The N-terminal Extensions of Deinococcus radiodurans Dps-1 Mediate DNA Major Groove Interactions as well as Assembly of the Dodecamer*\[^{\text{S}}\]

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Dps (DNA protection during starvation) proteins play an important role in the protection of prokaryotic macromolecules from damage by reactive oxygen species. Previous studies have suggested that the lysine-rich N-terminal tail of Dps proteins participates in DNA binding. In comparison with other Dps proteins, Dps-1 from Deinococcus radiodurans has an extended N terminus comprising 55 amino acids preceding the first helix of the 4-helix bundle monomer. In the crystal structure of Dps-1, the first ~30 N-terminal residues are invisible, and the remaining 25 residues form a loop that harbors a novel metal-binding site. We show here that deletion of the flexible N-terminal tail obliterates DNA/Dps-1 interaction. Surprisingly, deletion of the entire N terminus also abolishes dodecameric assembly of the protein. Retention of the N-terminal metal site is necessary for formation of the dodecamer, and metal binding at this site facilitates oligomerization of the protein. Electrophoretic mobility shift assays using DNA modified with specific major/minor groove reagents further show that Dps-1 interacts through the DNA major groove. DNA cyclization assays suggest that dodecameric Dps-1 does not wrap DNA about itself. A significant decrease in DNA binding affinity accompanies a reduction in duplex length from 22 to 18 bp, but only for dodecameric Dps-1. Our data further suggest that high affinity DNA binding depends on occupancy of the N-terminal metal site. Taken together, the mode of DNA interaction by dodecameric Dps-1 suggests interaction of two metal-anchored N-terminal tails in successive DNA major grooves, leading to DNA compaction by formation of stacked protein-DNA layers.

All aerobic microorganisms are exposed to reactive oxygen species (ROS)\(^2\) such as O\(_2^•\), H\(_2\)O\(_2\), and -OH that can damage cellular macromolecules, including proteins, lipids, and DNA. Therefore, they have developed a number of defense mechanisms to combat such stress conditions in the environment. One of the key components in the response to oxidative stress in prokaryotes is the nonspecific DNA-binding protein Dps (DNA protection during starvation).

Toxicity of the ROS H\(_2\)O\(_2\) itself is relatively weak, but it can form highly reactive hydroxyl radicals in the presence of transition metals such as Fe\(^2+\) according to the Fenton reaction (H\(_2\)O\(_2\) + Fe\(^2+\) → -OH + \(-\)OH + Fe\(^3+\)) (1). Thus, the presence of iron increases the probability of oxidative damage to cellular components. Dps, initially studied in Escherichia coli, was shown to protect DNA by its ability to chelate ferrous iron and also by its physical association with DNA (2–5).

Twelve Dps monomers form a spherical assembly similar to the spherical shell formed by 24 subunits of the iron storage protein, ferritin (6–9). Each Dps monomer adopts a four-helix (A–D) bundle conformation as seen for ferritin, but unlike ferritin, Dps possesses a short helix in the middle of the BC loop and lacks the C-terminal fifth helix present in the ferritin monomer (10–13). Secondly, the ferroxidase site in Dps is usually generated at the interface between two subunits and, with one reported exception, is not within the four-helix bundle as in the case of ferritin (14, 15). It is also notable that not all Dps homologs follow the same catalytic mechanism, as exemplified by the absence of a conserved ferroxidase center in Lactococcus lactis DpsB and the failure of Bacillus anthracis Dps1 to utilize H\(_2\)O\(_2\) in the ferroxidation reaction (16, 17).

In contrast to the highly conserved ferroxidase center, Dps homologs have a variable N-terminal extension. This N-terminal tail, which contains multiple positively charged residues, extends from the four-helix bundle core into the solvent (11, 18, 19). Because the surface of the Dps protein does not display “classical” DNA binding motifs and is dominated by negative charges, it has been proposed that the DNA binding properties of E. coli Dps, the family prototype, are associated with the presence of the lysine-rich N-terminal tail (11). Consistent with this notion, proteins that do not have an N-terminal extension such as the Dps homolog Hp–NAP from Helicobacter pylori or Dps from Agrobacterium tumefaciens, whose N-terminal tail is immobilized on the protein surface, fail to bind DNA (20, 21).

The mesophilic, non-spor-forming eubacterium Deinococcus radiodurans is known for its extraordinary ability to withstand the lethal and mutagenic effects of DNA damaging agents, such as ionizing radiation and desiccation, both conditions that are characterized by the presence of oxidative radi-
**D. radiodurans Dps-1 Binds Successive DNA Major Grooves**

**EXPERIMENTAL PROCEDURES**

**Mutagenesis, Overexpression, and Purification of Dps-dn and Dps-met**—Deletion of the entire N-terminal extension (Dps-dn) was accomplished by amplifying the Dps-1 gene lacking 165 bp at the N terminus from plasmid containing the entire Dps-1 gene (pET5a-dps1) using forward primer 5'-GAAAGAGCATATGACCCTTCGTC-3' and reverse primer 5'-CTTTAGAAGATCCCTTTCATTTCC-3'. The amplified PCR fragment was then cloned into the T7-NT/TOPO vector (Invitrogen).

Dps-1 retaining the N-terminal metal site was generated by amplification of the Dps-1 gene lacking 99 bp at the N terminus from plasmid containing the entire Dps-1 gene (pET5a-dps1) using forward primer 5'-GGCGGCCATTGCGAGTCAGCCTTC-3' and reverse primer 5'-CTTTAGAAGATCCCTTTCATTTCC-3'. The PCR product was then re-amplified using forward primer 5'-CACCATGACCCTTTCGTC-3' and reverse primer 5'-CTTTAGAAGATCCCTTTCATTTCC-3' to introduce the sequence necessary to clone it into the Champion pET100/D-TOPO vector (Invitrogen). The integrity of the constructs was confirmed by sequencing.

Each of the resulting plasmids was transformed into E. coli BL21(DE3)pLysS, and overexpression was induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside at an A600 of 0.3. Cells were pelleted 2 h after induction and stored at −80 °C. The cell pellet was resuspended in a lysis buffer, pH 8.0 (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 10% glycerol, 1 mM 2-mercaptoethanol, and 1 mM phenylmethlysulfonyl fluoride), and lysed by sonication. Nuclear acids were digested by the addition of DNase I followed by a 1-h incubation on ice. The cell lysate was centrifuged at 4 °C for 20 min at 5000 rpm. The supernatant was mixed with 5 ml of HIS-Select nickel affinity gel (Sigma) and incubated at 4 °C for 30 min. The mixture was then poured into a column and washed with 5 column volumes of wash buffer, pH 8.0 (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, 10% glycerol, 1 mM 2-mercaptoethanol, and 1 mM phenylmethlysulfonyl fluoride). Proteins were eluted with a 40-ml linear gradient from 20 mM imidazole (wash buffer) to 250 mM imidazole (elution buffer, pH 8.0, 50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, 10% glycerol, 1 mM 2-mercaptoethanol, and 1 mM phenylmethlysulfonyl fluoride) followed by 50 ml of elution buffer. Pure Dps-dn and Dps-met fractions were pooled, and protein concentrations were determined by quantification of Coomassie Blue-stained SDS-PAGE gels using bovine serum albumin as a standard. Untagged full-length Dps-1 was prepared as described (24). All protein preparations were judged to be >95% pure based on Coomassie-stained SDS-PAGE gels.

**Cleavage of the His Tag from Recombinant Dps-dn and Dps-met**—Fifty μg of protein was incubated with 1 unit of recombinant enterokinase (rEK) from Novagen at room temperature for 16 h in rEK cleavage buffer (50 mM NaCl, 20 mM Tris-HCl, 2 mM CaCl₂, pH 7.4) supplied with the enzyme. The cleavage reactions were judged by SDS-PAGE to be complete.

**Protein Cross-linking**—Proteins were cross-linked with 0.1% (v/v) glutaraldehyde in presence of 10 mM Hepes, pH 7.8, and 50 or 500 mM NaCl in a total reaction volume of 10 μl at room temperature for 30 min. The reaction was terminated by the addition of an equal volume of Laemmli sample buffer, and the cross-linked products were analyzed by SDS-PAGE followed by Coomassie Blue staining.

**Native Polyacrylamide Gel Electrophoresis**—The oligomeric state of wild type Dps-1 and the N-terminal deletion mutants was observed on 5% non-denaturing acrylamide gels. The gel recipe was the same as the running gel of SDS-PAGE according to the method of Laemmli, excluding the presence of SDS. The electrophoresis was carried out in 375 mM Tris-HCl, pH 8.7.

**FPLC and Gel Filtration**—All steps of gel filtration were carried out 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 A, pH 8.0 (50 mM NaH₂PO₄, 10 mM imidazole, 10% glycerol) and then with 2 column volumes of buffer B, pH 8.0 (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 10% glycerol). The gel filtration standard (Bio-Rad), which is a mixture of bovine thyroglobulin (170 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. The concentration of protein applied to the gel filtration column was 5 mg/ml for both wild type Dps-1 and Dps-dn. The proteins were run independently under the same conditions and were eluted with a flow rate of 0.5 ml/min.

**Ferroxidation by Dps-dn and Dps-met**—The kinetics of iron oxidation by Dps-dn and Dps-met was measured at 310 nm using an Agilent 8453 spectrophotometer. Proteins were diluted to 0.2 mg/ml in 20 mM Mops, pH 7.0, 100 mM NaCl. Solutions of ferrous ammonium sulfate, which were used as the
source of ferrous iron, were freshly prepared immediately before each experiment. The kinetic data were plotted using Prizm.

Electrophoretic Mobility Shift Assays—Superoiled pGEM5 (100 ng corresponding to 52 fmol of plasmid) was mixed with protein in 10 μl of binding buffer (20 mM Tris-HCl, pH 8.5, or 500 mM NaCl, 10 mM MgCl2, 0.1 mM Na2EDTA, 1 mM dithiothreitol, 0.05% Brij58, 100 μg/ml of BSA) and incubated at room temperature for 30 min. The entire reaction was then loaded onto a 1% (w/v) agarose gel in 0.5× TBE (45 mM Tris borate, pH 8.3, 1 mM EDTA). The gel was stained with ethidium bromide after electrophoresis.

Oligodeoxyribonucleotides used to generate short duplex DNA constructs were purchased and purified by denaturing polyacrylamide gel electrophoresis. The sequence of 26-, 22-, 18-, and 13-bp (all average G + C content) DNA is available as supplemental material. The top strand was 32P-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) before loading the gel.

Electrophoretic mobility shift assays were performed using 10% polyacrylamide gels (39:1 (w/w) acrylamide:bisacrylamide) in 0.5× TBE unless specified otherwise. Gels were pre-run for 30 min at 175 volts at room temperature before loading the samples with the power on, except for experiments with 18- and 13-bp duplex, which were performed at 4 °C to ensure stability of the duplexes. DNA and protein were mixed in binding buffer (containing 50 mM NaCl for dimeric Dps-1 and 500 mM NaCl for dodecameric Dps-1), and each sample contained 50 fmol (for dimeric Dps-1) or 2.5 fmol (for dodecameric Dps-1) of DNA in a total reaction volume of 10 μl unless indicated otherwise. 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 a complex to account for complex dissociation during electrophoresis. Data were fit to the Hill equation, where [Dps-1] is the protein concentration, f is fractional saturation, Kd reflects the apparent equilibrium dissociation constant, and n is the Hill coefficient. Fits were performed using the program KaleidaGraph, and the quality of the fits was evaluated by visual inspection, χ2 values, and correlation coefficients. All experiments were carried out at least in triplicate.

Effect of Divalent Metal Ions on DNA Binding—To remove the divalent cations from Dps-1, the protein was incubated with 50 mM bipyridyl for 20 min at 4 °C. The bipyridyl or metal-bipyridyl complex was then removed from the protein solution 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 phenylmethylsulfonyl fluoride) at 4 °C for 2 h. DNA (2.5 fmol) was then incubated with 0–12.4 nm bipyridyl treated protein with or without 80 nM CoCl2 (chosen as it is known from the crystal structure to bind the N-terminal metal site) at room temperature for 30 min. The reactions were analyzed on a 10% polyacrylamide gel (39:1 (w/w) acrylamide:bisacrylamide) in 0.5× TBE. After electrophoresis, the gel was dried, and protein-DNA complexes and free DNA were visualized by phosphorimaging.

Netropsin Assay—Eight nm dodecameric Dps-1 was incubated with 50 fmol of 26-bp double-stranded 32P-labeled DNA containing an 8-bp TATA box (the sequence available in the supplemental material) in 10 μl of binding buffer with 500 mM NaCl at room temperature for 45 min. Then the minor groove binding drug netropsin was added to the reaction to a final concentration of 1 μM for an additional 45-min incubation. The effect of netropsin on TATA box binding-protein (TBP)-DNA complex formation was studied in parallel where the same 26-bp duplex DNA was incubated with 1070 pmol of TBP in a reaction buffer (40 mM Tris-HCl, pH 8.0, 10 mM NaCl, 7 mM MgCl2, 3 mM dithiothreitol, 10 μg/ml BSA) followed by the addition of netropsin. All reactions were analyzed on a 10% polyacrylamide gel (39:1 (w/w) acrylamide:bisacrylamide) containing 2.5 mM MgCl2 in 0.5× TBE with 2.5 mM MgCl2. After electrophoresis, the gel was dried, and protein-DNA complexes and free DNA were visualized by phosphorimaging.

Methylation Interference Assay—26-bp 32P-labeled double-stranded DNA was methylated by treatment with 0.5% dimethyl sulfate (DMS) for 10 min at room temperature in a total reaction volume of 10 μl. The reaction was stopped by the addition of 2.5 μl of DMS stop solution (1.5 M sodium acetate and 1 M β-mercaptoethanol), and the DNA was recovered by ethanol precipitation. 8 nm dodecameric Dps-1 was incubated with 50 fmol of 26-bp labeled duplex DNA treated with or without DMS in binding buffer containing 500 mM NaCl at room temperature for 60 min. The reactions were analyzed on a 10% polyacrylamide gel (39:1 (w/w) acrylamide:bisacrylamide) in 0.5× TBE. After electrophoresis, the gel was dried, and protein-DNA complexes and free DNA were visualized by phosphorimaging.

DNA Cyclization—Plasmid pET5a was digested with BspHI to yield a 105-bp fragment, which was purified on a 2% agarose gel. Ligase-mediated DNA cyclization experiments were carried out with varying protein concentrations. Reactions were initiated by the addition of 80 units of T4 DNA ligase to a final volume of 10 μl. Reactions containing 10–100 fmol of DNA and the desired concentration of Dps-1 or Thermotoga maritima HU were incubated in 1× binding buffer with 200 mM NaCl and 1× ligase buffer at room temperature for 60 min. Reactions were terminated using 3 μl of 10% SDS followed by phenol-chloroform extraction and ethanol precipitation. Reactions were analyzed on an 8% polyacrylamide gel (39:1 (w/w) acrylamide:bisacrylamide) with 0.5× TBE as running buffer. After electrophoresis, gels were dried, and ligation products were visualized by phosphorimaging. All experiments involving protein-DNA interaction or native gel electrophoresis were performed at least in triplicate and with at least two different protein preparations.

RESULTS

Structural Considerations—A comparison of the Dps-1 amino acid sequence with that of other Dps proteins reveals that Dps-1 shares significant sequence homology with other Dps homologs, such as complete conservation of residues involved in assembly of the ferroxidase center, and that a main difference is the N-terminal extension (Fig. 1a). The crystal
of the four-helix bundle define a loop that harbors a unique metal ion-binding site (Fig. 1b). To specify the role of the N terminus in DNA binding and state of oligomerization of Dps-1, two deletion mutants, Dps-dn and Dps-met, were constructed. Dps-dn lacks the entire 55-amino acid N terminus (thus, comprising residues 56–207), whereas Dps-met lacks only the 33-residue flexible N-terminal region (and comprises residues 34–207). Dps-dn and Dps-met were expressed with an N-terminal His6 tag, whereas wild type Dps-1 is untagged (Fig. 2).

The N-terminal Metal Site Is Required for Oligomerization—Wild type Dps-1 can exist as a dimer at low salt concentrations, whereas assembly of a very stable dodecamer is favored upon exposure to divalent metals (24). Indeed, the vast majority of recombinant Dps-1 isolated from *E. coli* is in the dodecameric state as shown by glutaraldehyde-mediated cross-linking (24). In contrast, glutaraldehyde-mediated cross-linking of His6-tagged Dps-dn and Dps-met in buffer containing 50 or 500 mM NaCl yielded dimers as the only cross-linked species, with a significant proportion of protein appearing as monomer (data not shown). The addition of 2 mM MgCl2 or 2 mM ZnCl2 to the cross-linking reactions resulted in the formation of a few higher order oligomers, but dimer remained the predominant species along with uncross-linked monomers. However, removal of the N-terminal domain also removed the majority of the lysines from Dps-dn and Dps-met, which are necessary for glutaraldehyde-mediated cross-linking.

To determine rigorously the state of association of Dps-dn compared with Dps-1, FPLC-gel filtration experiments were carried out. From the gel filtration column, the major peak of wild-type Dps-1 eluted as a high molecular weight oligomer corresponding to *M*~r~ 310, consistent with the dodecamer mass (Fig. 3, b–c). An additional peak appeared at an elution volume of ~75 ml, indicating the presence of some lower oligomeric species. In contrast, His6*-tagged Dps-dn eluted as a single peak of *M*~r~ 44, corresponding to the dimer mass.
Consistent results were obtained by native PAGE analysis of Dps-1 and the mutant proteins. On a 5% non-denaturing polyacrylamide gel, His\textsubscript{6}-tagged Dps-dn, shown by FPLC to exist exclusively as a dimer, migrated as a single band (Fig. 3d, lane 3) with a molecular mass close to BSA (lane 1), whereas dodecameric Dps-1 remained very close to the well of the gel (lane 2). Dps-met, on the other hand, when electrophoresed on a 5% native gel, revealed the presence of two distinct species, one faint lower oligomeric species that migrates near BSA and a larger oligomeric species that runs much higher in the gel (lane 4). The mobility of the lower oligomer of Dps-met is consistent with a dimer mass. Removal of the His\textsubscript{6} tag from Dps-dn did not cause assembly of a dodecamer as seen by the even faster migration of the cleaved protein in the native gel (lane 5), whereas His-cleaved Dps-met migrated exclusively as a dodecamer after incubation at room temperature for half an hour in the presence of divalent cation (lane 6), suggesting that the His\textsubscript{6} tag of Dps-met hindered proper assembly of the dodecamer. Because the metal is coordinated in part by two histidine residues, these data also suggest that the reason for interference from the vicinal His\textsubscript{6} tag is that it impedes proper metal coordination. This interpretation is also consistent with the apparent slower mobility of His\textsubscript{6}-tagged Dps-met in SDS-PAGE (Fig. 2), which suggests that Dps-1 is more compact than Dps-met due to metal coordination at the N-terminal site. Evidently, retention of the N-terminal metal-binding site is essential for assembly of a dodecameric species.

Iron Oxidation Is Not Compromised in Dps-dn and Dps-met—In accordance with the presence of the ferroxidase center, both

FIGURE 2. Purified proteins. Coomassie Blue-stained 15% SDS-PAGE gel showing purified proteins. Lane 1, molecular weight markers in kDa; lanes 2–4, 1.5 μg of Dps-dn, Dps-met, and Dps-1, respectively.

FIGURE 3. State of association of Dps-1 variants. a–b, gel filtration analysis of Dps-1 and Dps-dn. Panel a shows molecular markers used to calibrate the FPLC column. Panel b depicts the gel filtration elution pattern of Dps-1 and Dps-dn. Peaks correspond to the dodecameric (X) and dimeric (Y) species. c, the linear calibration curve represents the logarithm of molecular mass as a function of elution volume; the elution volume of dodecameric Dps-1 and dimeric Dps-dn is indicated by arrows. d, 5% native gel showing oligomeric state of recombinant proteins. Lane 1, BSA (M, 66,000, pl 5.4); lane 2, Dps-1 (M, 23,020 for monomeric Dps-1, pl 5.3); lane 3, His-tagged Dps-dn; lane 4, His-tagged Dps-met; lane 5, His-cleaved Dps-dn; lane 6, His-cleaved Dps-met.
of the mutant proteins retained the ability to oxidize iron. A progress curve of iron oxidation was measured at 310 nm using His6-tagged proteins. As shown in Fig. 4, upon the addition of five Fe(II) per ferroxidase site, the absorbance gradually increased with time, which implies that Fe(II) was converted to Fe(III) by utilizing the molecular oxygen present in the air. Though Dps-dn was able to oxidize iron, as previously reported for dimeric wild type Dps-1, it did not exhibit significant absorbance at 300–400 nm after the ferroxidation reaction, indicating no mineralized iron core formation (26, 27). This is consistent with the exclusive existence of Dps-dn as a dimer. In contrast, Dps-met was able to assemble into a dodecamer after ferroxidation, as confirmed by native gel electrophoresis and the diagnostic absorbance at 300–400 nm due to formation of an iron core (data not shown).

The N-terminal Extension Is Required for DNA Binding—Both dimeric and dodecameric Dps-1 were previously shown to bind DNA, with dodecameric protein exhibiting ~1000-fold higher affinity compared with the Dps-1 dimer ($K_d \approx 1.3 \mu M$), and both proteins exhibiting positive cooperativity of binding (24). The binding of Dps-dn and Dps-met to DNA was analyzed in electrophoretic mobility shift assays using end-labeled DNA duplexes as well as supercoiled plasmid DNA. Although dimeric Dps-1 forms multiple complexes with DNA of 18 bp or longer (Fig. 5c and Ref. 24), no complex was observed when His6-tagged Dps-dn (Fig. 5a) or Dps-met (Fig. 5b) was incubated with 26-bp duplex DNA. Also, no protein-DNA complex was detected on agarose gels after incubation of up to 30 pmol of either of the mutant proteins with 100 ng of supercoiled pGEM5 (data not shown). To determine whether the mutant proteins form complexes with DNA that are electrophoretically too unstable for detection, a direct competition assay for DNA binding with wild type Dps-1 and Dps-dn or Dps-met was performed. The amount of Dps-1 used was selected to be just sufficient to saturate the DNA (24). The DNA-Dps-1 complex did not dissociate even in the presence of 25 pmol of either of the mutant proteins (Fig. 5d and data not shown), indicating the inability of both Dps-dn and Dps-met to bind DNA. Cleavage of the N-terminal His6 tag did not restore DNA binding to either protein (data not shown; we note that His-tagging of Mycobacterium smegmatis Dps was shown to
promote a DNA condensation not seen with untagged protein and that this evidently is not the case for Dps-1 (28)). These results show that the flexible lysine-rich region of the N terminus preceding the metal site is essential for association with DNA.

**Dps-1 binds DNA in the major groove—**Dps-1 contains no classical DNA binding motifs, and the mode of DNA interaction of Dps proteins is unknown. Netropsin, a reversible minor groove binding drug, was utilized to determine whether Dps-1 binds to the minor groove of DNA. If Dps-1 binds to the minor groove then in the presence of netropsin, the DNA-Dps-1 complex formation would be inhibited. A well characterized minor groove binder, the TBP, was used as a control to investigate the inhibitory effects of netropsin (29). In the standard assay DNA was incubated with the drug followed by protein addition. In the reverse assay, drug addition to the DNA followed the addition of the protein. In both assays, the presence of netropsin had no effect on DNA-Dps-1 complex formation. Dps-1 was able to bind DNA pretreated with netropsin (data not shown), and netropsin was unable to displace Dps-1 already bound to DNA (Fig. 6a, lane 5). This implies that DNA binding of Dps-1 does not occur through the minor groove. In contrast, DNA preincubated with netropsin cannot form a complex with TBP, as expected. Also, as shown in Fig. 6a, lane 3, netropsin can disrupt almost 95% of the preformed DNA-TBP complex, confirming activity of the drug.

Treatment of DNA with DMS methylates the N-7 of guanines in the major groove and the N-3 of adenosines in the minor groove (30, 31). As shown by the inability of netropsin to compete for Dps-1 binding, Dps-1 does not bind to the minor groove, and inhibition of DNA-Dps-1 complex formation by DMS would indicate that Dps-1 binding is specific for the major groove. As shown in Fig. 6b, DNA premethylated with DMS was unable to form a significant complex with Dps-1 (lane 4) compared with the DNA not treated with DMS (lane 3). Conversely, when Dps-1 was added to DNA before the addition of DMS, no dissociation of the complex was observed after DMS addition (data not shown). Hence, DMS has no effect on the already formed Dps-1-DNA complex. This suggests that the affinity of dodecameric Dps-1 for DNA is too high for DMS to displace the protein from the DNA. Note also the marked preference of Dps-1 for double-stranded DNA compared with single-stranded DNA (Fig. 6c). Taken together, these results show that Dps-1 binds preferentially to duplex DNA and that it does so through the major groove.

**The size of the Dps-1 binding site—**The affinity of Dps-1 for duplex DNA of decreasing length was measured to evaluate the binding site size of Dps-1 (32). Consistent with the previously reported ~0.5 nm affinity for 26-bp DNA (24), quantification of complex formation with 26-bp DNA yields a $K_d$ of $0.40 \pm 0.04$ nm for dodecameric Dps-1 (Fig. 7). Interaction with 22-bp DNA yields a $K_d$ of $0.45 \pm 0.03$ nm. But the affinity decreases very significantly as the duplex length is reduced to 18 bp; at the protein concentration where the dodecameric Dps-1 can saturate 22-bp DNA, barely discernable and electrophoretically unstable complex was observed with the 18-bp duplex (Fig. 7). Evidently, Dps-1 binds optimally to duplex DNA, presenting two complete helical turns. That dissociation of the short duplexes during the reaction is not responsible for the lack of binding is evidenced by the presence of duplex DNA after electrophoresis (Fig. 7c). For dimeric Dps-1, comparable low affinity binding to 26- and 76-bp DNA ($K_d \sim 1.3 \mu M$) was previously reported (24), and a significant further decrease in complex formation is observed only when the duplex length is reduced from 18 to 13 bp (Fig. 7c and data not shown).

**Dps-1 does not bend duplex DNA—**Considering the presence of multiple N-terminal extensions protruding from the core dodecamer, we investigated whether Dps-1 would be able to wrap DNA about itself. Accordingly, DNA bending by Dps-1 was assayed using a cyclization assay in which 105-bp linear duplex DNA was cyclized with T4 DNA ligase. In the presence of a DNA-bending protein, an increased rate of ligase-mediated DNA cyclization would be observed (33). However, no DNA circles were formed in the cyclization assay at a range of Dps-1 concentrations from 1 to 200 nM (Fig. 8, lanes 4–7, and data not shown). Evidently, Dps-1 is unable to cyclize 105-bp DNA, suggesting either its inability to introduce a DNA bend or its assembly of a DNA complex in which intramolecular ligation is otherwise hindered. That Dps-1 does not merely bind to DNA ends, thus preventing formation of ligation products, is observed by ligation of linearized plasmid DNA in the presence of Dps-1 (data not...
shown) and is supported by the observation that it binds single-stranded DNA with ~200-fold lower affinity compared with double-stranded DNA (Figs. 6c and 7).

The data so far suggest that a 22-bp unbent duplex allows for optimal interactions across one face of the Dps-1 dodecamer. An emerging question is, therefore, why the affinity of the dimer is so much lower than that of the dodecamer. Is it because the dimer has no metal bound at the N-terminal metal sites and its N-terminal extensions consequently are too flexible and/or not appropriately oriented in space? This is the most likely explanation, because the addition of divalent metal to dimeric Dps-1 causes it to oligomerize (24). Notably, dodecameric Dps-1 treated with bipyridyl fails to bind DNA with the same affinity as the untreated protein, and the addition of Co$^{2+}$ in the binding reaction restores high affinity binding (Fig. 9). The lower affinity is not due to dissociation of the dodecamer to some lower oligomeric species because the treatment with bipyridyl does not change the oligomerization state of the protein (as documented by glutaraldehyde-mediated cross-linking of the protein after removal of bipyridyl by microdialysis (see the supplemental material)).

**DISCUSSION**

The N-terminal Metal Site Is Required for Assembly of the Dps-1 Dodecamer—The overall quaternary structure formed by Dps homologs is highly conserved; an assembly of 12 four-helix bundle monomers. The main structural difference lies in the length of the N-terminal tails of the monomers. A multiple sequence alignment of Dps homologs (Fig. 1) shows that *D. radiodurans* Dps-1 has a longer N terminus, an extension of 55 amino acid residues preceding the first helix of the four-helix bundle monomer. Between residue 30 and the start of helix A, 25 amino acids define a loop harboring a metal-binding site (18, 19). This metal-binding site is coordinated by residues from a single protein subunit and is located at the external surface of the dodecameric sphere, accessible to the solvent. The tetragonal coordination includes three residues from the coiled N terminus (Asp-36, His-39, and His-50) and one residue from helix A (Glu-55). The presence of an intrasubunit metal ion-binding site is also observed in *L. lactis* Dps, where Zn$^{2+}$ is reported to be coordinated by two histidine residues and two water molecules (16). These two histidine residues are located at the end of an N-terminal helix (His-22) and...
the start of helix A (His-33). The presence or absence of bound metal does not appear to alter the conformation of the N-terminal helix of *L. lactis* Dps, and there is no sequence or structural homology between *D. radiodurans* and *L. lactis* Dps in this region.

The mutant protein Dps-dn, which lacks 55 amino acids at the N terminus including the metal site, fails to assemble into a dodecamer but exists as a stable dimer because of extensive interactions at the interface between monomers that also defines the ferroxidase center. The presence or absence of the N-terminal His<sub>6</sub> tag does not influence the oligomeric state of Dps-dn (Fig. 3d). For the other deletion mutant, Dps-met, which lacks only the first 33 amino acids at the N terminus and, hence, retains the metal coordination site, the N-terminal His<sub>6</sub> tag can interfere with assembly of the dodecamer. Removal of the His<sub>6</sub> tag causes Dps-met to exist exclusively as a dodecamer. This suggests that the presence of six additional histidine residues before the metal binding ligands may affect the proper coordination of a metal ion at the metal-binding site and that the presence of the metal in the dodecamer at the N terminus is necessary for dodecameric assembly of Dps-1. This is also in agreement with the previously reported observation that in the presence of divalent cations, dimeric Dps-1 immediately starts oligomerizing, ultimately to form a dodecameric superstructure (24). A bound metal ion at the N-terminal metal center may predispose the N-terminal loop in a conformation that allows residues from two distinct dimers to connect through hydrogen-bonding, thus nucleating assembly of the dodecamer. Specifically, Asn-44 can make hydrogen bonds with Asp-202 or Glu-204 of the adjacent dimer, and Asn-49 can connect with Arg-194 or Gln-198 via hydrogen bonds (Fig. 1b). Once assembly has occurred, stability of the dodecamer is accomplished through extensive interactions between neighboring four-helix bundles. An N-terminal deletion mutant of *L. lactis* DpsA (lacking residues 1–20), in which the N-terminal metal coordination was also disrupted by site directed mutagenesis, was unable to interact with DNA (16). But this DpsA variant eluted from a gel filtration column at a similar elution volume as native DpsA, suggesting that unlike Dps-1, disruption of the metal center at the N terminus has no effect on the dodecameric assembly of DpsA (16). We do not know which divalent cation would be necessary for dodecameric assembly of Dps-1. This is also in agreement with the previously reported observation that in the presence of divalent cations, dimeric Dps-1 immediately starts oligomerizing, ultimately to form a dodecameric superstructure (24). A bound metal ion at the N-terminal metal center may predispose the N-terminal loop in a conformation that allows residues from two distinct dimers to connect through hydrogen-bonding, thus nucleating assembly of the dodecamer. Specifically, Asn-44 can make hydrogen bonds with Asp-202 or Glu-204 of the adjacent dimer, and Asn-49 can connect with Arg-194 or Gln-198 via hydrogen bonds (Fig. 1b). Once assembly has occurred, stability of the dodecamer is accomplished through extensive interactions between neighboring four-helix bundles. An N-terminal deletion mutant of *L. lactis* DpsA (lacking residues 1–20), in which the N-terminal metal coordination was also disrupted by site directed mutagenesis, was unable to interact with DNA (16). But this DpsA variant eluted from a gel filtration column at a similar elution volume as native DpsA, suggesting that unlike Dps-1, disruption of the metal center at the N terminus has no effect on the dodecameric assembly of DpsA (16). We do not know which divalent cation would be necessary for dodecameric assembly of Dps-1.
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a conclusion that would be consistent with a binding mode involving the DNA axis lying parallel to the long axis of the Dps-1 dimer with the N termini extending from either end of the dimer contacting the DNA. However, the possibility exists that other pairs of N termini engage the DNA, resulting in curvature of the helix axis, and that the existence of multiple binding sites on the DNA leads to binding of additional protomers whose DNA bends are out of phase with the helical repeat and hence cancel.

We also infer that binding of a cation to the N-terminal metal site controls the relative orientation of the flexible N terminus and, hence, exerts a regulatory effect on the DNA/Dps-1 interaction; this inference is based on the observation that addition of divalent metal to dimeric Dps-1 causes its oligomerization (24), implying that the N-terminal metal site of dimeric Dps-1 is unoccupied. Notably, removal of metal by complexing with bipyridyl decreases the binding affinity of Dps-1, which indicates that occupancy of the N-terminal metal site is important for high affinity DNA binding (Fig. 9).

In conclusion, our present work reveals for the first time the involvement of the N-terminal extension in the dodecameric Dps-1 dimer with the N termini extending from either end of the dimer contacting the DNA. However, the possibility exists that other pairs of N termini engage the DNA, resulting in curvature of the helix axis, and that the existence of multiple binding sites on the DNA leads to binding of additional protomers whose DNA bends are out of phase with the helical repeat and hence cancel.

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