.000</td>
</tr>
<tr>
<td>C-terminal loop and adjacent subunit</td>
<td>8</td>
<td>1.000</td>
</tr>
<tr>
<td>Adjacent subunits at N-terminal three-fold axis</td>
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<td>0.070*</td>
</tr>
<tr>
<td>Metal at ferroxidase center/subunit 1</td>
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</tr>
<tr>
<td>Metal at ferroxidase center/subunit 2</td>
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<td>0.254</td>
</tr>
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<tr>
<td>Metal near C-terminal Dps-like three-fold axis</td>
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<tr>
<td>Metal at C-terminal loop/adjacent subunit</td>
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Figure 2.2: Oligomeric assembly of Dps-2. A. Native gel electrophoresis of Dps-dn (Mw 17 kDa, pI 4.7; lane 1), Dps-2 (lane 2), Dps-2 and CLess Dps-2 mixed together (lane 3), and CLess Dps-2 (Mw 23 kDa, pI 5.3; lane 4). B. Equilibrium sedimentation profile of CLess Dps-2. Lower panels shows the absorbance of 80 µM CLess Dps-2 as a function of the radial cell position. Data were fit to a model describing a single, non-associating species using the SEDFIT software. The upper panel shows the residuals to the fit, which were distributed randomly. C. Thermal stability of Dps-2 (open symbol) and CLess Dps-2 (closed symbol), measured as fluorescence emitted from SYPRO Orange upon binding to denatured protein as a function of temperature. D. Native gel electrophoresis. Lane 1 contains CLess Dps-2, lane 2 is CLess Dps-2 after bipyridyl treatment, and lane 3 bipyridyl-treated CLess Dps-2 with Mn$^{2+}$. Lanes 4-6 show dodecameric Dps-2, Dps-2 after bipyridyl-treatment, and bipyridyl-treated Dps-2 with Mn$^{2+}$, respectively.
form subassemblies corresponding to either dimers or trimers, we confirmed it using analytical ultracentrifugation (Figure 2.2B). Data were best fit to a model describing a single, non-associating protein species, yielding a molecular weight average of 21,881 Da for CLess Dps-2, consistent with a monomer. To assess the prediction from PISA analyses that dodecameric Dps-2 would be significantly more stable than an individual subunit, we compared thermal stability of Dps-2 and CLess Dps-2 using SYPRO Orange as a fluorescent reporter of protein unfolding (Figure 2.2C). Consistent with predictions, the melting temperature of Dps-2 is 68.1±0.0ºC, consistent with complete assembly (and comparable with the melting temperature for dodecameric Dps-1 (Grove & Wilkinson 2005), while that for CLess Dps-2 is 41.2±0.2ºC. Glutaraldehyde crosslinking also failed to detect any species other than the monomer for CLess Dps-2, while crosslinking of Dps-2 was very efficient, yielding no species smaller than a hexamer (data not shown). Glutaraldehyde reacts primarily with lysine to crosslink individual subunits. Inspection of the Dps-2 structure shows that most lysines extend from the ends of the four-helix bundle near the three-fold axis formed by three adjoining N-termini and that one lysine is located at the dimer interface, predicting crosslinking of dimeric species should they occur in solution. The failure of CLess Dps-2 to self-assemble is unexpected given that Dps homologs generally exist as dimers, trimers, or dodecamers. Taken together, these results indicate that Dps-2 exists primarily as a dodecamer in solution, and that the C-terminal extension is required for oligomeric assembly.

Considering that the ferroxidase center lies at the dimer interface, we considered the possibility that assembly of a CLess Dps-2 dimer requires the addition of divalent metal. As shown in Figure 2.2D, incubation of CLess Dps-2 with bipyridyl, which chelates divalent metal, has no effect on protein migration in a native gel (compare lanes 1 and 2). However, addition of
Mn\(^{2+}\) results in a marked reduction in electrophoretic mobility (lane 3). Since analytical ultracentrifugation suggests that CLess Dps-2 exists as a monomer, such change in electrophoretic mobility would be consistent with metal-dependent dimerization or slower migration of monomeric CLess Dps-2 due to binding of positively charged metal ions. By contrast, dodecameric Dps-2 fails to migrate from the well (lane 4), and no effect of either bipyridyl-treatment or metal-addition is evident (lanes 5 and 6).

**Ferroxidation by Dps-2**

A characteristic of essentially all Dps proteins is the ability to protect against ROS by oxidizing Fe\(^{2+}\) and sequestering it within its mineralized iron core. Dps-2 conserves all the residues that make up the ferroxidase center and would be predicted to exhibit ferroxidase activity. Both dodecameric and CLess Dps-2 were incubated with freshly prepared Fe(NH\(_4\))\(_2\)(SO\(_4\))\(_2\), and the kinetics of Fe\(^{2+}\) oxidation in molecular oxygen were measured at 310 nm (Figure 2.3). The significant change in absorbance shows the rapid production of Fe\(^{3+}\) by dodecameric Dps-2. Control reactions measuring Fe\(^{2+}\) oxidation in absence of protein showed no appreciable level of Fe\(^{3+}\) production. Notably, CLess Dps-2 did not exhibit significant ferroxidase activity, consistent with assembly of the ferroxidase center requiring dimer formation. As shown in the inset to Figure 2.3, the presence of Fe\(^{2+}\) or Fe\(^{3+}\) does not cause a change in electrophoretic mobility, supporting the conclusion that divalent metal is required for dimer assembly; Fe\(^{2+}\) bound at the ferroxidase center at the dimer interface would be oxidized to Fe\(^{3+}\), causing disassembly of the dimer. That no ferroxidation is observed for CLess suggests that any equilibrium between monomer and Fe\(^{2+}\)-mediated dimer lies in favor of the monomer or that dimerization is a slow process. Consistent with the latter interpretation, incubation with divalent metal (Mn\(^{2+}\)) for <30 min (the time scale of the ferroxidase experiment) fails to elicit a
change in electrophoretic mobility (data not shown). The failure of iron to elicit a change in electrophoretic mobility further indicates that such change in migration is not due simply to non-specific binding of positively charged metal ions.

DNA binding and protection

A second characteristic of several Dps proteins that contributes to protection of DNA from ROS is their physical association with DNA. To assess whether Dps-2 can bind DNA, agarose gel retardation assays were performed. Considering the effect of divalent metal on dimerization of CLess Dps-2, we compared DNA binding by bipyridyl-treated protein and protein to which Mn$^{2+}$ was added following bipyridyl-treatment (Figure 2.4A). As previously reported, chelation of divalent metal from dodecameric Dps-1 destroys its ability to bind DNA,

Figure 2.3: Iron oxidation by Dps-2. Dps-2 and CLess Dps-2 (0.2 mg/ml) were incubated with 50 µM Fe(NH$_4$)$_2$(SO$_4$)$_2$ in a 20 mM MOPS buffer, and the kinetics of iron oxidation in air were measured at 310 nm. Inset shows native gel of bipyridyl-treated CLess Dps-2 (lane 1) and CLess Dps-2 with Fe$^{2+}$, Fe$^{3+}$, or Mn$^{2+}$
Figure 2.4: DNA binding and protection by Dps-2. A. Binding of Dps homologs to plasmid DNA. Lane 1 is DNA only, lanes 2-3 DNA and 10 pmol Dps-1, lanes 3-4 DNA and 10 pmol CLess Dps-2, and lanes 5-6 DNA and 10 pmol Dps-2. Reactions in lanes 2, 4, and 6 contained bipyridyl-treated protein, and reactions in lanes 3, 5, and 7 bipyridyl-treated protein with Mn2+. B. Protection from hydroxyl-mediated DNA cleavage. Lane 1 shows DNA and lane 2 shows DNA degraded by hydroxyl radical. Reactions in lanes 3 and 5 contain DNA and 10 pmol Dps-1 and CLess Dps-2, respectively, and reactions in lanes 4 and 6 contain DNA and Dps-1 and CLess Dps-2, respectively, incubated with H2O2 and Fe2+. C. Protection from DNaseI-mediated DNA cleavage. Reaction in lane 1 contains DNA and reactions in lanes 2-3 DNA incubated with DNaseI for 10 and 5 min, respectively. Reactions in lanes 3-4 contain DNA and 10 pmol CLess Dps-2 incubated with DNaseI for 10 and 5 min, respectively, and reactions in lanes 6-7 DNA and 10 pmol Dps-2 incubated with DNaseI for 10 and 5 min, respectively.
while addition of Mn$^{2+}$ results in protein-DNA complexes that do not migrate from the well (lanes 2 and 3); for Dps-1, this metal-dependence of DNA binding is associated with occupancy of the metal-site within the N-terminal extension (Bhattacharyya & Grove 2007). For CLess Dps-2, no DNA binding is seen unless Mn$^{2+}$ is present (compare lanes 4 and 5); this suggests that dimerization of CLess Dps-2 is necessary for DNA binding. This result also indicates that while the C-terminal extension may contribute to DNA binding, it is not essential. Surprisingly, bipyridyl-treatment of dodecameric Dps-2 also eliminates DNA binding, while Mn$^{2+}$ addition restores complex formation (lanes 6 and 7). Since the ability of CLess Dps-2 to bind DNA implies that its N-terminal extension may participate in this interaction, we surmise that the inability of bipyridyl-treated dodecameric Dps-2 to bind DNA may be associated with interactions between the N- and C-terminal extensions that do not occur when the C-terminal metal-site is occupied and the C-terminal extension is properly folded. That complexes with dodecameric Dps-2 migrate much faster than those with Dps-1 may reflect that individual Dps-1 protomers are more prone to association with multiple DNA sites, (Nguyen et al. 2012) thus creating large networks of DNA and protein that cannot migrate from the well.

In order to determine if Dps-2 can protect DNA from ROS and DNaseI, in vitro DNA protection assays were conducted (Figure 2.4B-C). The incubation of pGEM with H$_2$O$_2$ and Fe$^{2+}$ leads to DNA degradation in the absence of either Dps-2 variant (Figure 2.4B lane 2). Both Dps-2 and CLess Dps-2, however, display a considerable level of DNA protection from ROS, with only a modest increase in nicked DNA species (lanes 4 and 6). Similarly, incubation of DNA with DNaseI resulted in complete DNA degradation (Figure 2.4C, lanes 2 and 3). CLess Dps-2 offers only modest protection, with the DNA being nearly completely degraded after a 10 min incubation with DNaseI (lane 4) and only modest levels of nicked and linear species visible after
5 min incubation (lane 5). Full-length Dps-2 is more effective, with nicked and linear DNA species detectable even after a 10 min incubation with DNaseI (lane 6). By comparison, Dps-1 affords efficient protection against DNaseI-mediated cleavage, but cannot protect against ROS-mediated degradation, an effect ascribed to its inability to sequester iron stably (Kim et al. 2006). The results suggest that Dps-1 and Dps-2 provide different levels of DNA protection, and only Dps-2 can provide efficient protection for DNA against ROS.

**Compaction of the nucleoid by Dps-1**

In *Deinococcus radiodurans*, the deletion of Dps-1 or Dps-2 does not have a major impact on the morphology of the nucleoid. On the other hand, the HU protein was shown to be essential in subaining the condensed nucleoid (Nguyen et al. 2009). In order to see if Dps-1 and Dps-2 are capable of condensing genomic DNA, DAPI experiments were conducted to view staining of *E. coli* cells expressing Dps-1, Dps-2 and HU. Overexpression of Dps-1 in the ΔHU strain leads to a major compaction of the nucleoid (Figure 2.5, middle panel). Cells expressing the HU protein does not show this level of compaction, but was able to restore the cell morphology to normal shape (Figure 2.5, bottom panel). Cells that are not expressing either Dps-1 or HU appear abnormal in that the nucleoid is elongating (Figure 2.5, top panel). When Dps-2 was overexpressed in WT cells, there was little or no effect on the nucleoid morphology (Reon et al. 2012)

**In vivo localization of Dps-2**

To determine the cellular localization in *D. radiodurans*, we fused eGFP directly to the Dps-2 signal peptide with the construct under control of the Dps-2 promoter. Further, to alleviate issues with slow folding of GFP resulting in poor fluorescence yield, (Andrews et al. 2008) cultures were grown at room temperature to reduce the growth rate. As shown in Figure
2.6, fluorescence may be seen around the perimeter of the cells, particularly visible at the junctions between cells within each tetrad. When cultures were grown at room temperature, the vast majority of cells express the reporter gene, while growth at 30°C does not yield significant expression of GFP. The observed pattern of GFP expression is again consistent with a non-cytoplasmic localization.

**Activity of Dps-1 and Dps-2 gene promoters in response to ROS**

Dps-1 is unusual in its inability to protect DNA from ROS (Kim et al. 2006; Bhattacharyya & Grove 2007) while Dps-2 conserves this characteristic of Dps homologs. We

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**Figure 2.5: Nucleoid Compaction in vivo:** E. coli BL21(DE3)pLyS containing a ΔHU expressing Dps-1 (middle panel) and *D. radiodurans* HU (bottom panel). Top panel is the control in which the cells are not expressing either protein. Images were viewed by DAPI staining. Overlay images combine both DIC and DAPI images.
Figure 2.6: *In vivo* localization of Dps-2. A. DIC image of wild type *D. radiodurans* (left panel) and cells expressing EGFP fused to the Dps-2 signal peptide under control of the Dps-2 promoter (right panel). B. GFP fluorescence of wild type and recombinant cells. C. Overlay of DIC and green fluorescence. D. Expanded view of *D. radiodurans* expressing EGFP fused to the Dps-2 signal peptide.
Figure 2.7: β-galactosidase activity reporting on promoter activity in response to H$_2$O$_2$ or Fe$^{3+}$. A-B. β-galactosidase activity of Dps-1 promoter in its forward and reverse orientations in untreated (white bars), H$_2$O$_2$-treated (gray bars) and iron-treated (hatched bars) cells. C-D. β-galactosidase activity of Dps-2 promoter in its forward and reverse orientations in untreated (white bars), H$_2$O$_2$-treated (gray bars) and iron-treated (hatched bars) cells. E. β-galactosidase activity of HU promoter in untreated (white bars) and H$_2$O$_2$-treated (gray bars) cells. Results are means + S.D. of three replicates.
therefore wondered if activity of the respective gene promoters is modulated by ROS. Reporter constructs were used in which lacZ is under control of the respective promoters in either their forward or reverse directions, and β-galactosidase activity was measured in response to H₂O₂ or Fe²⁺ (Figure 2.7). As shown in Figure 2.7A, the Dps-1 promoter does not respond to the addition of either H₂O₂ or Fe²⁺ to the media. In contrast, activity of the Dps-2 promoter is enhanced under both conditions; this enhancement is observed both during exponential and stationary phase growth (Figure 2.7C). The lower β-galactosidase activity measured during stationary phase can be attributed to a lower plasmid copy number; the plasmid used for these assays has been previously shown to exist in a copy number equivalent to the genome content, which is lower in stationary phase (Meima & Lidstrom 2000; Meima et al. 2001). As expected, little β-galactosidase is seen when promoters are in their reverse orientations (Figure 2.7B, D). As Dps-2 is expected to inactivate H₂O₂, we also monitored the response of the HU promoter as a control; as shown in Figure 2.7E, HU promoter activity is increased on addition of H₂O₂, indicating that H₂O₂ does enter the cytosol.

**Discussion**

**Assembly of Dps-2**

Extensions beyond the four-helix bundle monomer have been shown to participate in dodecameric assembly; for *D. radiodurans* Dps-1, dodecameric assembly is abolished on deletion of its N-terminal extension, and assembly of *M. smegmatis* Dps1 requires its C-terminal extension (Bhattacharyya & Grove 2007; Roy et al. 2007). Similarly, removal of the C-terminal extension from *D. radiodurans* Dps-2 results in a species that is no longer able to assemble into a dodecamer. More surprising is the observation that CLess Dps-2 also fails to form a dimer; the C-terminal extension does not make contact with the other monomer within a dimer, although the possibility exists for contacts with the N-terminal extension since this segment is not visible
in the structure. However, our data suggest that the presence of divalent metal induces dimerization of CLess Dps-2, as evidenced by its ability to bind DNA as well as by its migration in native gel electrophoresis (Figure 2.2). The alternative interpretation of CLess Dps-2 migration in native gels is that binding of positively charged metals reduce electrophoresis mobility of the monomer. Gel filtration experiments would be able to distinguish between these possibilities. The structure of Dps-2 was solved in an “apo” form as well as an iron-soaked form, and comparison of the two structures yields insights that are consistent with the observed metal-dependence of dimer formation. In apo-Dps-2, two of five distinct metal-sites are occupied, one of which is the metal-site at the C-terminus, which in the apo structure is filled with a metal other than iron (and speculated to be calcium) (Cuypers et al. 2007). Thus, occupancy of this metal-site may stabilize the structure of the C-terminal extension and promote oligomerization; similarly, occupancy of the N-terminal metal site of D. radiodurans Dps-1 was shown to be required for dodecameric assembly of Dps-1 (Bhattacharyya & Grove 2007). By contrast, apo-Dps-2 does not have metals bound at the ferroxidase center that lies at the interface between the two monomers that constitute the dimer (Figure 2.8). In the structure of iron-soaked Dps-2, ligands to iron at the ferroxidase center are His70 from one subunit and Asp97 and Gln101 from the other; by comparison, Asp97 and Gln101 form salt bridges with Lys77 and His174/Lys178, respectively, in apo-Dps-2; these salt bridges are disrupted on iron coordination. Further, a second metal site near the ferroxidase center is also occupied only in iron-soaked Dps-2, and this metal is loosely coordinated to the protein only by interaction with Asp98 (Cuypers et al. 2007). Thus, occupancy of metal sites at the dimer interface may not be required for oligomeric assembly, provided an intact C-terminal extension. In contrast, absence of the C-terminus appears to impose a requirement for metal-binding at the ferroxidase center for dimer
formation. Whether metal-bound dimers are a required intermediate in the assembly of dodecameric Dps-2, or if contacts mediated by the C-terminal extensions suffice, remains to be determined.

DNA binding and protection

Most frequently, the ability of Dps homologs to bind DNA is associated with an N-terminal extension of varying length. Consequently, Dps proteins lacking such an extension generally do not bind DNA, with the exception of HP-NAP from *Helicobacter pylori*, which has an unusual positive surface charge not seen in other members of the Dps family (Ceco et al. 2007). Another exception is Dps1 from *M. smegmatis*, which has a C-terminal extension that is necessary for DNA binding (Roy et al. 2007). The crystal structure of Dps-2 identifies a unique metal-bound C-terminal extension as well as an N-terminal extension that is not visible in the structure, most likely due to flexibility (Cuypers et al. 2007). Considering the content of positively charged residues in the C-terminal extension, this segment would be expected to contribute to DNA binding. It is, however, clear from the ability of CLess Dps-2 to engage DNA that while this extension may contribute to DNA binding, it is not essential. Dimeric Dps-1 binds DNA with the helix axis parallel to the dimer interface, allowing N-terminal extensions to engage consecutive DNA major grooves (Bhattacharyya & Grove 2007; Nguyen et al. 2012). For Dps-2, DNA binding is only observed in presence of divalent metal, which is required for dimerization (Figure 2.4). We therefore favor the interpretation that Dps-2, like Dps-1, binds DNA such that the helix axis is parallel to the dimer interface, with N-terminal extensions from each monomer engaging the DNA (Figure 2.8) (Bhattacharyya & Grove 2007; Nguyen et al. 2012). Consistent with this interpretation, the linker connecting the short BC helix to helix C of the four-helix bundle features an arginine that is surface-exposed and in the path of DNA aligned
with the long axis of the Dps-2 dimer; in Dps-1, a similarly positioned arginine within the BC helix (Arg132) has been shown to contribute to DNA binding (Nguyen et al. 2012).

Bipyridyl-treated dodecameric Dps-2 no longer binds DNA (Figure 2.4A). A similar phenomenon was observed for Dps-1; however, for Dps-1 it is removal of metal from its N-terminal extension that is responsible for the loss of DNA binding, as the N-termini are essential for interaction with DNA (Bhattacharyya & Grove 2007; Nguyen et al. 2012). For Dps-2, the N-termini are not visible in the structure, suggesting that they may be unstructured or flexible. Since the C-terminal extension is dispensable for DNA binding, a possible explanation for the failure of bipyridyl-treated Dps-2 to bind DNA is a loss of metal from the C-terminus that causes the now unstructured C-terminal extensions to interfere with DNA-binding by the N-termini, as the N- and C-terminal extensions of each monomer within the dimer are closely apposed (Figure 2.8).

**Figure 2.8: The Dps-2 dimer.** The two monomers are shown in light and dark blue. Metals at the ferroxidase center and at the C-terminus are indicated, along with the N- and C-termini of the protein (the structure does not include residues preceding the four-helix bundle). A. Top view of the dimer with the ferroxidase center at the dimer interface. B. End-on view of the dimer (90° rotation relative to panel A) with arginines near the short BC helices identified and an end-on view of a DNA duplex.
Although Dps-1 binds DNA with high affinity, it fails to protect it from ROS, a characteristic that is most likely caused by the continuous release of iron from the novel exit channel (Bhattacharyya & Grove 2007; Kim et al. 2006). On the other hand, high expression of Dps-1 led to a compacted nucleoid in *E. coli*, which may be a major mechanism for protecting DNA from damaging agents such as ROS (Reon et al. 2012; Frenkiel-Krispin *et al.* 2001). *In vivo*, deletion of Dps-1 was reported not to cause significant changes in nucleoid morphology as evidenced by DAPI staining of cells in exponential or early stationary phase, however, it is conceivable that other nucleoid-associated proteins such as HU may be upregulated to compensate for the loss of Dps-1 (Nguyen *et al.* 2009). Consistent with a role for *D. radiodurans* HU in nucleoid structuring, its deletion results in a disperse nucleoid (Nguyen *et al.* 2009). Unlike Dps-1, however, its overexpression in *E. coli* does not result in visible DNA compaction (Figure 2.5, bottom panel).

In contrast, Dps-2 is able to protect DNA from ROS-mediated cleavage. The remarkable differences in the ability of Dps-1 and Dps-2 to protect DNA suggest different functional roles *in vivo*. The primary role of Dps-1 may be involve in iron homeostasis or nucleoid organization, while the main function of Dps-2 may be to protect against exogenously derived ROS with the potential to damage cellular macromolecules. Consistent with this interpretation, we find that only the Dps-2 promoter is sensitive to ROS (Figure 2.7). The inability of H$_2$O$_2$ to upregulate the Dps-1 promoter was previously reported and suggested to be due to repression by OxyR (Chen *et al.* 2008). This study did not reveal upregulation of the Dps-2 promoter either, as measured by qRT-PCR, a difference perhaps due to different times of exposure to H$_2$O$_2$. 
**Dps-2 is non-cytoplasmically localized**

The N-terminal signal peptide of Dps-2 directs the EGFP reporter to a non-cytoplasmic localization in *D. radiodurans*, with fluorescence particularly visible at the interface between cells within a tetrad (Figure 2.6). The non-cytoplasmic localization of Dps-2 in *D. radiodurans* is intriguing in light of the reported sequestration of Fe$^{2+}$ in *D. radiodurans*; (Daly et al. 2007) while Mn$^{2+}$, which accumulates to mM concentrations is globally distributed, Fe is largely sequestered outside the cytoplasm in a region overlapping the septum between dividing cells. Such distribution of Fe would rationalize both the inability of cytoplasmically localized Dps-1 to protect DNA from •OH derived from Fenton chemistry and the non-cytoplasmic localization of Dps-2. Having a non-cytoplasmically localized Dps protein could afford *D. radiodurans* novel attributes. Importantly, Dps-2 may serve as a first line of defense against exogenously derived ROS. And while DNA binding may well be vestigial, we do note that *D. radiodurans* is naturally transformable (Tigari & Moseley 1980) raising the possibility that Dps-2 may participate in the uptake of foreign DNA. Only DpsA from *Synechococcus sp.* has been shown to have another than cytoplasmic localization, partitioning to the thylakoid membrane where a role in metal transport was proposed (Durham & Bullerjahn 2002).

The discovery that the iron in *D. radiodurans* is not located in the cytoplasm, but rather in the region between dividing cells brings about some interesting questions about iron homeostasis within *Deinococcus*. Firstly, it is important to note that in typical bacteria, iron is spread out in a relatively diffuse manner throughout the cytoplasm, whereas the iron in *D. radiodurans* is more concentrated, giving it the potential to cause a great deal of local oxidative damage. So, while it is undoubtedly beneficial to have Fenton reactive Fe$^{2+}$ away from DNA and critical cytoplasmic proteins, the Fe$^{2+}$ must be sequestered in some capacity, so as to prevent...
damage to membrane proteins and other periplasmic components. *D. radiodurans* does not encode genes corresponding to the two major iron storage proteins in bacteria, ferritin or bacterioferritin. However, it does encode the two Dps proteins. Furthermore, the presence of the signal peptide on Dps2 makes it the prime candidate for serving this iron-sequestering role in the cell.
CHAPTER 3: ON THE STOICHIOMETRY OF DEINOCOCCUS RADIODURANS DPS-1 BINDING TO DUPLEX DNA*

Introduction

Deinococcus radiodurans has a remarkable ability to withstand challenging environments in which it is exposed to nutritional stress, ionizing radiation, oxidation, or other agents with the potential to damage cellular components (Cox et al. 2010). Much emphasis has been placed on understanding how *D. radiodurans* repairs DNA breaks that would otherwise constitute irreversible and hence lethal damage to most microorganisms. Also, the existence of mechanisms that protect *D. radiodurans* from induced DNA damage have been proposed, including its toroidal nucleoid structure and its high intracellular ratio of manganese to iron (Levin-Zaidman et al. 2003; Frenkel-Krispin et al. 2004; Zimmerman & Battista 2005; Daly et al. 2007; Fredrickson et al. 2008; Slade & Radman 2011).

DNA protection during starvation (Dps) proteins constitute another mechanism for preventing DNA damage. Since the discovery of Dps in *Escherichia coli*, where it is upregulated during stationary phase and in response to stress, Dps proteins from many bacterial species have been characterized (Almirón et al. 1992; Bozzi et al. 1996; Martinez & Kolter 1997; Papinutto et al. 2002; Zhao et al. 2002; Halsey et al. 2004; Liu et al. 2006; Ceci et al. 2007). A distinctive feature of Dps proteins is to prevent hydroxyl radical formation by ferroxidation and sequestration of iron, and several also bind non-specifically to DNA (Ilari et al. 2002; Zhao et al. 2002; Pulliainen et al. 2003). DNA binding is usually associated with N- or C-terminal

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extensions preceding the conserved four-helix bundle subunits that assemble into a dodecameric sphere, the center of which harbors the distinctive iron core (Stillman et al. 2005; Ceci et al. 2007; Bhattacharyya & Grove 2007).

Dps-1 is one of two homologs encoded by *D. radiodurans* (Bhattacharyya & Grove 2007; Grove & Wilkinson 2007). The exact function of Dps-1 is still not known since it does not appear to sequester iron sufficiently avidly to avoid its spontaneous release from the protein core and subsequent participation in Fenton chemistry (Grove & Wilkinson 2005; Kim et al. 2006). Its depletion also does not result in overt changes in *D. radiodurans* nucleoid structure (Nguyen et al. 2001). Although a dimeric form of Dps-1 may be observed *in vitro*, the dodecameric assembly is highly favored (Grove & Wilkinson 2005); we have previously shown that both dimeric and dodecameric Dps-1 binds DNA, and that optimal binding interactions require 22 bp DNA. As DNA binding requires the N-terminal extensions interacting in DNA major grooves, a model for DNA binding was proposed in which DNA binds along the long axis of a Dps-1 dimer with N-terminal extensions from each monomer binding consecutive DNA major grooves (Grove & Wilkinson 2005; Bhattacharyya & Grove 2007). Since Dps-1 preferentially assembles into a dodecameric structure, its assembly from six dimers predicts a total of six possible DNA binding sites. We show here that while dodecameric Dps-1 can bind six 22 bp DNA duplexes, the stoichiometry of binding to longer duplexes is lower, suggesting that two Dps-1 dodecamers may bind opposite faces of duplex DNA sufficiently long to present consecutive DNA major grooves on each face. Mutation of surface-exposed arginines maps the path of DNA relative to the N-terminal extensions. Combined with the ability of Dps-1 to prevent cyclization of DNA, these data suggest a model for interaction with chromosomal DNA.
Materials and Methods

Mutagenesis, overexpression and purification of Dps-1 and Dps-R

Wild-type Dps-1 was purified and characterized as described (Grove & Wilkinson 2005) To introduce the Arg132Ser substitution into Dps-1, quick change mutagenesis PCR was performed by whole plasmid amplification of pET5a carrying the Dps-1 gene. The following primers were used to introduce the mutation (CGC→AGC); 5'-GCC AGC TAC AGC ACC-3' and 5'-GGT GCT GTA GCT GGC-3' (mutated codon in boldface). The PCR product was transformed into E. coli TOP10 cells and the resulting plasmid confirmed by sequencing. The mutant Dps-1 (Dps-R) was overexpressed in E. coli BL21(DE3)pLysS using 1.0 mM isopropyl-β, d-thigalactopyranoside (IPTG) for 2 hours. Cells were lysed in lysis buffer (50 mM Tris, 0.25 M NaCl, 5 mM Na₂EDTA, 5% glycerol, 5 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5 mg/mL lysozyme) and cell lysates were dialyzed against buffer A (20 mM Tris-HCl (pH 7.5), 50 mM KCl, 5% glycerol, 1 mM EDTA, 5 mM β-mercaptoethanol, 0.2 mM PMSF) and applied to a heparin-agarose column equilibrated in buffer A. The protein was eluted with a linear gradient from 50 mM to 1M KCl in buffer A. Elutions from the heparin column containing the mutant protein were collected, dialyzed against buffer A, and applied to a DEAE-Sepharose column equilibrated in buffer A and eluted with a linear gradient from 50 mM to 1 M KCl in buffer A. Protein concentrations were determined by staining of SDS polyacrylamide gels with Coomassie brilliant blue, using bovine serum albumin (BSA) as a standard, and by using the Micro BCA Protein Assay Kit (Pierce). The absence of dimeric Dps-1 was confirmed by electrophoresis on 5% non-denaturing acrylamide gels. The gel recipe was the same as the running gel of SDS-PAGE according to Laemmli, excluding SDS. The electrophoresis was carried out in 375 mM Tris-HCl, pH 8.7.
Electrophoretic Mobility Shift Assays

Complementary synthetic oligonucleotides used to generate 22 and 26 bp DNA were purchased and the concentrations determined using their extinction coefficients by spectrometry on a SmartSpec 3000 spectrophotometer (BioRad). The sequences of 22 and 26 bp DNA (average G+C content) are 5'-GGACTACTATAAATAGATGATC-3' (22 bp) and 5'-CGTGACTACTATAAATAGATGATCCG-3' (26 bp). The top strand was $^{32}$P-labeled at the 5'-end with phage T4 polynucleotide kinase. Equimolar amounts of complementary oligonucleotides were mixed, heated to 90°C and cooled slowly to room temperature (23°C) to form duplex DNA.

Electrophoretic mobility shift assays were performed using 10% polyacrylamide gels (39:1 (w/w) acrylamide:bisacrylamide) in 0.5x TBE (50 mM Tris borate, 1 mM EDTA). Gels were pre-run for 30 minutes at 175 volts at 23°C before loading the samples with the power on. For stoichiometry determinations, DNA and protein were incubated for 30 minutes at room temperature in binding buffer containing 20 mM Tris HCl (pH 8.0), 0.3 M NaCl, 0.1 mM Na$_2$EDTA, 1 mM dithiothreitol, 0.05% Brij58, and 10 μg/ml of BSA (under these conditions, the $K_d$ for Dps-1 binding to 22 bp DNA would be less than 0.5 nM, as the $K_d$ of 0.5 nM was measured in a buffer containing not 0.3 but 0.5 M NaCl) (Bhattacharyya & Grove 2007). Each reaction contained 100 fmol of DNA with increasing concentration of Dps-1 in a total reaction volume of 10 μl. For $K_d$ determination of Dps-R, DNA and protein were incubated for 1 hour at room temperature in binding buffer containing 200 mM Tris HCl (pH 8.0) and 500 mM NaCl (a 10-fold higher concentration of Tris compared to the stoichiometry measurements). Each reaction contained 5 fmol of DNA with increasing concentrations of Dps-1 or Dps-R in a total reaction of 10 μl. After electrophoresis, gels were dried and protein-DNA complexes and free
DNA were quantified by phosphorimaging, using software supplied by the manufacturer (ImageQuant 1.1). The region on the gel between complex and free DNA was considered as complex to account for complex dissociation during electrophoresis, and the fraction of bound DNA was calculated as the ratio of bound DNA to total counts in each lane. Total counts did not vary systematically as a function of protein concentration, indicating that recovery was independent of the extent of complex formation and that no material was selectively lost during handling. For $K_d$ determination, the percentage of complex formation was plotted against $[\text{Dps}]$ and data were fit to the Hill equation, $f=f_{\text{max}}[\text{Dps}]^n/(K_d+[\text{Dps}]^n)$, where $f$ is fractional saturation, $[\text{Dps}]$ is the protein concentration, $K_d$ reflects the apparent equilibrium dissociation constant and $n$ is the Hill coefficient. For measurements of stoichiometry, the percentage of complex formation was plotted against $[\text{Dps-1}]/[\text{DNA}]$ and fit to a smooth curve using the program KaleidaGraph. Tangents were generated from data points in the upward slope and in the plateau and the stoichiometry of Dps-1 complex formation was extrapolated algebraically. Experiments were carried out at least in triplicate, and several preparations of both DNA and proteins were used for which concentrations were independently determined. No difference between individual protein preparations were observed, and measured $K_d$ values are consistent with initially reported values (provided identical buffer conditions) (Bhattacharyya & Grove 2007; Grove & Wilkinson 2007). Also, Dps-1 is very stable, as previously reported (Grove & Wilkinson 2007), and no loss of activity (or dissociation of the dodecamer) was evident on storage.

**Intrinsic fluorescence measurements**

Fluorescence emission spectra from 280 nm to 500 nm were recorded on a Jasco FP-6300 spectrofluorimeter with an excitation wavelength of 280 nm at 23°C using a 0.5 cm path length.
cuvette. All experiments were performed with 0.03 mg/ml protein in 40 mM Tris-HCl (pH 8.0), 0.2 mM EDTA, 0.1% (w/v) Brij58 and 100 mM NaCl. Reactions were incubated at 23°C for 30 minutes before fluorescence was measured. The absorbance of each sample was measured from 280 nm to 500 nm to correct for the inner filter effect (Carpenter et al. 2001).

The corrected protein fluorescence at each wavelength ($F_{corr}(\lambda)$) was obtained from the observed fluorescence by first correcting for the background fluorescence to obtain ($F_c(\lambda)$). Inner filter effects were then resolved by the following correction factor, $F_{corr}(\lambda) = F_c(\lambda) \times 10^{(A_{ex}/2 + A_{em}/2)}$, where $A_{ex}$ and $A_{em}$ are the absorbances at the excitation and emission wavelengths, respectively (Carpenter et al. 2001). $F_{corr}(\lambda)$ is only reported for samples where both $A_{ex}$ and $A_{em}$ are less than 0.2. Fluorescence quenching ($Q$) on DNA binding was calculated by $Q = 1 - F_{corr}[X]/F_{corr}[0]$, where $F_{corr}[X]$ and $F_{corr}[0]$ are corrected fluorescence intensities with X μM and 0 μM DNA, respectively.

**DNA cyclization**

Plasmid pET5a was digested with BspHI to yield a 315 bp fragment, which was purified on a 2% agarose gel. The DNA fragment was $^{32}$P-labeled at the 5'-end with phage T4 polynucleotide kinase. Ligase mediated DNA cyclization experiments were carried out with varying protein concentrations. Reactions were initiated by addition of T4 DNA ligase to a final volume of 10 μl. Reactions containing 1000 fmol DNA and the desired concentration of Dps-1 were incubated in 1X binding buffer with 200 mM NaCl and 1X ligase buffer (New England BioLabs, Ipswich, MA) at 23°C for 60 minutes. Formation of circular ligation product was confirmed by the addition of 1 μl of exonuclease III followed by 30 minutes incubation at 23°C. Reactions were terminated using 3 μl of 10% SDS followed by phenol-chloroform extraction and ethanol precipitation. Reactions were analyzed on a 8% polyacrylamide gel (39:1 (w/w)
acrylamide:bisacrylamide) with 0.5X TBE as running buffer. After electrophoresis, gels were dried and ligation products were visualized by phosphormaging.

Plasmid DNA ligation

Plasmid pET5a was linearized with EcoRI. Reactions were initiated by addition of 200 units of T4 DNA ligase to a final volume of 10 μl. One hundred ng of DNA was incubated in 1X ligase buffer and 200 mM NaCl at room temperature for 60 minutes in the absence or presence of 1.3 pmole of Dps-1. Reactions were terminated with 10 μl of 75 mM EDTA, 6 mg/ml proteinase K, 15% glycerol, 1% SDS, bromophenol blue and xylene cyanol, followed by a 30 minute incubation at 55°C. Ligation products were resolved on 1% agarose gels in 0.5X TBE buffer at 110 volts for 2.5 hours. Gels were stained with ethidium bromide.

Results

Path of DNA relative to N-terminal extensions

Previous analyses of Dps-1 have shown that the N-terminal extension is important in DNA binding and that the main body of the protein contributes little to DNA binding in the absence of this extension (Bhattacharyya & Grove 2007). Once DNA binding has been initiated, the DNA presumably interacts with the body of the protein, but the exact path of DNA binding is still unknown. Looking at the structure of Dps-1, there is a single arginine residue that lies on the surface of Dps-1, in the short helix that connects the second and third helix of the four-helix bundle monomer, that has potential to interact with the DNA (Fig. 3.1a-b). To specify the role of this residue in DNA binding, a mutant was constructed in which this residue is changed to a serine. Electrophoretic mobility shift assays (Fig. 3.2a-b) show that this Dps-1 variant (Dps-R) binds DNA with lower affinity compared to wild type Dps-1. A complex is not apparent unless the DNA is incubated with 50 nM of Dps-R, and complexes are not stable and dissociate during
Figure 3.1: Possible DNA binding modes for dodecameric Dps-1. (a) The left side of the panel shows the structure of dodecameric Dps-1 (2F7N) (Kim et al. 2006) with a top view looking down on one of the six dimers (colored ribbons; blue to red starting at the N-terminus, with the short helices harboring R132 in yellow (perpendicular to the axis of the four-helix bundle)). The red spheres represent cobalt. The right side of the panel shows a side view of the identified dimer, with R132 in stick representation (yellow). Each Dps-1 dimer binds two consecutive major grooves in duplex DNA, identified by blue arrows. For 22 bp DNA (shown as the red segment of the double helix), only one face of the helix presents two consecutive major grooves. For 26 bp DNA (the entire duplex segment, red and gray) there are potential binding sites on both faces of the duplex (identified by all arrows, blue and gray). (b) The Dps-1 dimer with each chain colored as above with R132 in red stick representation. Metal ions in the ferroxidase center located at the interface of two monomers are in green; metal ions at the N-terminal loops are in blue. (c) Native gel showing N-terminally truncated Dps-1 (dimer; lane 1) and full-length Dps-1 (dodecamer; lane 2). N-terminally truncated Dps-1 has been previously shown by both gel filtration and native gel electrophoresis to exist as a dimer, while full-length Dps-1 exists as a dodecamer (Bhattacharyya & Grove 2007).
Figure 3.2: DNA binding by wild type Dps-1 and Dps-R. Electrophoretic analysis of 26 bp duplex DNA titrated with Dps-1 (a) and Dps-R (b) under equilibrium conditions ([DNA]<Kd). Experiments with Dps-1 and Dps-R were performed side-by-side with the same DNA preparations. For Dps-1, concentrations are 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 10, 20, 30, 40, 50, and 100 nM. For the mutant Dps-R, concentrations are 0, 0.25, 0.5, 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 nM. (c) Binding isotherm for Dps-1 binding to 26 bp (the presence of single-stranded DNA, to which Dps-1 does not bind, accounts for residual unbound DNA), (d) binding isotherm for Dps-R binding to 26 bp (a larger fraction of DNA migrates at the position of the DNA reference band after incubation with Dps-R suggests that it derives from complexes that dissociated during electrophoresis and not from DNA species that are inactive for binding). The best fit to the data were obtained using the Hill Equation (R²=0.9828, n=1.3±0.2 for Dps-1 and R²=0.9866, n=0.8±0.1 for Dps-R).
electrophoresis; this results in saturation at a level of bound DNA that is much lower than total DNA. In contrast, essentially complete complex formation is seen with only 5 nM of Dps-1. Quantification of complex formation with Dps-1 yields a $K_d$ of 4.0±0.6 nM (Fig. 3.2d), which is about six-fold lower compared to the mutant Dps-R ($K_d=24.0±5.0$ nM) (Fig. 3.2c). The $K_d$ value for Dps-1 is higher than the previously reported $K_d$ (~0.5 nM) (Bhattacharyya & Grove 2007). This is due to the higher ionic strength and different composition of the binding buffer compared to that used for previous determinations. Taken together, these data suggest that Arg132 in the helix connecting helices 2 and 3 of the four-helix bundle monomer contributes to DNA binding. We also note that the modest positive cooperativity of binding seen here and previously reported for wild-type Dps-1 is lost on removal of Arg132.

**Stoichiometric titrations reveal six DNA binding sites per Dps-1 dodecamer**

The mode of DNA binding by dodecameric Dps-1 involves interaction of two metal-anchored N-terminal extensions in consecutive DNA major grooves, a binding mode in part inferred from the observation that Dps-1 exhibits optimal binding to 22 bp DNA while interactions with shorter duplexes that do not present two consecutive DNA major grooves result in a significantly lower affinity (Bhattacharyya & Grove 2007). Since Dps-1 dimers can bind DNA, and since the Dps-1 dodecamer is assembled from six dimers, each Dps-1 dodecamer would have six potential binding sites for DNA. To assess this prediction, 22 bp DNA duplex was titrated with dodecameric Dps-1 under stoichiometric conditions ([DNA] > $K_d$) and complex formation measured using electrophoretic mobility shift assays; under the solution conditions used, the DNA would be in at least 20-fold excess above the $K_d$. As shown in Fig. 3.3, Dps-1 forms complexes with the DNA that are unable to enter the gel. When the percentage of complex formation was plotted against [Dps-1]/[DNA] and tangents were calculated from the
Figure 3.3: Stoichiometry of Dps-1 to DNA determined by electrophoretic mobility shift assays. (a)-(b) Stoichiometric titrations ([DNA]>K_d) of 100 fmol 22 bp and 26 bp DNA, respectively, with increasing concentrations of dodecameric Dps-1. Protein concentrations range from 0.15-2.1 nM for 22 bp DNA in increments of 0.15 nM and 0.3 – 4.2 nM for 26 bp DNA in increments of 0.3 nM. Complex and free DNA is identified at the right. (c)-(d) Percent complex formation with 22 bp and 26 bp DNA, respectively, as a function of [Dps-1]/[DNA]. The intercepts of tangents to the upward slope and the saturation plateau reflect saturation. DNA preparations used in these experiments contain no single-stranded DNA.
upward slope and the saturation plateau, a break point was observed reflecting saturation of Dps-1 (Fig. 3.3). The 22 bp DNA was seen to saturate Dps-1 at a [Dps-1]/[DNA] ratio of 0.17:1, i.e., a 5.9-fold molar excess of DNA is required to saturate the protein. This is consistent with the predicted existence of six DNA binding sites. We also note that dodecameric Dps-1 is the favored oligomeric assembly, and that no dimer was detectable in the protein preparations used for this experiment (Fig. 3.1c). Additionally, since an evaluation of stoichiometry requires knowledge of the active fraction of protein, we note the significant stability of Dps-1 (Grove & Wilkinson 2005) and the observation that no difference in activity is evident for different protein preparations or as a result of prolonged storage. The observed saturation of Dps-1 with a 5.9-fold molar excess of 22 bp DNA is consistent with expectations; indeed, it would be difficult to reconcile saturation at a higher ratio of DNA to protein with the assembly of Dps-1 from 6 dimers, each of which are capable of binding 22 bp DNA but not shorter duplexes (Grove & Wilkinson 2005). Therefore, we therefore have no indication that Dps-1 preparations are not essentially fully active.

As 22 bp DNA features two consecutive DNA major grooves only on one face of the DNA helix (Fig. 3.1a), a second Dps-1 protomer interacting with the opposite face of already bound DNA would be unfavorable (Grove & Wilkinson 2006). We therefore investigated the stoichiometry of Dps-1 binding to 26 bp DNA that would be sufficiently long to feature consecutive major grooves on opposite faces of the duplex (Fig. 3.3). The rationale for this experimental design was that if two Dps-1 dodecamers cannot bind simultaneously to opposite faces of the duplex, then a 6:1 stoichiometry of DNA to Dps-1 should still be observed, while simultaneous Dps-1 binding to opposite DNA faces would result in a lower ratio of DNA to Dps-1 at saturation. As shown in Fig. 3, 26 bp DNA duplex saturates Dps-1 at a [Dps-1]/[DNA] ratio
of 0.24:1, i.e., a 4.2-fold molar excess of DNA is required to saturate the protein, suggesting simultaneous binding of two Dps-1 dodecamers on either face of the 26 bp duplex.

Each Dps-1 monomer has two tryptophan residues; Trp84 at the end of $\alpha 1$ and Trp195 at the end of $\alpha 4$. An altered environment of tryptophan can be determined by changes in intrinsic fluorescence; we therefore measured the change in intrinsic tryptophan fluorescence of Dps-1 on interaction with DNA to corroborate stoichiometry determinations by EMSA. The intrinsic fluorescence spectrum of dodecameric Dps-1 is characterized by an emission maximum at 330 nm and a shoulder at 338 nm upon excitation at 280 nm (Fig. 3.4, black symbols). The most marked decrease in fluorescence is seen at a ratio of dodecameric Dps-1 to DNA of 1:1 (Figure 3.4, blue diamond) with a further decrease in fluorescence with increasing amounts of DNA. Equimolar amounts of DNA and Dps-1 effectively shift the emission maximum to 339 nm by reducing the peak fluorescence at 330 nm to that of the shoulder to create one broad peak spanning from about 330 nm to 342 nm; with increasing [DNA], fluorescence intensity in decreased asymptotically, but the wavelength of peak intensity remains constant. Notably, effectively no further fluorescence quenching is observed across the entire spectrum beyond a DNA:Dps-1 ratio of 4:1 for 26 bp DNA (Fig. 3.4b, purple symbols, and 3.4d), while fluorescence quenching on addition of 22 bp DNA continues until a ratio of 6:1 is reached (Fig. 3.4a, orange symbols, and 4c). These data suggest a different mode of binding of the two duplexes and are consistent with occupancy of 6 DNA binding sites by 22 bp DNA and with 26 bp DNA saturating Dps-1 at a lower stoichiometry. While the roles of receptor and titrant are reversed in this experiment and equivalent stoichiometries of binding are suggested, we note that simultaneous binding of two Dps-1 dodecamers to one DNA duplex may affect fluorescence quenching.
Figure 3.4: Stoichiometry of Dps-1 to DNA determined by quenching of intrinsic tryptophan fluorescence. (a) Intrinsic fluorescence of 0.03 mg/ml dodecameric Dps-1 titrated with 22 bp dsDNA. (ratio of protein:DNA: blue diamond=1:1; red square=1:2; green triangle=1:3; purple x=1:4; turquoise x=1:5; orange circle=1:6; light blue square=1:7; black symbol=Dps-1 only). (b) Intrinsic fluorescence of Dps-1 titrated with 26 bp dsDNA (protein:DNA: blue diamond=1:1; red square=1:2; green triangle=1:3; purple x=1:4; turquoise x=1:5; orange circle=1:6; light blue dot=1:7; red dot with yellow line=Dps-1 only). (c) Intrinsic fluorescence quenching of dodecameric Dps-1 as a function of the concentration of 22 bp dsDNA. (d) Intrinsic fluorescence quenching of dodecameric Dps-1 as a function of the concentration of 26 bp dsDNA. Fluorescence quenching at peak fluorescence is graphically represented only for illustrative purposes.
Dps-1 Restricts Cyclization of Short DNA Fragments by T4 DNA Ligase

The ability of Dps-1 to bend DNA was previously assessed using a cyclization assay in which DNA shorter than the persistence length was cyclized with T4 DNA ligase. Dps-1 was unable to promote cyclization of 105 bp DNA (Bhattacharyya & Grove 2007). The proposed organization of DNA-Dps-1 complexes suggests that DNA will not wrap around a single dodecamer, consistent with the lack of cyclization observed with 105 bp DNA, and that Dps-1 will instead “stiffen” the DNA. This mode of DNA-Dps-1 interaction should prevent cyclization of longer DNA duplexes. A ligase-mediated cyclization assay was therefore performed with a longer 315 bp DNA, which can cyclize in the presence of T4 DNA ligase without the help of any DNA bending protein. As shown in Fig. 3.5, 315 bp DNA can form minicircles in the absence of Dps-1; addition of ~1 nM Dps-1 results in significantly reduced yield of circular ligation product, while linear dimer may still be seen (lane 5), and higher concentrations of Dps-1 also inhibit formation of linear dimer (lanes 6-7). This is consistent with the interpretation that Dps-1 increases the persistence length at Dps-1 concentrations at which binding to multiple duplexes may not be the prevailing mode of binding, but the experiment also suggests that Dps-1 produces DNA complexes at higher concentrations that resist multimer-formation. This may be related to the binding of multiple DNA molecules per Dps-1 dodecamer that preclude proper alignment of DNA ends for ligation, as such duplexes would be bound at sites that are either at right angles to each other or at opposite faces of the dodecamer. This experiment also addresses the possibility that Dps-1 binds with significantly higher affinity to DNA ends compared to internal sites; such binding mode would have been expected to result in inhibition of DNA ligation at a Dps-1 concentration that would be sufficient to saturate DNA ends. This is not observed, as ligation of linearized plasmid pET5a by T4 DNA ligase was not inhibited by addition of 1.3 pmole of Dps-
which is more than sufficient to saturate the free DNA ends (130 nM protein, each featuring multiple DNA binding sites, to 7.6 nM DNA ends) (Fig. 3.5b, lane 3). Dps-1 binds dsDNA with very high affinity to internal sites ($K_d \approx 0.5$ nM under the ionic conditions of this experiment). The concentration of internal sites is much greater than the concentration of DNA ends (4134 bp plasmid featuring 188 contiguous 22 bp sites corresponding to 714 nM sites at saturation); assuming four DNA sites per Dps-1 dodecamer, 130 nM protein would feature 520 nM DNA-

![Figure 3.5: DNA end joining in the presence of Dps-1. (a) Dps-1 inhibits cyclization of 315 bp duplex DNA by T4 DNA ligase. Lane 1, 315 bp DNA alone. Lane 2, DNA and ligase. Lane 3, DNA, ligase, and exonuclease III. Lanes 4-7 show DNA incubated with ligase and increasing concentrations (0.8, 2.4, 8.0, and 24 ng) dodecameric Dps-1. Linear and circular ligation products are identified at the right. (b) Ligation of linearized plasmid DNA in the presence of Dps-1. Lane 1, linearized pET5a without the ligase. Lane 2, DNA after ligation in the absence of Dps-1. Lane 3, DNA after ligation in the presence of 1.3 pmoles of Dps-1. Lane 4, DNA and Dps-1 in the absence of ligase.](image-url)
binding site, comparable to the number of internal sites in the DNA. Therefore, this experiment suggests that Dps-1 does not prevent ligation by preferentially binding to the DNA ends or simply by interfering with ligase function.

**Discussion**

**The DNA binding site**

DNA binding by Dps proteins generally requires extensions beyond the four-helix bundle core. Dps-1 is no exception; truncation of the N-terminal extensions has been previously shown to result not only in a lack of DNA binding, but also in a failure to assemble into a dodecamer (Bhattacharyya & Grove 2007). Since high-affinity binding is observed only with DNA of sufficient length to feature two DNA major grooves on one face of the helix, and since Dps-1 dimers retain the ability to bind DNA, DNA binding may entail N-terminal extensions from each monomer of a Dps-1 dimer occupying consecutive DNA major grooves. Such binding mode would align the DNA helix axis with the long axis of a Dps-1 dimer (Fig. 3.1a). The surface-exposed helices α2 and α3 of the four-helix bundle monomer are connected by a short helix featuring Arg132, and further support of the proposed binding model is provided by the reduced DNA binding characteristic of a Dps-1 variant in which this residue is mutated (Fig. 3.2).

An explicit prediction from this binding model is that each Dps-1 dodecamer, which may be viewed as assembled from six dimers, should feature six binding sites, and that adjacent sites should be at right angles to each other, thus precluding DNA wrapping about the protein surface. These predictions are borne out by experiment: the stoichiometry of 22 bp DNA to dodecameric Dps-1 is 6:1 (Figs. 3.3 and 3.4) and DNA binding by Dps-1 may increase its persistence length, as evidenced by attenuated DNA cyclization by T4 DNA ligase at low concentrations of Dps-1 (Fig. 3.5a) and by the previously observed failure of Dps-1 to promote cyclization of shorter
duplexes (Bhattacharyya & Grove 2007). Taken together, these data suggest that the DNA
binding site in Dps-1 consists of N-terminal extensions from either monomer of a Dps-1 dimer
and arginine residues in the loops connecting helices two and three, and that Dps-1 features six
such sites.

A change in intrinsic tryptophan fluorescence signals an altered environment. In Dps-1,
Trp84 is at the end of α1, contributing to the dimer interface, and Trp195 is at the end of α4.
This helix leads to a loop in which Arg205 contributes to coordinating a metal ion at the
predicted iron exit pore (Kim et al. 2006). As neither tryptophan would be predicted to contact
DNA directly, an altered environment of one or both of these residues may arise due to a
propagation of conformational changes on DNA binding. This is consistent with the observation
that the greatest change in fluorescence quenching is seen at low DNA occupancy (Fig. 3.4),
with the observed positive cooperativity of DNA binding of dodecameric Dps-1, and with the
observation that dimeric Dps-1 does not bind DNA cooperatively (Bhattacharyya & Grove 2007;
Grove & Wilkinson 2005). In addition to the differential change in tryptophan fluorescence
observed at different levels of DNA saturation, there is no reason to expect both tryptophan
residues to respond equivalently to conformational changes induced on DNA binding. These
caveats notwithstanding, this experiment is consistent with the stoichiometries measured by
EMSA.

Proposed model for Dps-1 binding to chromosomal DNA

The packing of *E. coli* Dps dodecamers in the crystal is approximately hexagonal (Grant
et al. 1998). Likewise, hexagonal packing was seen in electron micrographs of isolated Dps
(Frenkiel-Krispin & Minsky 2006). While addition of DNA did not affect the in-plane lattice
spacing, it caused the rapid formation of sheet-like crystals, leading to the proposal that Dps
coordinates DNA in alternating layers of protein and DNA (Frenkiel-Krispin et al. 2004; Wolf et al. 1999; Frenkiel-Krispin & Minsky 2006). In such layers, the DNA would be buried and thus physically protected from damage. Our data further supports and extends this model: Combining the stoichiometry data with the observation that Dps-1 “stiffens” DNA as well as the hexagonal packing of *E. coli* Dps dodecamers observed in the crystal and the layered appearance of *E. coli* Dps with DNA observed in electron micrographs, we propose that Dps-1 can bind DNA along the axis of each of six dimers (Figure 3.1a; 22 bp DNA illustrated in red). Two Dps-1 protomers can bind simultaneously to opposite faces of a duplex, provided that it is sufficiently long and can present two consecutive major grooves, as illustrated for 26 bp DNA (Figure 3.1a; 26 bp DNA includes gray extension). Our binding model is consistent with a hexagonal packing of Dps-1 dodecamers and with a layered appearance in complex with genomic DNA, as simultaneous binding to opposite faces of a DNA duplex would require each “layer” of Dps-1 dodecamers to fit into a knobs-into-holes type arrangement with DNA passing between each Dps-1 layer (Figure 3.6a; DNA illustrated as red cylinders). According to this model, two of the six possible DNA binding sites per dodecamer would be occupied (rainbow-colored dimers along the x-axis, Figure 3.6a and blue and green dimers in Figure 3.6b). Notably, our data also suggest that the direction of DNA potentially bound at the remaining four sites would have to follow right angles relative to the sites parallel to the x-axis. Since sites parallel to the y-axis (gray dimers, Figure 3.6a and orange and purple dimers in Figure 3.6b) are not aligned between Dps-1 dodecamers in adjacent layers, DNA binding along the y-axis is likely unfavorable. In contrast, further pseudo-hexagonal packing along the z-axis would align binding sites from dodecamers in adjacent layers (blue dimers parallel to the z-axis, Figure 3.6a and red and yellow dimers in Figure 3.6b), potentially allowing DNA duplexes to bind along the z-axis, further
Figure 3.6: Model of dodecameric Dps-1 binding to genomic DNA. (a) A hexagonal arrangement of Dps-1 dodecamers with DNA (red cylinders) bound along the x-axes. (b) Dps-1 dodecamer with each dimer in a different color; DNA binds along the horizontal x-axis to blue and green dimers (top and bottom) with the DNA helix axis parallel to that of the four-helix bundle and along the z-axis to red and yellow dimers (left and right).
reinforcing the sheet-like packing, and allowing a maximal occupancy of 4 of 6 potential DNA binding sites per dodecamer. Such highly ordered assemblies would allow packing of the otherwise flexible DNA into the crystalline structures observed under conditions that promote upregulation of Dps.

Bacterial nucleoids are compacted by association with proteins such as HU, integration host factor (IHF), H-NS, Fis, and Dps that either promote and constrain DNA supercoiling or bridge distant DNA segments (Dorman 2009; Grove 2011). In *E. coli*, Dps is upregulated in stationary phase and is central to cellular stress responses (Almirón et al. 1992; Martinez & Kolter 1997). Under such circumstances, the ability to bridge DNA by association of each Dps protomer with multiple DNA segments is likely key to the observed DNA compaction and the formation of the crystalline arrays observed under severe stress conditions (Wolf et al. 1999; Frenkiel-Krispin & Minsky 2006). In *D. radiodurans*, the function of Dps-1 is less clear; although *D. radiodurans* does not encode homologs of many of the nucleoid-associated proteins characterized in *E. coli*, deletion of Dps-1 does not appear to cause overt changes in nucleoid morphology in exponential or early stationary phase cells as evidenced by DAPI staining of the nucleoids (with the caveat that other nucleoid-associated proteins such as HU may be upregulated to compensate for the loss of Dps-1) (Nguyen et al. 2009). However, it remains to be determined if *D. radiodurans* Dps-1 is likewise upregulated under the severe stress conditions under which *E. coli* Dps induces the formation of DNA-protective crystalline arrays.
CHAPTER 4: METAL BINDING AT THE DEINOCOCCUS RADIODURANS DPS-1 N-TERMINAL METAL SITE CONTROLS DODECAMERIC ASSEMBLY AND DNA BINDING*

Introduction

Oxidative stress is a condition that is generated by various environmental hazards such as radiation and desiccation as well as by endogenous conditions, including nutritional stress and starvation. Under these circumstances, large amounts of reactive oxygen species (ROS) such as H₂O₂, •OH, and •O₂⁻ are produced that can damage cellular components. Furthermore, these partially reduced oxygen species can be transformed into a more toxic form; for example, H₂O₂ can react with the transition metal Fe(II) to produce the highly toxic •OH. In bacteria, there are many different proteins that can protect against ROS. Some proteins, such as catalases and superoxide dismutases, can provide protection by detoxifying these species and other proteins also provide physical protection (Imlay & Linn 1988; Crichton et al. 2002; Imlay 2008). One prominent example of the latter is the Dps (DNA protection during starvation) protein.

Most Dps-family proteins protect the cells through two mechanisms: by physically binding and shielding the DNA against damaging agents and by sequestering and oxidizing Fe(II) to block the formation of •OH (Almirón et al. 1992; Martinez & Kolter 1997; Zhao et al. 2002; Su et al. 2005). Dps proteins possess a structure that is similar to that of the iron storage protein ferritin. However, Dps is smaller than ferritin in that it has only twelve subunits and assembles with 23 tetrahedral symmetry, compared to the twenty-four subunits of ferritin, which assemble with 432 octahedral symmetry. Furthermore, each ferritin subunit has its own

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*This chapter originally appeared as Nguyen, K. H. & Grove, A. (2012) Metal binding at the Deinococcus radiodurans Dps-1 N-terminal metal site controls dodecameric assembly and DNA binding. Reprinted with permission from Biochemistry, Volume 51(33), pages 6679-89, Copyright American Chemical Society Publication.
ferroxidase center while in most Dps homologs, an active ferroxidase center is localized between two subunits (Harrison & Arosio 1996; Grant et al. 1998). These ferroxidase sites catalyze the conversion of ferrous iron to Fe$^{3+}$, but unlike ferritin, Dps-family proteins generally prefer H$_2$O$_2$ over molecular oxygen as the oxidant; this distinction is important as it allows Dps to inactivate Fe$^{2+}$ and H$_2$O$_2$ simultaneously. The implication is also that the primary function of Dps proteins is to prevent the hydroxyl radical production that would occur by environmental oxidation of Fe$^{2+}$. Like ferritin, Dps ultimately accumulates iron in its central cavity to generate a hydrous ferric oxide mineral core; the smaller size of its internal cavity results in accumulation of ~500 Fe compared to the ~4,500 Fe per ferritin shell. Several residues are conserved among Dps proteins including the ones that are involved in coordinating the iron at the ferroxidase center; however, N- and C-terminal extensions beyond the four-helix bundle subunit have variable length. These extensions have been shown to contribute greatly to DNA binding and oligomerization of some Dps homologs (Stillman et al. 2005; Bhattacharyya & Grove 2007; Roy et al. 2007).

The Gram-positive bacterium *Deinococcus radiodurans* is known for its extreme resistance to various stress conditions including UV and ionizing radiation, a resistance thought to correlate with its resistance to desiccation (Mattimore & Battista 1996). It can survive up to thousand grays of radiation without losing viability. In other bacteria such as *E. coli*, such exposure would be lethal due to the massive damage to macromolecules (Battista 1997; Cox & Battista 2005). One potential contributor to the extreme resistance characteristic of *D. radiodurans* is its relatively high Mn/Fe ratio; (16) while Fe(II) can enhance the toxicity of ROS, Mn(II) has been proposed to provide protection by blocking the ROS–mediated oxidation of
proteins. In *D. radiodurans*, Mn(II) is found throughout the cells but Fe(II) is concentrated near the area between dividing cells (Daly et al. 2007).

*D. radiodurans* encodes two Dps proteins, Dps-1 and Dps-2 (Makarova et al. 2001). Both Dps-1 and Dps-2 have an N-terminal extension that is much longer than that of other Dps homologs; however, part of the N-terminal extension of Dps-2 is a predicted signal peptide. One of the unique features of Dps-1 is that it fails to protect against ROS despite being able to bind DNA with high affinity, a property inferred to derive from iron leaking from its mineral core (Grove & Wilkinson 2005; Kim et al. 2006). Such iron leakage would be expected to result in DNA damage were it to occur while Dps-1 is bound to DNA. Experiments reported below provide a possible mechanism by which the two functions of Dps-1 may be separated to prevent •OH-mediated damage to Dps-1-bound DNA. The crystal structure of Dps-1 (Figure 4.1) reveals not only the expected four-helix bundle subunit, but also four metal-binding sites (Table 4.1). For two of the metal-binding sites, the iron entry channel and the iron exit channel, divalent metals are coordinated mainly by negatively charged residues from three adjoining subunits. The 23 symmetry of Dps-1 leads to two non-equivalent three-fold interfaces. One such interface is the N-terminal ferritin-like channel that shares similar packing with ferritin and is lined with negatively charged residues; this interface corresponds to the iron entry channel. The other is the C-terminal pore that is unique to Dps proteins and is inferred to function as the iron exit channel in Dps-1. Another metal site is at the ferroxidase center with ligands from two subunits, created by two-fold rotational symmetry. Finally, Dps-1 contains a unique metal binding site at the base of the N-terminal extension (Kim et al. 2006, Romão et al. 2007). Here we show that this metal site is required to initiate dodecameric assembly and that it also contributes greatly to DNA binding. Our data suggest that disruption of the N-terminal metal site results in suboptimal
assembly of Dps-1 in which the protein forms a hemispherical hexamer and that occupancy of the N-terminal metal site is required for DNA binding.

**Figure 4.1: Sequence alignment.** (a) Alignment of *D. radiodurans* (*D. rad.*) Dps-1 and Dps-2 and *E. coli* Dps. Gray shading identifies conserved amino acids. The numbers 1-5 below the alignment represent the helical segments in each subunit. Residues involved in coordinating the metal at the ferroxidase center are highlighted in purple. Residues involved in coordinating the N-terminal metal in Dps-1 are highlighted in red. (b) Left panel shows the quaternary structure of Dps-1. Each monomer is represented by a different color. The right panel is an enlarged view of the N-terminal metal binding site with the residues coordinating the metal highlighted in black (Asp36, His39, His50 and Glu55). The metals at the N-terminus are represented by yellow spheres.
**Materials and Methods**

Cloning, overexpression and purification of Dps-1, Dps-dn and Dps-HE

Wild-type Dps-1 and Dps-dn lacking the N-terminal extension were purified and characterized as previously described (Grove & Wilkinson 2005; Bhattacharyya & Grove 2007). For the Dps-HE mutant, the following primers were used to introduce the mutations (CAC to AGC and GAA to CAA; mutagenic codons underlined) by whole plasmid amplification of plasmid harboring the Dps-1 gene: 5'-CACAGCCACTACCTGGAACAAAAAG-3' and 5'-ACGAGCGCGGTGGTCACG-3'. The PCR product was transformed into *E. coli* TOP10 cells and the resulting plasmid confirmed by sequencing. The mutant Dps-1 (Dps-HE) was

**Table 4.1.** Four metal sites in Dps-1

<table>
<thead>
<tr>
<th>Location</th>
<th>Symmetry axis</th>
<th>Coordinating residues</th>
</tr>
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<tbody>
<tr>
<td>1 N-terminus</td>
<td>N/A</td>
<td>D36, H39, H50, E55</td>
</tr>
<tr>
<td>2 Ferroxidase center</td>
<td>2-fold</td>
<td>H83, D110, E114</td>
</tr>
<tr>
<td>3 Iron entry</td>
<td>3-fold</td>
<td>D181</td>
</tr>
<tr>
<td>4 Iron exit</td>
<td>3-fold</td>
<td>E100</td>
</tr>
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</table>

Numbering of metal sites corresponds to their identification in the Dps-1 structure (PDB 2F7N). The symmetry leads to two types of non-equivalent 3-fold interfaces, corresponding to the iron entry and exit channels. Dps-1 subunits are represented by rectangles and filled circles represent metal sites.
overexpressed in *E. coli* BL21(DE3)pLysS grown in LB at 37°C with 1.0 mM isopropyl-β,D-thiogalactopyranoside for 2 h. Cells were lysed in lysis buffer [50 mM Tris, 0.25 M NaCl, 5 mM EDTA, 5% glycerol, 5 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5 mg/mL lysozyme], followed by dialysis against buffer A [20 mM Tris-HCl (pH 7.5), 50 mM KCl, 5% glycerol, 1 mM EDTA, 5 mM β-mercaptoethanol, 0.2 mM PMSF]. The dialysate was applied to a heparin–agarose column equilibrated in buffer A. The protein was eluted with a linear gradient from 50 mM to 1 M KCl in buffer A. The elutions from the heparin column that contain the mutant protein were collected and dialyzed against buffer A, and applied to a diethylaminoethyl (DEAE)–Sepharose column equilibrated in buffer A and eluted with a linear gradient from 50 mM to 1 M KCl in buffer A. The elutions from the DEAE–Sepharose column that contain the mutant protein were collected and dialyzed against buffer A, and applied to a carboxymethyl (CM)–Cellulose column equilibrated in buffer A and eluted with a linear gradient from 50 mM to 1 M KCl in buffer A. Protein concentration was determined by staining of SDS polyacrylamide gels with Coomassie brilliant blue using bovine serum albumin (BSA) as a standard and by using the Micro BCA Protein Assay Kit (Pierce). Purified proteins were judged to be >95% pure based on Coomassie-stained SDS-PAGE gels.

**Native polyacrylamide gel electrophoresis**

The oligomeric state of the mutant Dps-HE was visualized on a 5% non-denaturing acrylamide gels. The gel components were the same as the running gel of SDS-PAGE according to the method of Laemmli except SDS was not included. The gel was run in a buffer composed of 375 mM Tris-HCl, pH 8.7. At least three independent experiments were performed to confirm migration.
Gel Filtration

The entire gel filtration experiment was done at 4°C. HiLoad 16/60 Superdex 30 prep grade column (bed length 60 cm, inner diameter 16 mm; GE Healthcare) was first washed with 1 column volume of buffer AP, pH 8.0 (50 mM Na$_2$H$_2$PO$_4$, 10 mM imidazole, 10% glycerol) and then with 2 column volumes of buffer BP, pH 8.0 (50 mM Na$_2$H$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole, 10% glycerol). The gel filtration standard (Bio-Rad), which is a mixture of bovine thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa), and vitamin B-12 (1.35 kDa), was run to calibrate the column. Three mg/ml Dps-HE was applied to the gel filtration column and the protein was eluted with a flow rate of 0.5 ml/min.

Thermal stability

Dps-1 or bipyridyl-treated Dps-1 (Dps-1 incubated with 50 mM bipyridyl for 20 min at 4°C, followed by dialysis) was diluted to 10 μM in a buffer containing 50 mM Tris pH 8.0, 100 mM NaCl and 5X SYPRO Orange (Invitrogen). Fluorescence emission resulting from dye binding to unfolded protein was measured over a temperature range of 1°C to 90°C in 1 degree increments for 45 s using an Applied Biosystems 7500 Real-Time PCR System using the SYBR green filter. The correction for the total fluorescence yield was made using reactions without protein. The resulting data were exported to Sigma Plot 9 and the sigmoidal part of the curve was fit to a four parameter sigmoidal equation. Errors on the calculated Tm reflect standard deviation from two independent experiments.

Electrophoretic mobility shift assays

Oligonucleotides used to generate duplex DNA were purchased and purified by denaturing polyacrylamide gel electrophoresis. One strand was $^{32}$P-labeled at the 5'-end with T4
polynucleotide kinase. Equimolar amounts of complementary oligonucleotides were mixed, heated to 90°C and slowly cooled to 4°C to form duplex DNA.

Electrophoretic mobility shift assays (EMSA) were performed using 10% polyacrylamide gels [39:1 (w/w) acrylamide:bisacrylamide] in 0.5X TBE (50 mM Tris borate, 1 mM EDTA). Gels were prerun for 30 min at 175 V at 23°C before loading the samples with the power on. DNA and protein were incubated for 1 h at room temperature in binding buffer containing 20 mM Tris HCl (pH 8.0) and 500 mM NaCl. Each reaction contained 5 fmol of 26 bp DNA with increasing concentrations of Dps-1 or Dps-HE in a total reaction of 10 µL. The sequence of 26 bp DNA was 5’-CGTGACTACTATAAATAGATGATCCG-3’. After electrophoresis, gels were dried and protein–DNA complexes and free DNA were quantified by phosphorimaging using software supplied by the manufacturer (ImageQuant 1.1). For K_d determination, the percentage of complex formation was plotted against [Dps] and data were fit to the Hill equation, f=f_max [Dps]^n/(K_d + [Dps]^n), where f is fractional saturation, [Dps] is the protein concentration, K_d reflects the apparent equilibrium dissociation constant, and n is the Hill coefficient. Error bars represent standard deviation from four replicates.

**Effect of divalent metal ions on DNA binding**

In order to obtain a metal-free Dps-1 and Dps-HE, the proteins were incubated with 50 mM bipyridyl for 20 min at 4°C. The bipyridyl-treated protein was dialyzed against a high salt buffer (10 mM Tris-HCl, pH 8.0, 500 mM KCl, 5% (v/v) glycerol, 5 mM β-mercaptoethanol, and 0.2 mM PMSF) at 4°C for 2 hours to remove the bipyridyl. DNA (2.5 fmol) was then incubated with varying concentration of bipyridyl-treated Dps-1 with or without the addition of metals (80 nM Fe(NH_4)_2(SO_4)_2, MnCl_2 or CoCl_2). For Dps-HE, 1 µM of MnCl_2 was used. Where indicated, 1 mM H_2O_2 was added. The reactions were analyzed on a 10% polyacrylamide
gel (39:1 (w/w) acrylamide:bisacrylamide) in 0.5X TBE. After electrophoresis, the gel was dried, and protein-DNA complexes and free DNA were visualized by phosphorimaging. Experiments were repeated at least three times.

**Ferroxidation**

The kinetics of iron oxidation by Dps-1 was measured at 310 nm using an Agilent 8453 spectrophotometer. Dps-1 was diluted to 0.2 mg/ml and dialyzed against the reaction buffer, 20 mM Mops, pH 7.0, 100 mM NaCl. Prior to each experiment, fresh solution of ferrous ammonium sulfate and manganese chloride were prepared. Reactions contained 50 µM ferrous iron and 10 µM MnCl₂. Experiments were repeated three times. The kinetic data were plotted using Prizm.

**Metal binding by Dps-1 and Dps-HE**

Dps-1 and Dps-HE (50 µg/ml) previously treated with bipyridyl as described above was incubated with 1 µM CoCl₂ following which excess metal was removed by dialysis. To denature the proteins, SDS was added, followed by incubation at 90°C for 10 min. Samples were mixed with 125 µM of 4-(2-pyridylazo) resorcinol (PAR) in buffer A (20 mM Tris-HCl (pH 7.5), 100 mM KCl, 5% glycerol) and the absorbance from 320 to 675 nm was recorded using an Agilent 8453 spectrophotometer. Experiments were performed twice.

**Results**

The N-terminal metal-binding site is required for dodecameric assembly

Alignment of the amino acid sequence of Dps-1 with that of other Dps homologs reveals significant homology. For example, residues involved in assembling the ferroxidase center at the interface between two subunits are completely conserved (Figure 4.1a) and Dps-1 exhibits the associated ferroxidase activity (Grove & Wilkinson 2005). The main difference is the N-terminus that extends from the four-helix bundle core. Dps-1 has a lengthy N-terminal extension
that has been shown to play an important role in oligomeric assembly and DNA binding. A unique feature of this extension is a metal-binding site in which a divalent metal ion is coordinated by Asp36, His39 and His50 from the N-terminus and Glu55 from the α1 helix (Figure 4.1b). When the entire N-terminal extension including the metal-binding site is deleted, the protein exists exclusively as a dimer (this mutant was named Dps-dn). In contrast, when the first 33 amino acids of the extension are deleted with the metal-binding site remaining intact, dodecameric assembly is unaffected. Based on this observation, occupancy of the metal-binding site was inferred to stabilize the resulting loop that contacts the neighboring dimer, contacts that must be required for oligomeric assembly (Bhattacharyya & Grove 2007).

The Dps-1 interfaces were also analyzed using the Protein Interfaces, Surfaces, and Assemblies (PISA) server (Krissinel & Henrick 2007). The PISA server uses parameters such as hydrogen-bonding, interface area, and solvation energy gain to analyze a given structure and predict the most thermodynamically stable assemblies. The PISA algorithm predicts several interfaces that contribute significantly to assembly, with the most significant contributions deriving from metal-coordination at the N-terminal metal site, followed by interactions of the resulting loop with an adjacent subunit, and protein-protein contacts at the dimer interface (Table 4.2). Additional contributions derive from protein-protein interactions between subunits at the three-fold axis that passes through the iron exit channels, from coordination of metals at the ferroxidase center and the iron entry channels, and from coordination of a sulfate molecule adjacent to the metal at the iron exit channels. Based on these predictions, disruption of the N-terminal metal site should severely compromise oligomerization of Dps-1 and/or stability of the dodecameric assembly.
Removal of metals from dodecameric Dps-1 by bipyridyl does not alter the oligomeric state (Bhattacharyya & Grove 2007). To assess the prediction from the PISA analysis that it would destabilize the assembly, we compared thermal stability of Dps-1 and bipyridyl-treated protein using SYPRO Orange as a fluorescent reporter of protein unfolding (Figure 4.2). Consistent with predictions, the melting temperature of Dps-1 of 65.0±0.0ºC is reduced to 52.2±0.1ºC on removal of metals.

Table 4.2. PISA analysis predicts several interfaces that contribute to assembly of Dps-1

<table>
<thead>
<tr>
<th>Type of interaction</th>
<th>HB+SB</th>
<th>CSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-terminal Co²⁺ and protein subunit</td>
<td>0</td>
<td>0.415</td>
</tr>
<tr>
<td>N-terminal loop and adjacent subunit</td>
<td>18</td>
<td>0.385</td>
</tr>
<tr>
<td>Dimer interface</td>
<td>26</td>
<td>0.326</td>
</tr>
<tr>
<td>Adjacent subunits at 3-fold exit channel</td>
<td>16</td>
<td>0.101</td>
</tr>
<tr>
<td>Co²⁺ at iron entry channel</td>
<td>0</td>
<td>0.129</td>
</tr>
<tr>
<td>Co²⁺ at iron exit channel</td>
<td>0</td>
<td>0.094a</td>
</tr>
<tr>
<td>Co²⁺ at ferroxidase center/subunit 1</td>
<td>0</td>
<td>0.201</td>
</tr>
<tr>
<td>Co²⁺ at ferroxidase center/subunit 2</td>
<td>0</td>
<td>0.148</td>
</tr>
</tbody>
</table>

HB + SB, number of hydrogen bonds and salt bridges. CSS; Complex Formation Significance Score, an indicator of interface relevance to complex formation (Krissinel & Henrick 2007). In addition to the listed interfaces, contacts to a sulfate molecule near the metal at the iron exit channel is also predicted to contribute to complex stability. aNot a significant contribution. Calculations done using the PISA server at www.ebi.ac.uk/msd-srv/prot_int/pistart.html in auto processing mode with PDB 2F7N (Kim et al. 2006). Metal at the ferroxidase center is coordinated by two subunits, generating two distinct interfaces.
Structuring of the metal-bound loop may also be required for DNA binding as evidenced by the failure of dodecameric Dps-1 to bind DNA when metals have been removed by bipyridyl treatment (Bhattacharyya & Grove 2007). To address the function of the N-terminal metal-binding site directly, a mutant was created in which two of the four residues that coordinate the metal were mutated (His50 to Ser and Glu55 to Gln). The mutant protein named Dps-HE was successfully purified (Figure 4.3b, inset). To define the role of the N-terminal metal site in Figure 4.2: Thermal stability of Dps-1. Fluorescence emitted from SYPRO Orange upon binding to denatured protein measured as a function of temperature. Untreated Dps-1 (open symbol) and Dps-1 treated with bipyridyl (closed symbol).
Figure 4.3: Oligomeric state of Dps-HE. (a) The molecular mass standards used to standardize the FPLC column. (b) The elution pattern of Dps-HE. Inset shows SDS PAGE of purified Dps-HE (lane 2). (c) The logarithm of molecular mass as a function of elution volume. The arrow represents the elution volume of Dps-HE. (d) Native gel showing the oligomeric state of Dps-1, Dps-HE and Dps-dn. Lane 1 contains Dps-1, lanes 2 and 8 contains Dps-HE, lanes 3 and 4 contain Dps-HE with 800 and 400 nM Mn, respectively, lanes 5 and 7 contain bipyridyl-treated Dps-HE, lane 6 contains Dps-dn and lane 9 contains bipyridyl-treated Dps-HE followed by addition of 800 nM Mn.
dodecameric assembly, gel filtration was performed. These experiments reveal that Dps-HE (Mw ~23 kDa) elutes as an oligomer with a molecular weight of approximately 150 kDa, suggesting that it exists as a hexamer (Figure 4.3b-c). This is unusual because Dps homologs generally exist as a dimer, trimer or dodecamer (Gupta & Chatterji 2003; Grove & Wilkinson 2005; Bhattacharyya & Grove 2007). The incomplete oligomeric assembly of Dps-HE was confirmed by native gel electrophoresis. Full-length Dps-1 and Dps-dn (the mutant Dps-1 that lacks the first 55 amino acids, including the metal-binding site) were used as controls because both proteins were previously shown by gel filtration to exist exclusively as a dodecamer and a dimer, respectively (Bhattacharyya & Grove 2007). As seen in Figure 3d, dodecameric Dps-1 fails to migrate from the well (lane 1) while dimeric Dps-dn migrates a significant distance (lane 6). Dps-HE migrates in between Dps-1 and Dps-dn, which is consistent with a hexameric assembly (lanes 2 and 8).

An excess of Mn(II) was added to Dps-HE to see if it can restore dodecamer assembly. Mn(II) was chosen because *D. radiodurans* has an unusually high ratio of manganese to iron, a feature proposed to contribute to its radiation resistance. However, addition of Mn(II) did not cause Dps-HE to oligomerize to a dodecamer (lanes 3-4). Instead, removal of metals by chelation with bipyridyl causes significant disassembly into dimers (lanes 5 and 7); longer incubation of Dps-HE with bipyridyl increases the fraction of protein that exists as a dimer (data not shown), suggesting slow disassembly on removal of metals. Notably, when metals were added to the bipyridyl-treated Dps-HE, the dimers re-oligomerize to a hexamer (lane 9), indicating that hexameric assembly of Dps-HE is metal-dependent. Furthermore, glutaraldehyde-mediated crosslinking of Dps-HE and Dps-dn in the presence of excess metals also did not result in higher oligomeric states than a hexamer for Dps-HE and a dimer for Dps-dn.
(data not shown), suggesting that the failure to observe such higher oligomeric assemblies is not
due to their instability during electrophoresis. While we cannot rule out the existence of
oligomeric assemblies in solution that cannot be cross-linked, we consider this possibility
unlikely since crosslinking of full-length Dps-1 reveals the expected dodecamer (Grove &
Wilkinson 2005). Collectively, these experiments point to a requirement for metal-binding at the
N-terminal metal site for complete oligomeric assembly. That neither Dps-HE nor Dps-dn
assemble into higher-order oligomers in presence of Mn(II) also suggests that subunit
coordination around a metal site at the three-fold axes, whether at the iron entry or exit sites
(Table 4.1), is insufficient to drive assembly in absence of other stabilizing interactions.

Dps-HE binds metals

Substitution of residues that coordinate metal at the N-terminal metal site results in
incomplete oligomeric assembly, attesting to the involvement of this site in dodecamer
formation. Yet the presence of divalent metal is still required for dimeric Dps-HE to form the
larger oligomeric species seen in both gel filtration and native gels. To confirm that added metal
indeed binds to the proteins, we used 4-(2-pyridylazo) resorcinol (PAR), which binds various
divalent metals resulting in a diagnostic absorbance of the metal-PAR complex (McCall &
Fierke 2000) to evaluate metal-binding by Dps-1 and Dps-HE.

As shown in Figure 4.4a-b, uncomplexed PAR has an absorbance maximum at 416 nm
(gray line), and addition of bipyridyl-treated native or denatured Dps-1 (Figure 4.4a) or Dps-HE
(Figure 4.4b) (continuous and broken cyan lines, respectively) has no effect on the wavelength of
maximal absorbance, indicating the absence of divalent metals from bipyridyl-treated proteins
that can form a detectable complex with PAR. After incubation of bipyridyl-treated proteins
with Co^{2+}, a shift in the peak absorbance to 508 nm is seen on incubation of PAR with denatured
Figure 4.4: Metal binding by Dps-1 and Dps-HE. (a) Absorption spectrum of native, untreated Dps-1 (black continuous line), denatured, untreated Dps-1 (black broken line), bipyridyl-treated native Dps-1 (cyan line), bipyridyl-treated denatured Dps-1 (cyan broken line), metal-containing native Dps-1 (red line), metal-containing denatured Dps-1 (red broken line) and buffer with PAR (gray line). (b) Absorption spectrum of bipyridyl-treated native Dps-HE (cyan line), bipyridyl-treated denatured Dps-HE (cyan broken line), metal-containing native Dps-HE (red line), metal-containing denatured Dps-HE (red broken line) and buffer with PAR and SDS (gray line).
proteins (red broken line) while no absorbance peak at 508 nm is seen on incubation of PAR with native proteins (red continuous line). This absorbance maximum is diagnostic of the Co(II)-PAR complex and shows that both proteins bind the divalent metal. We attempted to assess metal-binding specifically to the N-terminal metal site; as this would require monomeric Dps-1 we conducted an experiment in which Dps-1 and Dps-HE were run on an SDS-PAGE gel followed by staining in a buffer containing PAR (addition of guanidine failed to disassemble the very stable Dps-1). This attempt was unsuccessful because the metals were no longer associated with the monomeric proteins, indicating that the N-terminal metal site was unfolded by exposure to SDS (data not shown). Significantly, the evidence for metal-binding by both bipyridyl-treated Dps-1 and Dps-HE supports the observed metal-dependent assembly of Dps-HE hexamers and the metal-dependent DNA binding discussed below.

The N-terminal metal-binding site affects DNA binding

Both dimeric and dodecameric forms of Dps-1 bind DNA, but dimeric Dps-1 has very low affinity (Grove & Wilkinson 2005). Deletion of the N-terminal extension obliterates the ability of Dps-1 to bind DNA whether or not the N-terminal metal site is retained, indicating that sequence preceding the metal site is required for DNA binding (Bhattacharyya & Grove 2007). If occupancy of the N-terminal metal site contributes to structuring the N-terminal extension for optimal DNA binding, then DNA binding by Dps-HE should be compromised. Electrophoretic mobility shift assays (EMSA) show that Dps-HE indeed binds DNA with a much lower affinity compared to full length Dps-1. There is no clear complex formation unless DNA is incubated with 50 nM of Dps-HE (Figure 4.5b). Furthermore, the complexes appear to be unstable and dissociate during electrophoresis as evidenced by the smeared appearance of protein-bound DNA. In contrast, Dps-1 forms complexes with DNA at ~0.5 nM dodecameric protein (Figure 4.5a). Quantitation of binding isotherms show that Dps-HE binds DNA with a K_d of 120±8 nM,
Figure 4.5: DNA binding by wild-type Dps-1 and Dps-HE. Electrophoretic analysis of 26 bp duplex DNA with Dps-1 (a) and Dps-HE (b) under equilibrium conditions ([DNA] < Kd). For Dps-1, concentrations range from 0-4 nM dodecamer. For the mutant Dps-HE, concentrations range from 0-800 nM hexamer. (c) Binding isotherm for Dps-1 binding to 26 bp DNA. (d) Binding isotherm for Dps-HE binding to 26 bp DNA. The Hill equation was used to obtain the best fit data (R²= 0.9677, n=1.4±0.1 for Dps-1 and R²=0.9726, n=0.7±0.1 for Dps-HE).
which is more than 100-fold higher compared to wild type Dps-1 ($K_d=0.5\pm0.1$ nM) (Figure 4.5c-d). The Hill coefficient for hexameric Dps-HE is <1, suggesting that there is no positive cooperativity as opposed to wild-type Dps-1, which binds DNA cooperatively. These data are consistent with the hypothesis that occupancy of the metal sites is required to orient the N-terminal extensions for optimal DNA binding.

Effect of metals on DNA-binding by Dps-1 and Dps-HE

Removal of metals from dodecameric Dps-1 destroys DNA binding (inferred to be due to removal of metal from the N-terminal site) but it does not disrupt oligomeric assembly, and subsequent addition of metal (Co(II)) restores DNA binding (Bhattacharyya & Grove 2007). This finding prompts additional questions. First, does bipyridyl-treated Dps-HE emulate Dps-1 in terms of metal-dependent DNA binding? Second, can any metal restore DNA binding to bipyridyl-treated Dps-1? Can the metals restore DNA binding in any condition, such as during oxidative stress? Finally, is there a preference for a particular metal at the metal binding sites?

To address the first question, an EMSA was conducted with bipyridyl-treated Dps-HE to which divalent metal was subsequently added. Results show that bipyridyl-treated Dps-HE lost its ability to bind DNA (Figure 4.6, lane 2) and that the addition of Mn(II) restored DNA binding (Figure 4.6, lane 3). This is similar to the previous finding with Dps-1, except that bipyridyl-mediated removal of metal from wild-type Dps-1 does not alter oligomeric state, whereas hexameric Dps-HE disassembles following incubation with bipyridyl (Figure 4.3d) (Bhattacharyya & Grove 2007).

To address the subsequent questions, EMSAs were conducted using different metals. Co(II) was used because it was seen to bind at all metal-binding sites in the crystal structure of
Dps-1, (Kim et al. 2006) Fe(II) was chosen because Dps is an iron storage protein, (Ilari et al. 2002) and Mn(II) was selected due to the high Mn:Fe ratio in *D. radiodurans* (Daly et al. 2004). Results confirm that removal of metals from Dps-1 by treatment with bipyridyl abolishes DNA binding (Figure 4.7a, lanes 2-4), suggesting that metal-binding is needed to orient the N-terminus in a way that allows it to bind DNA (an inference based on retention of the dodecameric superstructure and the absolute requirement for the N-terminal extensions for DNA binding) (Bhattacharyya & Grove 2007). Conversely, addition of either Co(II), Fe(II) or Mn(II) to bipyridyl-treated Dps-1 restores DNA binding (Figure 4.7a, lanes 5-13). Notably, Fe(II) fails to restore DNA binding to Dps-1 in the presence of hydrogen peroxide, conditions under which Dps-1 would also exhibit ferroxidase activity (Figure 4.7b, lanes 11 and 12). It is only at a high concentration of Dps-1 that DNA binding is restored in the presence of peroxide (Figure 4.7b, lane 13; this reaction corresponds to a condition where the concentration of N-terminal binding

![Figure 4.6](image)

**Figure 4.6: Effect of metal on DNA binding by bipyridyl-treated Dps-HEx.** DNA is incubated without Dps-HEx (lane 1), 60 nM bipyridyl-treated Dps-HEx (lane 2), 60 nM bipyridyl-treated Dps-HEx followed by the addition of 1 µM MnCl₂ (lane 3) and 60 nM of untreated Dps-HEx (lane 4). Equivalent results obtained with 400 nM Dps-HEx (data not shown).
Figure 4.7: Effect of different metals on DNA binding by bipyridyl-treated Dps-1. (a) DNA incubated with increasing concentration of bipyridyl-treated Dps-1 (1-10 nM); lanes 2-4, in the absence of metal; lanes 5-7, with 80 nM CoCl\(_2\); lanes 8-10, with 80 nM MnCl\(_2\); lanes 11-13, with 80 nM Fe(NH\(_4\))\(_2\)(SO\(_4\))\(_2\). (b) Same reactions as panel (a) with the addition of 1 mM H\(_2\)O\(_2\) (lanes 2-13). (c) Competition assay between Mn and Fe in the presence of H\(_2\)O\(_2\). Lane 2 has equal ratio of Mn:Fe, lanes 3-10 have varying ratio of Mn:Fe (2:1, 3:1, 7:1; 10:1, 1:2, 1:3, 1:7, and 1:10), lane 11 contains Mn only and lane 12 contains Fe only. Reactions in lanes 2-10 contain 1 mM of H\(_2\)O\(_2\) and reactions in lanes 2-12 contain 1 nM Dps-1. (d) Recovery assay using Mn(II). DNA binding of 1 nM bipyridyl-treated Dps-1 with Fe(II) (lane 2), Mn(II) (lane 3), Fe(II) with H\(_2\)O\(_2\) (lane 4) and Fe(II) with H\(_2\)O\(_2\) followed by addition of Mn(II) (lane 5). (e) Metal effect on DNA migration. Lane 2, Dps-1 with Fe(II); lane 3, Dps-1 with Fe(III); lane 4, Fe(II) only; lane 5, Fe(III) only; lane 6, Mn(II) only; lane 7, Co(II) only. In all five panels, the first lane corresponds to free DNA.
sites (120 nM) exceeds that of Fe\textsuperscript{2+} (80 nM). In contrast, H\textsubscript{2}O\textsubscript{2} has no effect on DNA binding in the presence of Co(II) and Mn(II) (Figure 4.7b, lanes 5-10). Since different metals can bind at the metal sites, and given the high Mn:Fe ratio characteristic of \textit{D. radiodurans}, we investigated if there is a preference for a particular metal using a competition assay. At equal ratio of Mn:Fe, DNA binding is not restored to bipyridyl-treated Dps-1 in the presence of peroxide (Figure 4.7c, lane 2). When the [Mn] is much higher (Mn:Fe is 7:1), DNA binding is restored (Figure 4.7c, lane 5). This suggests the occurrence of redox reactions that reduce the concentration of Mn(II) available to bind the site when Fe(II) is being oxidized by H\textsubscript{2}O\textsubscript{2} (and expected due to the higher reduction potential of Mn).

Since Fe(II) cannot restore DNA binding to bipyridyl-treated Dps-1 in the presence of peroxide, this suggests two possibilities. First, when Fe(II) binds to the N-terminal metal sites with peroxide present, the histidine residues that coordinate that site might get oxidized. A study by Lee and Helmann shows that the transcriptional regulator PerR fails to bind DNA when Fe(II) is bound at the metal site in the presence of peroxide. They also show that the loss of DNA binding is due to the histidine residues that coordinate the metal getting oxidized. The oxidation of those histidines prevents any metal from binding (Lee & Helmann 2006). Second, peroxide might oxidize Fe(II) to Fe(III), which can no longer bind at the site. To address which scenario is correct, a recovery assay was conducted in which Mn(II) was added to Fe(II)-treated Dps-1 in the presence of peroxide. As shown in Figure 4.7d, when Mn(II) is added to Dps-1 after Fe(II), DNA binding is restored (lane 5) compared to the lack of DNA binding when Dps-1 is incubated with Fe(II) and H\textsubscript{2}O\textsubscript{2} (lane 4) or with Fe(III) (Figure 4.7e, lane 3). This suggests that it is most likely the Fe(II) that is being oxidized as His-oxidation would have resulted in reduced metal-binding and hence significantly attenuated DNA-binding (Figure 4.5b). Finally, to rule out that
the metals affect the migration of the DNA, an EMSA was carried in which the metals (Fe(II), Fe(III), Mn(II), and Co(II)) were incubated with free DNA. Results show that the metals have no effect on DNA migration (Figure 4.7e).

**Mn(II) inhibits ferroxidase activity**

For some Dps homologs, certain metals can inhibit ferroxidase activity. For example, Zn(II) has been shown to block ferroxidase activity by competing with Fe(II) for binding at the ferroxidase site (Stefanini et al. 1999). *D. radiodurans* accumulates a high level of Mn(II); therefore, we determined the effect of this metal on ferroxidase activity. Using equivalent concentrations of monomer, each protein sample contains the same concentration of ferroxidase centers (but not the same molar concentration of dodecamer or hexamer, respectively). Both Dps-1 and Dps-HE exhibit ferroxidase activity, consistent with a hexameric assembly of Dps-HE containing three ferroxidase centers. Interestingly, there appears to be a delay at the start of the ferroxidation reaction for Dps-HE before a gradual increase in absorbance is visible, perhaps reflecting assembly issues that are delaying the ferroxidase activity of this mutant. However, the ferroxidase activity of both Dps-1 and Dps-HE is completely abolished in the presence of Mn(II), suggesting that Mn(II) competes for binding to the ferroxidase center (Figure 4.8).

**Discussion**

**Ferroxidase activity of Dps-1 may be prevented in vivo**

Mn(II) inhibits ferroxidase activity of both Dps-1 and Dps-HE at a Mn:Fe ratio of 0.2, comparable to the ratio reported in *D. radiodurans* (Daly et al. 2004). This inhibition is likely due to Mn(II) binding at the ferroxidase site. Although we cannot rule out the possibility that Mn(II) binds other sites, resulting in an altered conformation of the ferroxidase center, this is unlikely for a couple of reasons. First, altering the metal site at the N-terminus has little impact.
on the ferroxidase activity (Figure 4.8). This is also evidenced by a previous report that Dps-dn, the mutant that lacks the entire N-terminus, exhibits ferroxidase activity (Bhattacharyya & Grove 2007). Second, mutations in the metal site at the iron exit channel did not affect the ferroxidase activity or the core formation of Dps-1 (Kim et al. 2006). Therefore, even if Mn(II) binds at those sites, it would not be expected to affect the ferroxidase activity. *D. radiodurans* accumulates manganese, which is globally distributed, and it sequesters iron in a region overlapping the septum between dividing cells (Daly et al. 2007). Dps-1 is associated with the

![Figure 4.8: Effect of Mn(II) on ferroxidase activity of Dps-1 and Dps-HE.](image)

Iron oxidation by Dps-1 and Dps-HE in 20 mM MOPS buffer. The concentration of both protein were 0.2 mg/ml. The kinetics of iron oxidation by Dps-1 and air were measured at 310 nm. Ferroxidase activity of Dps-1 with Fe(II) (diamond), Dps-HE with Fe(II) (square), Fe(II) only (circle), Dps-1 with Fe(II) and Mn(II) (triangle) and Dps-HE with Fe(II) and Mn(II) (X).
nucleoid (Nguyen et al. 2009). The physiological implication, therefore, is that ferroxidase activity of Dps-1 may be prevented in a cellular environment where [Mn]>[Fe] (Figure 4.9).

The N-terminal metal site affects DNA binding

Dodecameric Dps-1 may be thought of as composed of six dimers, each composed of two subunits oriented in an antiparallel fashion (Kim et al. 2006, Romão et al. 2007). DNA binding has been proposed to involve contact with the N-terminal extensions protruding from either end of a dimer and with Arg132 in the short helix between the second and third helix of the four-

![Figure 4.9: Model of Dps-1 function. Left panel indicates Dps-1 binding to DNA under normal physiological conditions where [Mn^{2+}]>[Fe^{2+}]. One Dps-1 dimer is shown in cyan with metal bound at the N-terminal metal site in magenta. Arrows represent N-terminal extensions interacting with DNA major grooves. When Fe^{2+} and ROS are present, Dps-1 catalyzes the oxidation of Fe^{2+} to Fe^{3+} to prevent hydroxyl radical formation by Fenton chemistry, but with the potential for iron subsequently leaving the mineral core. Without metal bound at the N-terminal metal site, the N-terminal extensions are not structured properly for DNA interaction.](image)

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helix bundle subunit (Figure 4.9, left panel) (Bhattacharyya & Grove 2007, Nguyen et al. 2012). Positive cooperativity of DNA binding by Dps-1 is seen only with dodecameric protein, which features six DNA sites, (Nguyen et al. 2012) and not with dimeric Dps-1 or hexameric Dps-HE, pointing to conformational changes on DNA binding to dodecameric protein that propagate to other DNA sites. Dimeric Dps-1 features only one DNA site, while the lack of cooperativity of DNA binding by Dps-HE, which would be expected to have three DNA sites, suggests that the connectivity between DNA sites that is evidenced by the positive cooperativity of DNA binding by dodecameric Dps-1 is lost in Dps-HE, perhaps related to its suboptimal assembly.

DNA binding by Dps-HE is significantly reduced (Figure 4.5). Dodecameric Dps-1 has been shown to feature six DNA sites, with N-terminal extensions preceding the metal site required for DNA binding, along with Arg132 in the middle of the four-helix bundle subunit (Nguyen et al. 2012). This suggests that DNA binds with its axis parallel to the dimer interface. If hexameric assembly of Dps-HE were the only difference compared to dodecameric Dps-1, with individual DNA sites otherwise remaining unaltered, a change in DNA-binding affinity reflecting half the number of DNA sites would be expected for Dps-HE. Instead, we observe a >100-fold decrease in affinity. An interpretation more consistent with the observed change in DNA binding is therefore that metal-coordination aids in structuring the N-termini, thereby mediating optimal DNA contacts. Since a Dps-1 mutant containing the N-terminal metal site but lacking the flexible N-terminal extension fails to bind DNA, the metal site is likely required only for structuring the N-terminus and not for direct DNA contacts. However, as for Dps-1, DNA binding by Dps-HE is metal-dependent. Inspection of the Dps-1 sequence suggests the presence of several alternate metal-coordinating residues in the N-terminus. For example, Glu21, Asp33, His34, His51, Glu54 and Glu57 might be able to participate in this coordination; as there are
several His and Glu residues in the N-terminus that could substitute for the His and Glu that were mutated in Dps-HE, the chemistry of coordination is likely unaltered. If the original metal site is disrupted and a metal is coordinated by other residues, then that would change the conformation of the loop as well as the disposition of the N-terminus. As a result, the N-terminal extension may not be oriented such that optimal DNA binding can occur. That removal of metals by bipyridyl treatment led to a complete loss of DNA binding for both Dps-1 and Dps-HE is likely due to excessive flexure of the N-terminal extension.

**Differential metal effects on DNA binding**

Experiments with bipyridyl-treated Dps-1 show that different metals ((Co(II), Mn(II) and Fe(II)) can restore DNA binding to metal-free Dps-1. Notably, Fe(II) is unable to restore DNA binding in the presence of hydrogen peroxide. Since addition of Mn(II) to the peroxide-treated, Fe(II)-bound Dps-1 restores DNA binding, Fe(II) is likely oxidized to Fe(III), which cannot bind the metal site (Figure 4.7). We also note that since Dps-1 binds DNA in the presence of Fe$^{2+}$ (Figure 4.7a), the interpretation is that Fe$^{2+}$ already bound is protected from oxidation by molecular oxygen on the time scale of the DNA-binding assay, but not from oxidation by H$_2$O$_2$. The physiological relevance of this observation is that Dps-1 may be prevented from binding DNA under conditions of ongoing ferroxidation, conditions that might also lead to subsequent release of iron from the core and the consequent production of •OH through Fenton chemistry (Figure 4.8). Thus, differential metal-binding at the N-terminal metal site provides a possible mechanism by which the two functions of Dps-1 may be separated to prevent •OH-mediated damage to Dps-1-bound DNA.
Proposed assembly of hexameric Dps-HE

Dps-1 exists almost exclusively as a dodecamer. Only at low salt concentrations can dimeric Dps-1 be detected following expression in *E. coli* (Grove & Wilkinson 2005). No monomer may be detected, even for Dps-dn that lacks the entire N-terminal extension (Bhattacharyya & Grove 2007). This is consistent with the PISA algorithm, which uses thermodynamics calculations to predict which assemblies are likely to be most stable in solution; PISA predicts that both protein-protein contacts at the dimer interface as well as metal coordination at the ferroxidase center contribute significantly to thermodynamic stability of the dodecameric superstructure (Table 4.2). As these contacts would also be present in dimeric Dps-1, it is reasonable to infer that this interface is responsible for stability of the Dps-1 dimer. Assembly of dodecameric Dps-1 is therefore likely to occur by association of six dimers. This is different from the reported assembly of *M. smegmatis* Dps1, for which trimers were suggested to be assembly intermediates (Roy et al. 2007; Chowdhury et al. 2008). That disruption of the N-terminal metal site to generate Dps-HE compromises assembly clearly shows that proper conformation of the metal-bound loop is a prerequisite for dodecamer formation (Figure 4.3). The structure of Dps-1 shows that the metal-bound loop contacts a neighboring dimer, and evaluation of Dps-1 using PISA also points to this interface participating significantly in assembly; for instance, His51 within the loop forms a salt bridge with Asp191 of the adjacent subunit and Tyr52 forms a hydrogen bond to Arg165 (Kim et al. 2006). Consistent with the important contribution of such interactions, we also note that Arg165 corresponds to one of two residues that when substituted together in *E. coli* Dps prevent dodecamer formation (Zhang et al. 2011). That metals may be removed from dodecameric Dps-1 without effect on oligomeric state, but with severe consequences to DNA-binding, also suggests that contacts between the metal-
bound loop and the adjacent dimer are destabilized on removal of metal, but no longer needed to maintain the oligomeric state. The destabilization predicted by PISA analyses to result from removal of metals is evident in the significantly reduced thermal stability of bipyridyl-treated Dps-1 (Figure 4.2). Taken together, our data suggest that occupancy of the N-terminal metal site is required for initial contacts between associating dimers, but not to maintain the dodecameric assembly once attained.

For Dps-HE, the N-terminal loops are not properly folded, which means that optimal interactions between adjacent dimers cannot occur. The outcome is a hexameric assembly that is incompatible with further assembly into a dodecamer. The delay at the start of the ferroxidation reaction with Dps-HE likely also reflects its suboptimal assembly, since the ferroxidase center resides between two subunits. That Dps-HE does not exist as a dimer in the presence of divalent metal suggests that alternate residues coordinate the metal, as discussed above. This is also supported by the previous observation that a mutant Dps-1 in which the first 33 amino acids are removed could not form a dodecamer unless the N-terminal His-tag was cleaved. This finding suggests that the extra histidine residues interfered with proper metal coordination to prevent oligomerization (Bhattacharyya & Grove 2007). Evidently, coordination of metal by alternate residues promotes some interaction between dimers to yield the observed hexamer.

Dps-1 contains two types of three-fold axes that pass through either the iron entry or exit channels, the ferritin-like and Dps-like axes, respectively (Table 4.1). There is a metal site at the iron entry channel, and this metal is coordinated by Asp181 from three subunits. In one of the two published Dps-1 structures, this site is not filled unless the crystals are soaked in a solution containing Fe$^{2+}$ (Romão et al. 2007). This suggests that occupancy of this site is unnecessary for dodecameric assembly. Likewise, a metal near the iron exit channel is also coordinated by
residues from three subunits. However, for the metal site at the iron exit channel to have a structural role is also unlikely since mutations in this site did not affect dodecameric assembly (Kim et al. 2006). Considering the building blocks of dodecameric Dps-1 to be a dimer, a hexamer centered around the iron entry site would be primarily stabilized by the N-terminal loops interacting with the neighboring dimer, while a hexamer assembled around the three-fold axis that passes through the iron exit site would in addition be stabilized by protein-protein contacts between adjoining subunits and the embedded sulfate ion. Consistent with the contribution of these interfaces, PISA analysis predicts that a trimer assembled around the exit pore would be more stable than one assembled around the entry site. We therefore propose that the Dps-HE hexamer may correspond to three dimers assembled about the iron exit (Dps-like) channel.

In conclusion, our data show that metal-binding at the N-terminus is required to initiate dodecameric assembly, and that incomplete structuring of the N-terminal loop leads to a hexameric assembly intermediate. After assembly, metal-binding is no longer required to maintain the oligomeric state, instead it impacts DNA interactions, particularly under conditions of oxidative stress. This affords a mechanism by which DNA-binding is avoided under conditions where Dps-1 would engage in ferroxidation. Combined with the efficient inhibition of ferroxidation by Mn(II) at the Mn:Fe ratio observed in D. radiodurans, this regulation may serve to separate the two functions of Dps-1 to prevent damage to Dps-1-bound DNA deriving from iron leaking from the core followed by generation of •OH through Fenton chemistry.
CHAPTER 5: SUMMARY AND CONCLUSIONS

The dodecameric protein Dps has been reported to contribute significantly to the survival during oxidative stress of several bacteria including *E. coli*, in which deletion of this protein leads to higher sensitivity to H$_2$O$_2$. Protection involves interacting with the DNA and oxidizing and sequestering iron to prevent hydroxyl radical formation (Almirón et al. 1992; Calhoun & Kwon 2010; Chiancone & Ceci 2010). In *D. radiodurans*, there are two Dps homologs: Dps-1 and Dps-2. Dps-1 is different from other Dps homologs because it is unable to protect DNA from ROS due to the release of iron from the protein (Grove & Wilkinson 2005; Kim et al. 2006). Dps-2 is the first Dps homolog reported to have a signal peptide, suggesting that it is not localized in the cytoplasm (Cuypers et al. 2007; Reon et al. 2012). In this study, I examined the functions of the two Dps homologs. Furthermore, I identified the role of the N-terminal metal site of Dps-1 and the C-terminal extension of Dps-2 in DNA binding and oligomerization.

**The Functions of Dps-1 vs. Dps-2**

DNA binding and protection assays show that Dps-2 is capable of protecting DNA against ROS, a trait that Dps-1 lacks (Figure 2.3). This is consistent with the results from the β-galactosidase assay, in which the Dps-2 promoter is strongly expressed in the presence of H$_2$O$_2$ while Dps-1 promoter is not (Figure 2.5). When the Dps-2 signal peptide is fused with EGFP, the majority of the fluorescence is found on the outer region of the cells, suggesting that Dps-2 is non-cytoplasmically localized (Figure 2.4). EMSA results show that Dps-2 does not bind DNA as well as Dps-1 (Figure 2.3 and data not shown). If Dps-2 is not localized near the nucleoid, then there is no reason why it would need to bind DNA strongly, unless it participates in uptake of foreign DNA. Taken together, these data suggest that Dps-2 is found outside of the cytoplasm and is providing some level of protection against extracellular H$_2$O$_2$. On the other hand, Dps-1 is
probably found in the cytoplasm and its role might be to participate in organizing genomic DNA and iron homeostasis (Reon et al. 2012).

**The Role of the C-terminal Extension in Dps-2**

When the C-terminus is removed from Dps-2, the mutant protein (CLess) exists as a monomer (Figure 2.1), suggesting that this C-terminal extension is required for oligomerization. However, this extension is not needed for DNA binding as CLess is still capable of binding DNA. CLess is also capable of providing protection against ROS and to a lesser extent, provide protection against DNase. In the presence of divalent metals, data suggest that CLess can oligomerize to a dimer, suggesting that the ferroxidase center might aid in dimer formation when there is a metal bound, an inference that is supported by PISA analyses. Since metal binding to monomeric CLess Dps-2 would also be predicted to reduce electrophoresis mobility, conformation by gel filtration is required, although the ability to protect against ROS would be difficult to reconcile with a monomeric species (Reon et al. 2012).

**The Mode of DNA Binding of Dps-1**

Wild type Dps-1 contains six dimers, indicating that there might be six DNA binding sites. This inference is confirmed with the results from electrophoretic mobility assays and intrinsic tryptophan fluorescence, which reveal that the stoichiometry of Dps-1 with 22-bp DNA substrate is 1:6 (Figure 3.3). When the 26-bp DNA substrate is used, the stoichiometry is only 1:4 (Figure 3.3). This finding suggest that the dodecameric Dps-1 can bind on both faces of the DNA if the duplex is long enough to feature two consecutive major grooves on each face (Figure 3.1). The structure of Dps-1 reveals an arginine residue (Arg132) on the surface of the short helix in the four helix bundle. When this residue is mutated, there is a major decrease in the
affinity of the protein for the DNA. These results indicate the DNA interacts with this residue on the protein body after the first contact with the N-terminus and position at the dimer interface (Nguyen et al. 2012).

**The Importance of the N-terminal Metal Site in Dps-1**

There is a metal site at the N-terminus of Dps-1 that is coordinated by four residues from one subunit. When this site is disrupted, the mutant protein (Dps-HE) becomes a hexamer and DNA binding is decreased dramatically. When the metals are removed from Dps-HE using bipyridyl, it breaks down further into dimers while addition of metals back to the bipyridyl treated Dps-HE restores hexamer formation. This suggests that hexamers assemble around of the two metal-bound channels. Nevertheless, the addition of surplus metals was unable to restore dodecameric formation, suggesting that this metal site is needed for proper oligomerization (Nguyen & Grove 2012).

**Impact of Different Metals on DNA Binding and Ferroxidation of Dps-1**

When Mn(II), Co(II) or Fe(II) is added to the bipyridyl treated Dps-1, the protein is able to bind DNA again. However, in the presence of H₂O₂, Fe(II) is unable to restore DNA binding. The results from the recovery assay indicate that the Fe(II) is probably oxidized to Fe(III) by H₂O₂, which can no longer bind at the metal site and orient the N-terminus in a proper orientation to allow DNA binding. Also, the presence of Mn(II) hinders ferroxidase activity of Dps-1, indicating that this metal competes with Fe(II) for binding at ferroxidase site. The significance of these observations is that DNA binding and degradation is prevented should Dps-1 bind Fe³⁺ in place of the more abundant Mn²⁺ (Nguyen & Grove 2012).
Future Studies

Mutagenesis of the Dps-1 N-terminal metal site did not cause the protein to break down completely into a monomer or dimer. Instead, the protein can still exist as a half shell (Nguyen & Grove 2012), which means that there other factors that contribute to the assembly. In addition to the N-terminal metal site, Dps-1 has three other metal sites. The second site is localized at the ferroxidase center and is coordinated by residues from two subunits (Figure 1.2a). The other two sites are found at the iron entry (Figure 1.2b) and iron exit channel (Figure 1.2c) and these sites are made up of residues from three subunits (Kim et al. 2006). Therefore, by disrupting these other three metal sites, it would shed more lights on the assembly process of Dps-1.

The mechanism on which Dps-2 binds DNA is not well-studied. It would be interesting to see if DNA interacts with Dps-2 along the dimer interface in a similar fashion as Dps-1 which can be done by conducting stoichiometry experiments. In addition to the C-terminal extension, Dps-2 also has an N-terminal extension (Cuypers et al. 2007) so it would be logical to truncate the entire N-terminal extension to see what effect that will have on oligomerization and DNA binding of Dps-2. Also, the different migration pattern of CLess Dps-2 in the presence of metals (Figure 2.2d) opens up the question on whether CLess can oligomerize to a dimer in the presence of excess metals; therefore, gel filtration or analytical ultracentrifugation experiments using CLess with excess metals would be helpful in answering this question.
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Author: Khoa Huynh Nguyen and Anne Grove

Publication: Biochemistry

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VITA

Khoa Huynh Nguyen was born in Saigon, Vietnam to Ut Van Nguyen and Mui Huynh. He attended Broadmoor High School where he graduated as Salutatorian in May 2004. After high school, he attended Louisiana State University. He graduated in May 2008 as Magna Cum Laude with a Bachelor in Biological Sciences. He then enrolled in graduate school to do research under Dr. Anne Grove’s supervision. During his time in graduate school, Khoa serves as a teaching assistant for various lab and lecture courses including: introductory biology, microbiology, genetics and molecular genetics. After finishing his Ph.D. degree, Khoa plans to apply for an instructor position at a university.