N-terminal Amino Acid Residues Mediate Protein-Protein Interactions between DNA-bound α/β-Type Small, Acid-soluble Spore Proteins from Bacillus Species

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The binding of α/β-type small, acid-soluble spore proteins (SASP) to DNA of spores of Bacillus species is the primary mechanism by which spore DNA is protected from the damaging effects of heat, peroxides, and UV radiation (1, 2). The α/β-type SASP are nonspecific DNA-binding proteins, whose synthesis is developmentally regulated such that these proteins are only synthesized within the developing forespore during sporulation (3, 4). The α/β-type SASP accumulate to very high levels, which are sufficient to saturate the spore chromosome, and DNA within this nucleoprotein complex is protected from a variety of environmental insults (1, 2, 4). Accordingly, the α/β-type SASP are important determinants of long term spore survival, and spore resistance to heat, peroxides, and UV radiation. Spores of Bacillus subtilis that lack the majority of their α/β-type SASP, termed α-β spores, are much more sensitive to these treatments than are wild-type spores (1, 2). The α/β-type SASP are encoded by a number (4–7) of monocistrionic genes in Bacillus species. Generally, in each species there are two α/β-type SASP that are expressed at very high levels (the major α/β-type SASP), and a variable number of other α/β-type SASP expressed at much lower levels (the minor α/β-type SASP) (4). The amino acid sequences of these proteins are highly conserved, both within and between species; however, significant differences in DNA binding affinity between α/β-type SASP have been demonstrated (5–7). During spore germination, the α/β-type SASP are rapidly degraded by a sequence-specific endoprotease, termed the germination protease (GPR), which recognizes and cleaves within a pentapeptide sequence found within all α/β-type SASP (4).

The interaction between α/β-type SASP and DNA has been studied in detail, and several features of α/β-type SASP-DNA binding have been characterized (5, 6, 8–10). DNA structure changes from B-DNA to an A-like conformation upon binding to α/β-type SASP (7, 8), whereas α/β-type SASP undergo a transition from random coil to a largely α-helical conformation upon binding to DNA (6). The binding interaction is significantly cooperative, with α/β-type SASP having a 50- to ~600-fold greater affinity for contiguous DNA binding sites than for noncontiguous sites depending upon the bound polynucleotide and the salt concentration (5, 6). The binding cooperativity is thought to be due at least in part to protein-protein interactions between adjacent, DNA-bound α/β-type SASP (8, 11). Contacts between the α-amino group and the carboxylate side chains of three acidic residues found within the N-terminal 40–50% of a variety of α/β-type SASP have been identified previously using a zero-length cross-linking reagent (11). The N-terminal third of α/β-type SASP varies both in length and amino acid sequence, and its sequence is generally less well conserved than the C-terminal two-thirds of these proteins (Fig. 1) (4). Because individual α/β-type SASP have significantly different affinities for DNA, it seems reasonable to speculate that these differences in binding affinity could be due to differences in the length and sequence of the N-terminal third of these proteins. Consequently, we have investigated the effects of modifications of the N-terminal sequence upon the DNA binding properties of α/β-type SASP. This analysis has further

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Printed in U.S.A.

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led to a model for α/β-type SASP protein-protein interaction, which involves an electrostatic interaction between the positively charged N-terminal region of one protein and an acidic region on an adjacent DNA-bound protein.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—The Escherichia coli strains used were JM83 (araD139lac-proAB) RpsL 680 lacZAM15 (12) and BL21(D30) (T7 RNA polymerase under control of the lac promoter) (13). The B. subtilis strains used were all derivatives of strain 168. E. coli strains were routinely grown in Terrific broth (24 g of yeast extract, 12 g of tryptone, and 1% of glycerol prepared in 100 ml of 100 mM KH2PO4, 720 mM KH2PO4) at 37 °C with shaking. For the overexpression of cloned genes encoding α/β-type SASP from pET11d vectors (13), the medium was supplemented with 200 μg/ml ampicillin and 0.5% glucose. B. subtilis was transformed to kanamycin resistance with pUB110 derivatives as described previously (14).

**Construction of Genes Encoding SspC N-terminal Variants**—The genes encoding all SspC deletion variants were generated by the polymerase chain reaction (PCR) using plasmid pPS708 (10) as the template and the following oligonucleotide primers. The upstream primers were: SSCP 5'-CGATGTGTAAGCTTTTTTTATT-sspB (16). An sspC restriction site was first introduced at the initiating methionine codon of the sspB strong, sporulation-specific promoter of the B. subtilis strain BL21(DE3) from pET11d vectors (13), the medium was supplemented with 200 μg/ml ampicillin and 0.5% glucose. B. subtilis was transformed to kanamycin resistance with pUB110 derivatives as described previously (14).

**Purification of α/β-type SASP and Polynucleotides**—All SspC variants were overexpressed in E. coli strain BL21(DE3) from pET11d-derived plasmids as described previously (13). SspC and SspCα1-13-D13K were extracted from dry ruptured E. coli cells with 3% acetic acid, 30 mM HCl and purified as described previously (19). SspCα11-D13N and SspCα7 were acid-extracted as described above, but were purified by ion exchange chromatography on a CM-cellulose column equilibrated in 10 mM NaOAc (pH 5.5) at 4 °C. Proteins were eluted by a linear salt gradient from 0 to 200 mM NaCl in 10 mM NaOAc (pH 5.5) at 4 °C. Column fractions containing purified α/β-type SASP were pooled, concentrated by lyophilization, and dialyzed exhaustively against 10 mM sodium phosphate (pH 7.5). All proteins were >95% pure, as judged by SDS-PAGE and staining with Coomassie Blue. The concentrations of protein stock solutions were determined by quantitative amino acid analysis.

Plasmid DNA (pUC19) was purified by two rounds of CsCl equilibrium density centrifugation, and linearized by digestion with EcoRI. Poly(dG)-poly(dC) and poly(dA)-poly(dT) were obtained commercially (Sigma). All polynucleotides were dialyzed exhaustively in Spectra/Por 3 tubing (molecular mass cut-off 3500 Da) against 10 mM sodium phosphate (pH 7.5).

**Assay of α/β-type SASP—α/β-type SASP were chemically deaminated as follows; 120 μl of purified protein (1 mg/ml in 10 mM sodium phosphate (pH 7.5)) was slowly added to 350 μl of 2.5 mM NaOAc (pH 5.2), 50 mM sodium glycolate, 5 mM CuSO4 (20, 21). The resulting solution was incubated at 22 °C for 20 min, followed by dialysis in Spectra/Por 3 tubing (molecular mass cut-off 3500 Da) against three 1-liter changes of distilled water at 4 °C for 8 h each. Dialyzed, deaminated protein was frozen, lyophilized, dissolved in ~50–100 μl of 8 M urea, and dialyzed exhaustively against 10 mM sodium phosphate (pH 7.5) at 4 °C. This protein was used for in vitro DNA binding assays. SspC (1 mg/ml) was digested with endoproteinase Asp-N (Sigma) in 10 mM sodium phosphate (pH 7.5) at an enzyme to substrate ratio of 1:200 (w/w) at 22 °C for 6 h. SspC (1 mg/ml) was digested with the germination protease (recombinant GPR from Bacillus megaterium) (22) at an enzyme to substrate ratio of 1:500 (w/w) in 10 mM Tris-HCl (pH 7.4), 2 mM CaCl2 at 22 °C for 3 h. The C-terminal proteolytic fragments from both digests were purified by reverse phase-HPLC as described (11).

**Analysis of SspC Variants in Vitro and in Vivo—DNA binding was assessed in vitro by DNase I protection assays (5) and by circular dichroism (CD) spectroscopy (6). DNA binding by the α/β-type SASP was assessed by the ability of protein to protect EcoRI-linearized pUC19 plasmid from DNase I digestion as described previously (5, 19). All CD measurements and spectra were obtained on a Jasco 715 spectropolarimeter with a Jasco PS-150–5 power supply, and all recorded spectra were the average of three scans (6). Far UV (protein conformation) spectra were obtained from solutions containing 25 μM α/β-type SASP and 115 μM (in bp) poly(dG)-poly(dC) (5, 23) at 21 °C. Difference spectra corresponding to the α/β-type SASP component of the complex were obtained by subtracting the spectrum of free DNA from the spectra of α/β-type SASP-poly(dG)-poly(dC) complexes as described (6, 23, 24). Secondary structure deconvolution was carried out on data from difference spectra using a web-based neural network algorithm, K2D (25).

The thermal denaturation of SspC variant-DNA complexes was performed on pre-equilibrated, stirring solutions containing 5 μM SspC variant complexed with 25 μM (in bp) poly(dG)-poly(dC) in 10 mM sodium phosphate (pH 7.5) (6). This amount of DNA is sufficient to bind all wild-type SspC (6). Denaturation was monitored by measuring ellipticity at 222 nm at 0.5 °C intervals as a function of temperature from 20 °C to 90 °C. The midpoint of each transition (defined as Tm) was determined by taking the first derivative of ellipticity at 222 nm with respect to the inverse of the absolute temperature as described (26). Experimental error was estimated at ±1 °C, based on duplicate measurements of poly(dG)-poly(dC) complexes with SspCα11-D13K, SspCα7, and SspCα1-13-D13K.

Equilibrium binding titrations by CD spectroscopy were performed as described using EcoRI-linearized pUC19 plasmid, poly(dG)-poly(dC), and poly(dA)-poly(dT) DNA. A DNA-binding site was defined when the α/β-type SASP was bound directly determined for SspCα11-D13K, SspCα7, SspCα1-13-D13K, and SspCα7-D13K from stoichiometric forward titrations of poly(dG)-poly(dC) in 10 mM sodium phosphate (pH 7.5) at 21 °C, as was found previously for a number of α/β-type SASP with several DNAs (5, 6). Stoichiometric DNA binding conditions could not be obtained for SspCα11-D13K, SspCα7, Asp-N fragment, and SspCα7-D13K, although data from forward titrations of...
poly(dG)poly(dC) with these proteins under no salt conditions were entirely consistent with the same 4-bp site size. Mean residue ellipticity, \([\theta]_{222}\), values for DNA-bound protein were determined from complexes containing 25 \(\mu\)g protein and 115 \(\mu\)M (in bp) poly(dG)poly(dC) under no salt conditions after correction for DNA contributions to ellipticity at 222 nm. Further addition of poly(dG)poly(dC) above 115 \(\mu\)M resulted in no additional change in \(\alpha\) in vitro type SASP ellipticity at 222 nm. The binding site size \((n)\) and the mean residue ellipticities at 222 nm for free \((\langle\theta\rangle_{222}\rangle\), and DNA-bound \((\theta)_{222}\), \(\alpha\) in vitro type SASP were used to calculate the percentage of bound and free protein at each point in forward titrations according to a two-state model as described (6). The McGhee-von Hippel model was fit to these data by an iterative least squares method (using KaleidaGraph 3.0.2) to obtain apparent binding constants, \(K_a\), for the interaction with pUC19, and intrinsic binding constants \((K)\) and cooperativity factors \((\omega)\) for the interaction with poly(dA-dT)poly(dA-dT) (6). Errors in the fits of \(K_a\) and \(\omega\) were determined by the model fitting program. The experimental error in \(K_a\) determinations was estimated to be \(\pm 15\%\) as determined by duplicate titrations of pUC19 DNA with SspC and poly(dA-dT)poly(dA-dT) with SspC(11-135).

SspC variants were chemically cross-linked in the presence of EcoRI-linearized pUC19 plasmid DNA with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and cross-linked products purified by SDS-PAGE as described (11). Purified cross-linked and monomeric proteins were digested with trypsin as described (11) and the digests (100 \(\mu\)l) dialyzed in 100-Da cut-off DispoBiodialyzer\textsuperscript{TM} (Spectrum) against two 1-liter changes of 50 mm NH\(_4\)HCO\(_3\) at 4 °C for 18 h each. Dialyzed tryptic digests were directly analyzed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry to identify cross-linked peptide species (11). Spores of \textit{B. subtilis} that overexpress variants of SspC were prepared by nutrient exhaustion in 2 \(\times\) 2269-Phosphate minimal medium (27). Spores were purified by sonication and distilled water washing at 4 °C (28), and all spores used were >98% pure. Purified spores were analyzed for resistance to both wet heat and UV radiation as described (19, 29).

RESULTS

\textit{SspC} N-terminal Variants Bind to DNA in Vitro—\textit{Amino acid sequence alignment of all identified \(\alpha/\beta\)-type SASP from \textit{Bacillus}, \textit{Sporosarcina}, and \textit{Thermoactinomyces} species show that these proteins are highly conserved with variations in both length and amino acid sequence (4). The identification of protein-protein contacts between amino acid residues found within the N-terminal \(21\)–\(25\) of \(\alpha/\beta\)-type SASP in the DNA-bound state (11) suggests that the N termini of \(\alpha/\beta\)-type SASP mediate protein-protein interactions which may be important for binding cooperativity, and therefore the apparent binding affinity of the DNA binding interaction. To explore this question in more detail, a number of N-terminal variants of SspC, a minor \(\alpha/\beta\)-type SASP from \textit{B. subtilis}, were produced and their DNA binding properties analyzed. SspC was chosen for this study because it is easily overexpressed and purified from both \textit{B. subtilis} spores and \textit{E. coli}, and its interaction with and effects upon DNA have been studied extensively both in \textit{vivo} and in \textit{vitro} (5, 9, 10). Furthermore, SspC binds to DNA with a higher affinity than many other \(\alpha/\beta\)-type SASP, and also has one of the longest N termini of all identified \(\alpha/\beta\)-type SASP (4, 15).

Variants were designed in which blocks of approximately five amino acid residues were progressively removed from the N terminus of SspC (Fig. 1). In addition, SspC(11-135) was constructed to assess the effect of an N-terminal deletion that removes an asparagine residue conserved in all \(\alpha/\beta\)-type SASP from \textit{Bacillus} species and their close relatives (Fig. 1). Each deletion variant was designed such that the protein would contain a N-terminal alanine residue after post-translational removal of the initiating methionine residue (Fig. 1), since alanine is the N-terminal residue of most \(\alpha/\beta\)-type SASP (4, 31). It was possible to overexpress and purify SspC(11-25), SspC(11-10), and SspC(11-14) from either \textit{E. coli}, or from spores of \textit{B. subtilis}. However, smaller SspC variants (SspC(11-21) and SspC(22-25)) could not be overexpressed in \textit{E. coli} nor in spores of \textit{B. subtilis} (data not shown); the reason for this is not clear, but these proteins may not bind to DNA well and therefore they may be very unstable \textit{in vivo}, since DNA binding greatly stabilizes \(\alpha/\beta\)-type SASP against protease digestion (32). In addition to these genetically engineered N-terminal SspC variants, the C-terminal fragments of SspC from endopeptidase Asp-N and GPR digests were also purified and analyzed (Fig. 1). The ability of SspC N-terminal variants and C-terminal proteolytic fragments to bind to DNA was first assessed by DNase I protection assays. All SspC N-terminal variants as well as the C-terminal Asp-N fragment of SspC gave approximately the same degree and pattern of DNase protection to linear pUC19 plasmid DNA as did wild-type SspC, whereas the C-terminal GPR fragment of SspC gave no DNase protection to pUC19 (data not shown). These data indicate that up to 14 residues of SspC (\(-20\%\) of
FIG. 2. CD difference spectra of SspC N-terminal variants bound to poly(dG)-poly(dC). Far UV difference spectra of SspC N-terminal deletion variants bound to poly(dG)-poly(dC). The CD spectrum of poly(dG)-poly(dC) was subtracted from the CD spectra of complexes of SspC N-terminal deletion variant (25 μM) and poly(dG)-poly(dC) (115 μM in bp), as described under “Experimental Procedures” to obtain the presented difference spectra.

The thermal stability of SspC N-terminal variant complexes with poly(dG)-poly(dC) was determined by monitoring the CD at 222 nm for each protein-DNA complex as a function of temperature. Each α/β-type SASP-poly(dG)-poly(dC) complex underwent a sharp thermal dissociation transition over a characteristic temperature range, and the midpoint of each transition (defined as T_M) was determined (Table I) (6). The thermal stability hierarchy was as follows: SspC > SspCΔ14 > SspCΔ10 > SspCΔ11 > Asp-N C-fragment > SspC (Table I). This result was unexpected in that the deletion of additional residues from SspCΔ11 to SspCΔ14 actually increased the stability of the α/β-type SASP-poly(dG)-poly(dC) complex.

Table I: Thermal stability of SspC N-terminal variant-poly(dG)·poly(dC) complexes

<table>
<thead>
<tr>
<th>SspC N-terminal variant</th>
<th>T_M (°C)</th>
</tr>
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<tbody>
<tr>
<td>SspC</td>
<td>71.5</td>
</tr>
<tr>
<td>SspCΔ5</td>
<td>65.5</td>
</tr>
<tr>
<td>SspCΔ10</td>
<td>55.5</td>
</tr>
<tr>
<td>SspCΔ11</td>
<td>57.0</td>
</tr>
<tr>
<td>Asp-N fragment</td>
<td>56.5</td>
</tr>
<tr>
<td>SspCΔ14</td>
<td>63.5</td>
</tr>
<tr>
<td>SspCΔ11-D13N</td>
<td>67.5</td>
</tr>
<tr>
<td>SspCΔ11-D13K</td>
<td>80.5</td>
</tr>
</tbody>
</table>

*The midpoint temperature (T_M) of the thermal dissociation transition was determined as described under “Experimental Procedures.”

used to determine the thermal stabilities (T_M) and equilibrium binding constants (K_α) for α/β-type SASP-DNA interactions (6).

The thermal stability of SspC N-terminal variant complexes with poly(dG)-poly(dC) was determined by monitoring the CD at 222 nm for each protein-DNA complex as a function of temperature. Each α/β-type SASP-poly(dG)-poly(dC) complex underwent a sharp thermal dissociation transition over a characteristic temperature range, and the midpoint of each transition (defined as T_M) was determined (Table I) (6). The thermal stability hierarchy was as follows: SspC > SspCΔ5 > SspCΔ14 > SspCΔ11 > Asp-N C-fragment > SspC (Table I). This result was unexpected in that the deletion of additional residues from SspCΔ11 to SspCΔ14 actually increased the stability of the α/β-type SASP-poly(dG)-poly(dC) complex.

We next wanted to determine the apparent equilibrium constants (K_α) for the interaction between SspC N-terminal deletion variants and DNA to confirm the relative binding affinities suggested by the thermal stability studies. Intrinsic binding constants (K) and cooperativity factors (ω) may be extracted from equilibrium binding titrations of homogeneous synthetic polynucleotides by fitting the McGhee-von Hippel model of cooperative, nonspecific protein-nucleic acid binding to the experimental data (6, 33). The cooperativity factor (ω) is a dimensionless thermodynamic parameter, which describes the relative affinity of a protein ligand for a ligand-contiguous binding site versus an isolated binding site (33). Therefore, differences between SspC variants in both their intrinsic binding constants and binding cooperativity may be detected and quantitated; this is potentially important as the N termini of α/β-type SASP are thought to be involved in DNA-dependent protein-protein interactions (11). Initial equilibrium binding studies were conducted with the N-terminal variants and poly(dG)-poly(dC); however, because the binding cooperativity is low with this polynucleotide and the interactions are very tight (6), it was impossible to accurately determine K and ω values. The interaction between α/β-type SASP and poly(dA-dT)poly(dA-dT) is much more cooperative than with poly(dG)-poly(dC) (6). Unfortunately, most of the SspC N-terminal variants did not bind to poly(dA-dT)poly(dA-dT) at concentrations that could be accurately measured by CD spectroscopy. However, we were able to conduct titrations of poly(dA-dT)poly(dA-dT) with SspC and SspCΔ5, and determined a significant decrease in both K and ω for the interaction with SspCΔ5 compared with that with SspC (Table II). To obtain binding constants for all the N-terminal variants, additional equilibrium titrations were conducted using linear pUC19 plasmid DNA, which tends to bind to α/β-type SASP more tightly.
than poly(dA-dT)-poly(dA-dT). Apparent equilibrium binding constants ($K_\omega$) from these titrations confirmed the hierarchy of binding affinities as determined by thermal stability assays, although individual fits of $K$ and $\omega$ were not possible because the McGhee-von Hippel model was not designed to describe binding to polynucleotides of heterogeneous sequence (Table II).

**Protein-Protein Contacts between DNA-bound SspC N-terminal Variants**—Previously, we used chemical cross-linking to identify amino acid residues that form close contacts between adjacent DNA-bound $\alpha/\beta$-type SASP (11). These residues are located within the N-terminal 40–50% of the proteins, and all identified cross-links occurred between the $\alpha$-amino group of the protein and either of the two glutamate residues of the GPR recognition sequence, or a relatively nonconserved acidic residue (aspartate 13 in SspC) found closer to the N terminus of $\alpha/\beta$-type SASP (11) (Fig. 1). Therefore, the N terminus of a DNA-bound protein appears to be interacting with negatively charged residues on an adjacent DNA-bound protein. This proposed protein-protein interaction could partially explain the unexpected increase in binding affinity seen in SspC N-terminal variants as in wild-type SspC, although individual fits of $K$ and the total digests analyzed by MALDI-TOF mass spectrometry. MALDI maps of tryptic digests of wild-type SspC EDC dimer showed three additional peptides not seen in tryptic digest mass maps of EDC-treated SspC monomer (Fig. 4, A and B). The mass of one of these peptides (Fig. 4, B, labeled 2745.12 Da) corresponded to that of a previously characterized EDC cross-linked peptide in SspC (Ala$^1$-Arg$^6$×Ser$^5$-Lys$^{27}$), which contains an isopeptide cross-link between the $\alpha$-amino group of alanine 1 and the $\beta$-carboxyl of aspartate 13 (Table III) (11). The mass of a second unique peptide (Fig. 4, labeled 2490.75 Da) corresponded to that predicted for a peptide containing a cross-link between the $\alpha$-amino group of alanine 1 and the $\gamma$-carboxyl of either glutamate 29 or 33 (Ala$^1$-Arg$^6$×Leu$^{28}$-Arg$^{65}$) (Table III); these glutamate residues are located within the GPR recognition and cleavage site (Fig. 1). This type of cross-link has previously been identified in other $\alpha/\beta$-type SASP (SASP-A and SASP-C from B. megaterium, and Bee1$^{1010}$K from Bacillus celer) treated with EDC (11), and therefore it was presumed that this peptide has the same structure. The mass of the third unique peptide was consistent with a peptide containing a cross-link between the $\alpha$-amino group of alanine 1 and the C-terminal carboxyl group (Fig. 4, labeled 1802.55 Da). This third cross-linked species is probably a minor cross-linking product, as it has never been detected in reverse phase-HPLC tryptic maps of cross-linked SspC (11); therefore, we have not been able to purify this peptide nor confirm its structure. MALDI tryptic digest maps of EDC-cross-linked SspC$^{15}$ and SspC$^{311}$ indicated that the same amino acid residues were cross-linked as in wild-type SspC (Table III and data not shown). The MALDI tryptic map of SspC$^{311}$ dimer only showed two cross-linked peptides corresponding to cross-links between the $\alpha$-amino group and aspartate 13 and the C terminus (Table III). It is possible that the other expected cross-linked peptide from SspC$^{311}$ failed to ionize efficiently and therefore was not detected by mass spectrometry. Only two cross-linked peptide species were expected in SspC$^{311}$ dimer trypsin digest because the aspartate residue involved in cross-linking in the other proteins (aspartate 13 in wild-type SspC) has been deleted in this variant (Fig. 1). We were only able to detect one cross-linked peptide, corresponding to a cross-link between the $\alpha$-amino group and the C terminus, in the trypsin digest of SspC$^{311}$ dimer. However, we hypothesize that additional cross-linking occurs between the $\alpha$-amino group and the glutamates

### Table II

<table>
<thead>
<tr>
<th>Polynucleotide</th>
<th>Protein</th>
<th>$K_\omega$</th>
<th>$K_\varepsilon$</th>
</tr>
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<tr>
<td>Poly(dA-dT) - poly(dA-dT)</td>
<td>SspC</td>
<td>1.0 $\times$ 10$^6$</td>
<td>2100 (± 100)</td>
</tr>
<tr>
<td></td>
<td>SspC$^{15}$</td>
<td>9.6 $\times$ 10$^4$</td>
<td>960 (± 66)</td>
</tr>
<tr>
<td></td>
<td>SspC$^{311}$-D13K</td>
<td>4.6 $\times$ 10$^6$</td>
<td>ND$^d$</td>
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<tr>
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<td>SspC</td>
<td>8.3 $\times$ 10$^5$</td>
<td>ND$^d$</td>
</tr>
<tr>
<td></td>
<td>SspC$^{310}$</td>
<td>3.8 $\times$ 10$^5$</td>
<td>ND$^d$</td>
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<td>SspC$^{311}$</td>
<td>1.6 $\times$ 10$^4$</td>
<td>ND$^d$</td>
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<td>Asp-N peptide</td>
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<td>Deaminated SspC</td>
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<td>SspC$^{311}$-D13N</td>
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<tr>
<td></td>
<td>SspC$^{311}$-D13K</td>
<td>&gt; 10$^6$</td>
<td>ND$^d$</td>
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</table>

$^a$ Apparent binding constant. Experimental error was estimated at ±15% as described under “Experimental Procedures.”

$^b$ Intrinsic binding constant for isolated, non-contiguous DNA binding site. Errors were obtained from the fitting program.

$^c$ Dimensionless cooperativity factor. Errors were obtained from the fitting program.

$^d$ Not determined. The error of the fit was >50% due to relatively high binding affinity.

$^e$ Not determined as the McGhee-von Hippel model is not intended to describe ligand binding to heterogeneous polynucleotides.
of the GPR sequence, and that this cross-linked peptide probably does not ionize efficiently during mass spectrometry. These data, in conjunction with the estimates of DNA-bound N-terminal variant secondary structure content, suggest that the nature of the protein-protein interactions formed by the N-terminal variants while bound to DNA is very similar to those formed by wild-type SspC.

The α-Amino Group and N-terminal Charge α/β-Type SASP Are Important for DNA Binding—Amino acid residues within the N-terminal half of α/β-type SASP have been shown to be in close contact with one another while in the DNA-bound state by cross-linking and all identified cross-links involved the α-amino group of the N terminus (11). The data presented thus far in this report also suggest that residues in the N-terminal region of α/β-type SASP are important in determining the strength of protein-protein interactions and therefore DNA binding affinity. Therefore, we sought to determine whether the α-amino group of α/β-type SASP is important for binding of these proteins to DNA. The N-terminal α-amino group of proteins can be specifically converted to an α-keto group by a nonenzymatic transamination reaction with glyoxylate and copper(II) ions, resulting in an oxidative deamination of the α-amino group (20, 21). Several α/β-type SASP were deaminated quantitatively as determined by polyacrylamide gel electrophoresis at acid pH (34), which demonstrated a net loss of positive charge (data not shown). The transamination reaction was specific as no other modifications were detected by reverse phase-HPLC peptide mapping of trypsin and endoproteinase...
Glutamic acid residues of unmodified and deaminated α/β-type SASP (data not shown). DNAase I protection assays showed that deaminated SspC conferred somewhat less protection to plasmid DNA than did untreated SspC (Fig. 5, lanes 3 and 6). CD-based equilibrium binding studies also demonstrated that N-terminal deamination significantly reduced the affinity of SspC for pUC19 with a >6-fold decrease in the apparent binding constant (Table III). The effect of N-terminal deamination was even more dramatic for another α/β-type SASP, SASP-A, a major α/β-type SASP from *B. megaterium*, which binds less tightly to DNA than does SspC; deaminated SASP-A provided much less DNase I protection to plasmid DNA than untreated SASP-A (Fig. 5). N-terminal deamination of SspC demonstrated and SspC11 had the same effect upon the ability to provide DNase I protection to plasmid DNA as was seen with SASP-A (Fig. 5 and data not shown), whereas the effect was less dramatic comparing SspC30 and deaminated SspC36 (Fig. 5).

To further confirm that charged residues at the N terminus of α/β-type SASP are important for high affinity DNA binding, the aspartate 13 residue of SspC11 was changed to either an asparagine or a lysine residue. These changes were chosen because asparagine (or glutamine) and lysine residues are found at this position in other α/β-type SASPs (4, 31). Far UV CD spectroscopy of the resulting proteins, SspC11-Arg13, and SspC11-Lys13, bound to an excess of poly(dG)/poly(dC) indicated that these proteins contain essentially the same amount of secondary structure as SspC31 in the DNA-bound state (data not shown). As predicted, SspC11-Arg13 and SspC11-Lys13-poly(dG)/poly(dC) complexes were significantly more thermostable than the SspC31-poly(dG)/poly(dC) complex (Table I). Accordingly, SspC11-Arg13 and SspC11-Lys13 also bound to DNA with significantly higher affinity than SspC31 (Table II). SspC11-Arg13 had ~10-fold greater affinity for pUC19 plasmid DNA than did SspC31, whereas SspC11-Lys13 bound to pUC19 and poly(dA-dT)/poly(dA-dT) more tightly than even wild-type SspC (Table III). EDC cross-linking of SspC11-Arg13 and subsequent MALDI mass spectrometry analysis confirmed that this protein (and presumably also SspC11-Lys13) makes the same DNA dependent protein-protein contacts as the other SspC N-terminal variants (Table III).

**Analysis of the Effects of SspC N-terminal Variants on Spore Properties**—The *in vitro* analysis of SspC N-terminal variants described above demonstrates that up to 14 amino acid residues may be deleted from the N terminus of SspC while still maintaining DNA binding ability, albeit at lower affinities than full-length protein. Therefore, we wanted to determine whether SspC N-terminal variants are able to confer heat and UV resistance on spores *in vivo*, as does the full-length protein. SspC N-terminal variant proteins were overexpressed in *B. subtilis* spores expressing SspC11 (Table II). SspC11 was expressed at similar levels in these spores. Spores expressing SspC, SspC35, and SspC36 were essentially equally resistant to UV radiation, whereas spores expressing SspC11 were slightly, yet significantly, more sensitive to UV radiation (Fig. 6A). Similar results were obtained when heat resistance at 85 °C was determined, except that spores expressing SspC35 and SspC36 appeared to be less resistant to heat killing than spores expressing wild-type SspC (Fig. 6B). The resistance properties of spores expressing SspC31 could not be directly compared with the other spore strains because SspC31 was only expressed at ~50% of the levels of wild-type SspC (data not shown). These data indicate that, although SspC35 and SspC36 have a lower affinity for DNA *in vitro*, they still function nearly as well as wild-type SspC *in vivo*.
the failure of the GPR C-terminal fragment of SspC to bind overexpress shorter SspC N-terminal deletion variants, and significantly to the SspC-DNA binding interaction. Our inability to form DNA, indicating that the deleted residues contribute significantly to the N-terminal regions. These residues are therefore probably not directly involved in contacting the DNA, consistent with the low level of sequence conservation in this region of α/β-type SASP. However, SspC N-terminal deletion variants do show reduced binding affinity for DNA, indicating that the deleted residues contribute significantly to the SspC-DNA binding interaction. Our inability to overexpress shorter SspC N-terminal deletion variants, and the failure of the GPR C-terminal fragment of SspC to bind DNA, suggest that proteins smaller than SspC do not interact with DNA very strongly, and are presumably rapidly degraded in vivo. Additionally, it appears that the N terminus of wild-type SspC is considerably flexible because protein cross-linking demonstrated that the N termini of the SspC N-terminal variants all make similar close contacts with acidic residues on adjacent DNA-bound proteins. Indeed, heterotypic cross-links have also been identified between SASP-A and SASP-C from B. megaterium, whose N termini vary greatly in length (4, 11). These conclusions are consistent with the variability in N-terminal length and sequence seen in the amino acid alignment of all identified α/β-type SASP; in fact, the first significantly conserved amino acid residue in wild-type SspC is asparagine 12 (Fig. 1). However, although not conserved or required for DNA binding, residues glutamine 2 through asparagine 11 in wild-type SspC are important in that they significantly increase the affinity of this α/β-type SASP for DNA.

Thermal stability and equilibrium binding analysis of the SspC N-terminal variant-DNA complexes indicate that SspC binds to DNA with higher affinity than SspC, SspC, and the large Asp-N fragment of SspC. The DNA binding affinity of these proteins seems to be related to the net charge in the N-terminal regions. These findings, in conjunction with the EDC cross-linking data showing that the N terminus of each protein is in close contact with negatively charged residues on adjacent DNA-bound protein (11), may be explained by a model of DNA-dependent protein-protein interaction which involves a significant electrostatic interaction. According to this model, a flexible and positively charged N terminus from each DNA-bound protein interacts with a proposed acidic patch (formed by aspartate 13 and glutamates 29 and 33) found on a neighboring DNA-bound protein (Fig. 7). If the N terminus is arbitrarily defined as the first five amino acid residues, the net charge of the N terminus of wild-type SspC at pH 7.0 is +2, and is conferred by arginine 5 and the α-amino group (Fig. 1). By the same criteria, most of the N-terminal deletion variants of SspC have a less positively charged N terminus: SspC is +2, SspC is 0, SspC is 0, Asp-N C-fragment is 0, and SspC is +1 (Fig. 1). These changes in N-terminal charge are due to the removal of arginines 5 and 7, and the increasing proximity of aspartate 13 to the N terminus as amino acid residues are deleted from wild-type SspC (Fig. 1). In particular, the presence of the negatively charged aspartate 13 residue at the N terminus (in SspC, SspC, and the Asp-N fragment of SspC) appears to interfere with protein-DNA interaction and therefore with DNA binding affinity, because when this residue is removed (along with leucines 14 and 15) in SspC, the DNA binding affinity is significantly increased. Therefore, we hypothesize that electrostatic repulsion between the now N-terminal aspartate 13 and the acidic...
patch on an adjacent DNA-bound SspC$^{11}$ or SspC$^{10}$ contributes to the lower DNA binding affinity. Consistent with this model, derivatives of SspC$^{11}$ that contain either an asparagine or a lysine residue in place of aspartate 13 have significantly higher affinity for DNA than SspC$^{11}$.

The model presented above (Fig. 7) is probably applicable to all α/β-type SASP; the evidence for this is as follows. First, DNA-dependent EDC cross-links identical to those found in SspC have been identified in three other α/β-type SASP from different species (11). Second, higher α/β-type SASP-DNA binding affinity roughly corresponds to a net positively charged N terminus (5, 6, 11). Third, deamination of SASP-A and SspC and its variants reduces the N-terminal net positive charge and results in significantly lower DNA binding affinity. Of course, we cannot exclude the possibility that the N-terminal α-keto group in deaminated α/β-type SASP actually disrupts binding interactions. However, the electrostatic component of α/β-type SASP-protein interactions has been shown to be thermodynamically significant because the cooperativity parameter ($\omega$) of the α/β-type SASP-DNA interaction decreases with increasing salt concentration (6), suggesting that DNA-dependent protein-protein interactions are destabilized by salt.

Although the deletion of 10 amino acid residues from the N terminus of SspC resulted in a protein (SspC$^{10}$) that showed significantly lower affinity for DNA in vitro, it was still able to confer almost full resistance to UV radiation and significant protection from heat to α' β' spores. This is not particularly surprising because SASP-α, the α/β-type SASP in B. subtilis most responsible for sporé UV resistance, has a much lower affinity for DNA than does wild-type SspC (5, 7). In fact, it is thought that high affinity for DNA is not necessary for α/β-type SASP function because the major α/β-type SASP are present at very high concentrations (~1–2 mM) within the spore core (4). At these concentrations, ~80–90% of α/β-type SASP would be DNA-bound at binding constants as low as 1.0–2.0 $\times 10^4$ M$^{-1}$. In addition, the spore core is very dehydrated compared with the corresponding cell cytoplasm, and most of the divalent cations are probably chelated by the enormous level of dipicolinate in the spore core (1); both of these spore core environmental factors should tend to favor α/β-type SASP-DNA interaction. However, although SspC$^{11}$ has a similar or even higher affinity for DNA in vitro compared with SspC$^{10}$, it was less able to confer UV and heat resistance to α' β' spores. The reason for this is not clear, but may be due to the removal of the very highly conserved asparagine 12 residue, which is found in all α/β-type SASP identified from Bacillus species (4, 31). Perhaps this conserved asparagine residue plays an significant structural role in vivo.

It appears that the function of amino acid residues glutamine 2 through asparagine 11 in wild-type SspC is to increase the DNA binding affinity of the protein. Although this increased affinity is not necessary for α/β-type SASP function in vivo, it may reflect the actual role SspC plays in wild-type (α' β') spores. SspC is a minor α/β-type SASP and is only present as ~10% of total α/β-type SASP within wild-type B. subtilis spores (4), with SASP-α and SASP-β comprising most (~80%) of the remaining α/β-type SASP (18). SspC has a higher affinity for DNA than both SASP-α and SASP-β, and therefore SspC may have been selected to bind regions of the spore chromosome that are not bound efficiently by SASP-α or SASP-β. Although there is no significant decrease in spore heat or UV radiation resistance in the laboratory when the sspC gene is inactivated, the role for SspC proposed above could confer a selective advantage to spores. This proposed function for SspC may be generalizable because all Bacillus examined to date contain a number of minor α/β-type SASP (4, 31).

The results in this report also have practical value in aiding in the designing of a minimal high affinity α/β-type SASP for biophysical studies. Because all α/β-type SASP are largely unstructured in the absence of double-stranded DNA (6), a well defined α/β-type SASP-oligonucleotide complex will be required to obtain a high resolution structure. This complex should be small to avoid potential problems with degeneracy arising from the nonspecific nature of α/β-type SASP-DNA binding. In addition, only small complexes (<25 kDa) are routinely tractable by multidimensional NMR methods. For these reasons, the identification of minimal α/β-type SASP that maintain high affinity for DNA is desirable. This study indicates that ~1.2 kDa of SspC may be removed while retaining DNA binding, although there is an attendant loss of binding affinity. However, we have been able to produce a truncated α/β-type SASP, SspC$^{11}$-D$^{13K}$, which binds to DNA with higher affinity than even wild-type SspC. Small, double-stranded oligonucleotides that bind to α/β-type SASP have previously been identified by CD spectroscopic studies (6), and these oligonucleotides are currently being tested with SspC$^{11}$-D$^{13K}$ in an attempt to determine the solution structure of an α/β-type SASP-DNA complex by multidimensional NMR studies.

Acknowledgment—All mass spectrometry was performed by Dr. John Leszyk at the Laboratory for Protein Microsequencing and Mass Spectrometry at the University of Massachusetts School of Medicine.

REFERENCES