**Structure of Two Iron-binding Proteins from Bacillus anthracis**

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**Bacillus anthracis** is currently under intense investigation due to its primary importance as a human pathogen. Particularly important is the development of novel anti-anthrax vaccines, devoid of the current side effects. A novel class of immunogenic bacterial proteins consists of dodecamers homologous to the DNA-binding protein of Escherichia coli (Dps). Two Dps homologous genes are present in the *B. anthracis* genome. The crystal structures of these two proteins (Dlp-1 and Dlp-2) have been determined and are presented here. They are sphere-like proteins with an internal cavity. We also show that they act as ferritins and are thus involved in iron uptake and regulation, a fundamental function during bacterial growth.

A novel class of bacterial proteins with architecture and properties similar to ferritins has been recently described (1, 2). They consist of 12 identical subunits, each of them with a four-helix bundle folding similar to that of ferritins. These dodecamers form a shell with a large central cavity. Despite this structural similarity and the fact that some of these proteins are capable of incorporating iron in vitro, their function in vivo is still unclear. The family members expressed by *Escherichia coli* and *Bacillus subtilis* bind and protect DNA from oxidative damage (Dps, DNA protecting protein under starved conditions) (3–5), whereas the *Listeria innocua* protein (Flp) is believed to be a cold shock protein (7), and FtpA from *Hemophilus ducreyi* is a structural protein of fine tangled pili (8).

One of the most interesting members of the Dps-like family, termed HP-NAP, has been discovered in *Helicobacter pylori*. It is a molecule that activates different human inflammatory cells and is a major antigen in the human immune response to this bacterium and, as such, is a component of an anti-*H. pylori* vaccine currently under trial (11). In addition, the homologous proteins from *Treponema pallidum* and *Borreliaburgdorferi* are also highly immunogenic (12, 13).

*Bacillus anthracis*, the causative agent of the various forms of the anthrax disease, possesses two different genes encoding for proteins (Dlp-1 and Dlp-2) with amino acid sequences similar to those of the other members of this family (www.tigr.org). In consideration of the high immunogenicity of some of the proteins of the Dps family and of the side effects associated with the presently available anti-anthrax vaccine (14, 15), we have cloned, expressed, and purified Dlp-1 and Dlp-2. Here, we report on their crystal structure, and we show that they have properties compatible with ferritin activity.

**EXPERIMENTAL PROCEDURES**

Bioinformatics—A TBLASTN search of the unfinished *B. anthracis* genome available from the Institute for Genome Research (TIGR) at www.tigr.org was performed using the complete amino acid sequence of HP-NAP (HP0243) (16). Theoretical molecular weights and isoelectric point values of the proteins were calculated using the ProtParam tool available at www.expasy.ch.

Cloning of the *dlp* Gene from *B. anthracis* into the Expression Vector pSM214G—*Both dlp-1* and *dlp-2* were amplified by PCR from *B. anthracis* 9131 template DNA using primers BA1 (5'–caggatcaagttgataactg-3') and BA2 (5’–ggaagttattgattcaaggaag-3') for *dlp-1* and BA3 (5’–ggaagttattgattcaaggaag-3') and BA4 (5’–ggaagttattgattcaaggaag-3') for *dlp-2*, respectively. The restriction sites for SacI and HindIII are underlined. Each PCR was carried out using standard methods, and the thermal cycling parameters were as follows: 1 cycle of 94 °C (5 min) followed by 30 cycles of 94 °C (1 min), 55 °C (1 min), 72 °C (2 min), and with a final elongation step at 72 °C (10 min). The amplified fragments (474 bp for *dlp-1* and 477 bp for *dlp-2*) were cloned into pCR2.1 using the TA cloning kit (Invitrogen) resulting in plasmid pCR2.1-Dlp1 and pCR2.1-Dlp-2, respectively. The cloned fragments were then excised from both pCR2.1-Dlp1 and pCR2.1-Dlp-2 by digestion with SacI and HindIII and ligated into the pCR and HindIII sites of the expression vector pSM214G resulting in plasmid pSM214G-Dlp1 and pSM214G-Dlp-2. pSM214G contains an artificial constitutive promoter, a chloramphenicol resistance cassette, and two origins of replication that allow expression of cloned genes both in *E. coli* and *B. subtilis* (17). Both plasmids were sequenced to confirm the presence of the correct gene. The sequences have been deposited with GenBank™ under accession numbers AF374268 and AF374269 for *dlp-1* and *dlp-2*, respectively.

Purification of *Dlp-1* and *Dlp-2*—*E. coli* strain XL1-blue (supE44 hsdR17 recA1 endA1 gyrA46 thi-relA lac F- proAB lacI lacZM15 Tn10 (Tet’)) (Stratagene) containing the appropriate plasmid, i.e. either pSM214G-Dlp1 or pSM214G-Dlp2 was grown for 16 h in YT (15 g/liter yeast extract, 16 g/liter bacitracyn, 5 g/liter NaCl medium) with 20 μg/ml chloramphenicol. After three passages through a French press and removal of bacterial debris by centrifugation at 32,000 × g, ammonium sulfate was added to the supernatant (60% v/w) for *Dlp-1* and 65% v/w for *Dlp-2*. At these percentages of ammonium sulfate both proteins remained in solution. The resulting supernatant was dialyzed overnight in buffer A (Tris-HCl 30 mM, pH 7.8, NaCl, 0.1 mM) and then loaded onto a Mono Q FPLC column (Amersham Biosciences) equilibrated with buffer A. The proteins were eluted by high perform-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF374268 and AF374269.

The atomic coordinates and structure factors (code 1JJ5 and 1JBI) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org) .

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Table I
Data collection and refinement statistics

|                | Dlp-1 (λ = 1.20 Å) | Dlp-2 (λ = 0.93 Å) |
|----------------|---------------------|---------------------|
| Data collection| Res. (Å)            | 50–2.5 (2.64–2.5)   | 50–1.46 (1.54–1.46) |
|                | Ind. reflections    | 20,677 (3107)       | 105,192 (13,823)    |
|                | Multiplicity        | 2.8 (2.7)           | 3.0 (1.8)           |
|                | Completeness (%)    | 95.8 (98)           | 98.2 (88.2)         |
|                | (I/σ(I))            | 4.7 (1.8)           | 12.7 (9.0)          |
|                | R<sub>free</sub> (%)| 0.10 (0.38)         | 0.092 (0.068)       |
| Refinement     | Protein atoms       | 4612                | 4656                |
|                | Solvent molecules   | 153                 | 328                 |
|                | R<sub>free</sub> (%)| 22.6 (32.0)         | 18.6 (22.4)         |
|                | R<sub>p</sub> (%)   | 23.1 (32.6)         | 20.5 (25.1)         |
|                | r.m.s. deviation on | 0.009               | 0.007               |
|                | bond distances (Å)  | 1.3                 | 1.1                 |

![RNAse](image.png)

Fig. 1. Primary sequence alignment of Dlp-1 and Dlp-2 with HP-NAP, Flp, and Dps. Identities are shown on a black background and conserved residues on a gray background. Residues involved in metal binding are indicated by an asterisk.

The iron binding ability of Dlp-1—expressed recombinant Dlp-1 model as a template (Protein Data Bank code 1JI4).<sup>2</sup> Model visualization and rebuilding was performed with QUANTA software (21). The 3-fold molecular axis is coincident with a crystallographic axis of the R3 space group, and the content of the asymmetric unit corresponds to one monomer. A perfect hemihedral twinning was revealed by the analysis of the intensity distribution of Dlp-2 data, the twinning operator being (h, h, k, l). Consequently, refinement was performed using the twinning procedure of the CNS package (22). Initial refinement was carried out using one single monomer and imposing a strict noncrystallographic symmetry restraint. The four monomers were refined independently only in the final stages of refinement, but imposing noncrystallographic symmetry restraints. One metal site per monomer was visible. The final model of Dlp-2, composed of 4656 protein atoms, four cations, and 324 solvent molecules, presents a crystallographic twinned R factor of 0.186 (R<sub>free</sub> = 0.205) and an R factor of 0.191 (R<sub>free</sub> = 0.208) after detwinning of the data (23). In Dlp-1, an electron density accounting for an MPD molecule was visible in the difference-Fourier map in proximity of a hydrophobic pocket, and it was introduced and refined. In the final model it is close to hydrophobic residues (Phe-20 of two different subunits and Ile-53) and makes two hydrogen bonds with OG1 of Thr-21 and O of Ser-17 of the same monomer.

The quality of the models was tested with the PROCHECK software (24). Both final models present an overall G-factor of 0.3 and a good stereochemistry. The presence of a “perfect” twinning for Dlp-2 did not hinder the refinement process, allowing a smooth convergence. The Dlp-1 model is less accurate than the Dlp-2 one not only because the large r.m.s. deviation on bond angles of 0.191 (<i>R</i><sub>free</sub> = 0.237) but also due to the large diffuse scattering present in the spectrum.

Staining of Iron-binding Proteins—The iron binding ability of Dlp-1 and Dlp-2 was determined by resolving the E. coli-expressed recombinant Dlp-1 and Dlp-2 on nondenaturing (native) polyacrylamide gels.
and staining according to Ref. 25. The bacteria were grown in LB medium overnight at 37 °C in the presence or absence of 1.0 mM FeCl₂. Total protein extracts were then prepared by resuspending pelleted bacteria in phosphate-buffered saline, freeze/thawing (liquid N₂/37 °C) three times, and then centrifuging for 10 min at 16,000 × g. The total protein content of the resulting supernatants was quantified using the Bradford assay and bovine serum albumin as standard. Polyacrylamide gels (8%) were prepared without the addition of SDS, and the samples (in 10% w/v sucrose) were electrophoresed at 15 mA until the bromphenol blue tracking dye (bromphenol blue in 10% w/v sucrose) reached a position 1–2 cm from the bottom of the gel. The gels were then stained with potassium ferricyanide solution (100 mM K₃[Fe(CN)₆] in 50 mM Tris-HCl, 100 mM NaCl, pH 7.5) for 10 min in the dark and destained with a 10% trichloroacetic acid/methanol solution until clearing of the background. An image of the stained gel was recorded using a digital scanner (Epson), and the gel was then subjected to Coomassie staining using standard techniques. Horse ferritin (Sigma) and bovine serum albumin were used as positive and negative controls, respectively.

RESULTS AND DISCUSSION

Using the amino acid sequence of HP-NAP (HP0243) (16), a search of the available genome sequence of B. anthracis (www.tigr.org) revealed the presence of two Dps-like genes designated dlp-1 and dlp-2 that encode for Dlp-1 (146 amino acids) and Dlp-2 (147 amino acids), respectively (Fig. 1). The pI and Mᵣ, respectively, of each, predicted using the ProtParam program, are 4.76 and 16910 Da for Dlp-1 and 4.79 and 16649 Da for Dlp-2.

Oligonucleotides were designed based on the available nucleotide sequence data, and the dlp genes were amplified by PCR, cloned into the expression vector pSM214G, and transformed into E. coli XL1-blue. The proteins were purified to homogeneity using a two-step purification protocol for Dlp-1 and a three-step purification protocol for Dlp-2, as described under “Experimental Procedures.”

Structure of the Dlp Monomer—Dlp-1 and Dlp-2 are dodecamers of 12 identical subunits, folded in a four-helix bundle. Helices A and B are connected to helices C and D via a 25-residue-long segment. This fold is similar to that of Dps and Flp (1, 2). The major difference with respect to ferritins (26) is the lack of the C-terminal helix E and the presence of a short helix.
in the middle of the BC connecting segment. This short helix appears to play an important structural role, as it is involved in the hydrophobic subunit-subunit interaction, related by a 2-fold symmetry axis, and mediated by two Leu and two Met residues (Fig. 2A). Dlp-1 and Dlp-2 present a sequence identity of 58% between themselves and of 39 and 41%, respectively, with Flp (Fig. 1). The superposition of the equivalent Ca’s of the monomers yields a root mean square (r.m.s.) deviation of 0.5 Å (Dlp-1/H11002 Dlp-2), of 0.9 Å (Dlp-1/H11002 Flp), and of 0.8 Å (Dlp-2/H11002 Flp) (Fig. 2B). At variance, the sequence identity between Dlp-1 and Dlp-2 and Dps decreases to 18 and 21%, respectively, and the superposition of the models yields a r.m.s. deviation of 1.5 and 1.4 Å, respectively. These data support the possibility that Dlp-1, Dlp-2, and Flp are mini-ferritins, whereas Dps has diverged to fulfill a different biological function. In fact, Dps lacks the specific entry portal of the cation into the cavity (see below), and it protects bacterial DNA from oxidative damage (3).

Structure of the Dodecamer—The arrangement of the 12 monomers generates a nearly spherical shell, with 32 symmetry (Fig. 3). In both cases, one of the 3-fold axes of the molecule is coincident with a crystallographic axis. The internal cavity, where iron is likely to be stored, is about 45 Å in diameter. It should be noted that in the two structures presented here no iron was observed in the internal cavity, which is probably filled with unordered solvent molecules. This absence does not necessarily reflect the in vivo situation, but may result from the heterologous expression and/or the biochemical operations to which the proteins were submitted during extraction, purification, and crystallization. In fact, the proteins expressed in B. subtilis do contain iron (not shown). These macromolecules possess four 3-fold axes, each of them passing through the shell in two different 3-fold environments, organized as pores. One of the two 3-fold pores corresponds to the postulated iron entry channel of Flp (2). This channel in Flp has a negatively charged environment, with positive charges lining the pore surface. In Dlp-1, the negatively charged environment is contributed by residues Asp-124 and Glu-120, pointing their carboxylates into the interior of the cavity, and by Glu-112 and Asp-118 located around the pore. At variance, in Dlp-2, Glu-114, Glu-118, and Asp-126 form the negatively charged environment of the iron entry pore, and only one positively charged residue is present on the external surface of the pore (Lys-109 in Dlp-1, Lys-110 in Dlp-2).

The second of the 3-fold pores is the smaller of the two, in both Dlp-1 and Dlp-2. Moreover, it is closed by the side chains of Thr-37 and Gln-34 in Dlp-1 and of Thr-39 and His-36 in Dlp-2.

The quaternary structures of Dlp-1 and Dlp-2 are very stable and as much as 6 molar guanidinium chloride is necessary to denature them (not shown). Such stability is mainly due to the large number of intersubunit interactions. Each monomer of both proteins in engaged in 19–20 hydrogen bond interactions with the surrounding subunits. In addition, metal coordination contributes to the oligomer stability, since each cation binding site is formed by two contiguous monomers.

Metal Binding Site—The iron binding sites of Dlp-1 and Dlp-2 are illustrated in Fig. 4. The cation environment is quite...
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another water molecule, which is also hydrogen-bonded to His-41. At variance, the conserved Glu-45 residue of Dlp-2 is positioned further away, and as such it does not interact with the ligand site.

Dlp-1 and Dlp-2 Act as Ferritins—Dlp-1 and Dlp-2 do not activate human neutrophils and do not bind to bacterial DNA (not shown). To determine whether these proteins are capable of binding iron, both purified proteins and total protein extracts of E. coli strains expressing Dlp-1 and Dlp-2 grown in the presence and absence of 1 mM FeCl$_2$ were stained for iron binding by the potassium ferricyanide method. Potassium ferricyanide reacts with protein-bound iron atoms to form royal blue complexes, and it is believed that the intensity of the staining is dependent in part on the number of iron atoms present per molecule of protein (25). No iron was detected in the purified proteins or in the bacterial extracts grown in the absence of iron. On the contrary, when iron was included in the culture medium, both Dlp-1 and Dlp-2 were stained (Fig. 5, A and B), clearly revealing that Dlp-1 and Dlp-2 bind iron. To study the role of this property in vivo, the ability of both proteins to confer resistance to iron overload when overexpressed in E. coli was investigated as previously done for HP-NAP and Pfr (27).

The E. coli recombinants expressing Dlp-1 and Dlp-2 and the parental XL1-blue strain were grown in triplicate in LB medium supplemented with 5.5 mM FeCl$_2$, a concentration previously determined to be toxic for E. coli XL1-blue (27). The growth of each strain was monitored over an 8-h period. As it can be seen from Fig. 5C, the recombinants expressing Dlp-1 and Dlp-2 grew under iron overload conditions, while the parental strain did not grow. Therefore both Dlp-1 and Dlp-2 are capable of binding and sequestering free iron, allowing for bacterial growth under iron overload conditions by reducing the amount of free iron in the growth medium. Altogether these data indicate that Dlp-1 and Dlp-2 act as ferritins in B. anthracis. Their value as antigens remains to be studied, but the present work provides the structural basis for mapping the surface epitopes of these proteins.

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Fig. 5. Iron binding of Dlp-1 and Dlp-2. A, native acrylamide gels (8%) were stained with potassium ferricyanide as described under “Experimental Procedures” and then subjected to Coomassie staining. B, lane 1, horse ferritin (10 µg); lane 2, Dlp-1 purified from E. coli (10 µg); lane 3, Dlp-2 purified from E. coli (10 µg); lane 4, total protein extract (75 µg) from E. coli expressing Dlp-2; lane 5, total protein extract (75 µg) from E. coli expressing Dlp-1 grown overnight in the presence of 1.0 mM FeCl$_2$; lane 7, total protein extract (75 µg) from E. coli expressing Dlp-2 grown overnight in the presence of 1.0 mM FeCl$_2$; lane 8, bovine serum albumin (10 µg). C, iron resistance in E. coli XL1-blue. The growth of E. coli XL1-blue in the presence (●) or absence of iron (5.5 mM FeCl$_2$) (●), and of the recombinant strains expressing Dlp-1 (▲) and Dlp-2 (■) in the presence of iron, was monitored in triplicate for 8 h. Error bars represent S.D. values.

similar in the two proteins and corresponds to a tetrahedral coordination. It is made up of two oxygen atoms (from an aspartate and a glutamate), one nitrogen of a histidine, and an oxygen atom of a water molecule, as identified by its thermal parameter and interatomic distance. The two acidic residues and the His belong to two different monomers. In Dlp-1, the solvent molecule filling the fourth coordination position is kept in place by Glu-43 and by a second solvent molecule, which is also hydrogen-bonded to His-39. In Dlp-2 the solvent molecule coordinated to the metal ion forms a hydrogen bond only with

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