Equilibrium and Kinetic Binding Interactions between DNA and a Group of Novel, Nonspecific DNA-binding Proteins from Spores of *Bacillus* and *Clostridium* Species*

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Binding of α/β-type small acid-soluble spore proteins (SASP) is the major determinant of DNA resistance to damage caused by UV radiation, heat, and oxidizing agents in spores of *Bacillus* and *Clostridium* species. Analysis of several α/β-type SASP showed that these proteins have essentially no secondary structure in the absence of DNA, but become significantly α-helical upon binding to double-stranded DNAs or oligonucleotides. Folding of α/β-type SASP induced by a variety of DNAs and oligonucleotides was measured by CD spectroscopy, and this allowed determination of a DNA binding site size of 4 base pairs as well as equilibrium binding parameters of the α/β-type SASP-DNA interaction. Analysis of the equilibrium binding data further allowed determination of both intrinsic binding constants (K) and cooperativity factors (ω), as the α/β-type SASP-DNA interaction was significantly cooperative, with the degree of cooperativity depending on both the bound DNA and the salt concentration. Kinetic analysis of the interaction of one α/β-type SASP, SspC17γ, with DNA indicated that each binding event involves the dimerization of SspC17γ monomers at a DNA binding site. The implications of these findings for the structure of the α/β-type SASP-DNA complex and the physiology of α/β-type SASP degradation during spore germination are discussed.

Dormant spores of *Bacillus* and *Clostridium* species are extremely resistant to a number of environmental insults compared with the corresponding vegetatively growing cells (1–3). A family of abundant spore proteins termed the α/β-type small acid-soluble spore proteins (SASP)1 that saturates the dormant spore chromosome is primarily responsible for the resistance of spore DNA to lethal damage caused by heat, oxidizing agents, and UV radiation (4–8). Indeed, spores of *Bacillus subtilis* that lack the majority of their α/β-type SASP are much more sensitive to these treatments than are wild-type spores (4, 5, 8). The α/β-type SASP are small (6.2–7.6 kDa) nonspecific DNA-binding proteins that are synthesized only within the developing forespore compartment during sporulation (7). The amino acid sequences of α/β-type SASP are highly conserved both within and between species (~70% identity and ~80% similarity, without gaps for *Bacillus* species); however, these proteins show no sequence similarity to any other protein family and do not contain any motifs characteristic of other DNA-binding proteins (7). In all *Bacillus* species studied thus far, two major α/β-type SASP accumulate to high levels, whereas other minor α/β-type SASP are present at lower levels (7). During spore germination and outgrowth, α/β-type SASP are rapidly cleaved into two peptides by a sequence-specific endoprotease, the germination protease; and these oligopeptides are then degraded to amino acids by other spore peptidases (7).

Previous studies suggest that α/β-type SASP undergo a significant change in structure upon binding to DNA. First, α/β-type SASP are extremely sensitive to proteases, but become much more resistant to proteases such as trypsin, chymotrypsin, and germination protease when bound to DNA (9). Second, α/β-type SASP are very susceptible to two forms of spontaneous covalent protein damage, asparagine residue deamidation and methionine residue oxidation (10, 11), indicating that the α/β-type SASP peptide backbone is flexible and accessible to solvent (12, 13). However, the rates of asparagine residue deamidation and methionine residue oxidation in α/β-type SASP are substantially reduced when these proteins are bound to DNA (10, 11). Increased resistance to proteolysis, asparagine residue deamidation, and methionine residue oxidation is consistent with α/β-type SASP becoming structurally more compact upon binding to DNA.

One established aspect of the structural change in α/β-type SASP is the oligomerization of these proteins upon binding to DNA. Equilibrium ultracentrifugation studies have demonstrated that α/β-type SASP are monomeric in solution (14). However, electron micrographs of α/β-type SASP-plasmid DNA complexes show clearly that these proteins bind in long clusters along the DNA backbone, indicating that DNA binding may be cooperative and that the cooperativity could be due to protein-protein interactions between adjacent bound α/β-type SASP (15). DNase I protection studies also indicate that α/β-type SASP-DNA binding is cooperative (16). DNA-dependent protein-protein contacts between α/β-type SASP have been confirmed in other studies, and the interacting amino acid residues have been identified (17).

Clearly, there is considerable evidence that substantial conformational changes occur in α/β-type SASP upon binding to DNA. In this study, we report the use of CD spectroscopy to more directly detect and quantify these structural changes. Spectroscopic signals arising from these structural changes were used to obtain thermodynamic and kinetic parameters for the α/β-type SASP-DNA interaction. The significance of these findings is discussed with respect to the role of α/β-type SASP in the mature spore and their degradation during spore germination and outgrowth.
**Thermodynamics of α/β-Type SASP-DNA Binding**

**EXPERIMENTAL PROCEDURES**

Expression and Purification of α/β-Type SASP—SspCTyr (1.28Y variant of SspC from *B. subtilis*) (18) was overexpressed in *Escherichia coli* strain BL21(DE3) (T7 RNA polymerase under the control of the lac promoter) (19) from the pETT3d-derived plasmid pPS2896. The α/β-type SASP Bef was expressed in *Bacillus cereus* T (17, 20) and ChiC from *Clostridium bifermentans* (21) were overexpressed in *E. coli* strain BL21(DE3) from pET-derived plasmids pPS2734 and pPS2896, respectively. SASP-A was purified from dormant spores of *Bacillus megaterium* strain QMB1551. SASP-α and SASP-β (22) were purified from dormant spores of *C. bifermentans* (a gift of W. M. Waite).

*E. coli* strains were routinely grown in Terrific broth (24 g of yeast extract, 12 g of Tryptone, and 4 ml of glycerol per 900 ml plus 100 ml of 170 mM KH₂PO₄ and 720 mM K₂HPO₄ supplemented with 200 μg/ml ampicillin and 50 μg/ml chloramphenicol at 37 °C shaking. *E. coli* containing plasmids pPS2734, pPS2315, and pPS2896 were grown to an absorbance of 600 nm of 2.5, and α/β-type SASP synthesis was induced by adding isopropyl-β-D-thiogalactopyranoside to 0.5 mM. Cells were harvested by centrifugation after 2 h of further incubation, washed once with 20 mM Tris-HCl (pH 7.4) and 150 mM NaCl, frozen, and lyophilized. *B. megaterium* was sporulated at 30 °C in supplemented nutrient broth, and spores were harvested, purified, and lyophilized as described previously (14, 23).

Lyophilized *E. coli* cells (100 mg at a time) were digested by dry rupture for 2 min in a dental amalgamator (Wig-L-Bug) with glass beads (150 mg) as the abrasive. Lyophilized *B. megaterium* spores (100 mg) were broken similarly, but by 8 min of dry rupture. All α/β-type SASP except SASP-α, SASP-β, and αSASP-β were extracted twice with cold 10 mM sodium phosphate (pH 7.5) at 30°C. Lyophilized *E. coli* containing plasmids pPS2734, pPS2315, and pPS2896 were dialyzed against 10 mM sodium phosphate (pH 7.5). All proteins were dialyzed in Spectrapor 3 tubing at 4 °C against 1% acetic acid (three changes of 4 h). Acid extracts (40–80 ml) were dialyzed in Spectrapor tubing (molecular mass cutoff of 3500 Da) at 4 °C against 1% acetic acid (three changes of 4 h).

Agarose gel electrophoresis was performed on 0.8% agarose gels in 1× TBE buffer. Bands were visualized by staining with ethidium bromide and were excised from the gels. DNA mixtures were averages of three scans acquired at a rate of 0.25 nm/min, a bandwidth of 1 nm, and a response time of 8 s, using a 1-mm path length cuvette. All equilibrium, kinetic, and thermal dissociation data were obtained with a 1.0-cm path length cuvette. Stoichiometric reverse titrations of SspCTyr and ChiC with poly(dG)–poly(dC) were conducted in 1.2 mM of 10 mM sodium phosphate (pH 7.5) at 21 °C with stirring. Poly(dG)–poly(dC) was added sequentially, and the CD signals at 222 nm were measured for 3 min at 5-s intervals. The CD values at 222 nm for each titration point were averaged, and corrections were made for dilution and the contribution of DNA to the CD signals at 222 nm. Corrected CD values are expressed as mean residue ellipticity, [θ]₂22. Secondary structure deconvolution was carried out using a web-based neural network algorithm, K2D (26).

Equilibrium binding forward titrations to determine binding constants were carried out with 15–17 μM (in base pairs) DNA at 21 °C in 5 mM sodium phosphate (pH 7.5) with 20, 40, or 80 mM NaCl or in 10 mM sodium phosphate (pH 7.5) without added NaCl. Each forward titration consisted of 15–20 separate solutions with increasing amounts of α/β-type SASP, which were allowed to equilibrate for 15 h prior to CD measurements. The concentrations of all protein stock solutions were determined by quantitative amino acid analysis. The CD signal of each forward titration solution was measured at 222 nm for 3 min at 5-s intervals, and a spectrum from 300 to 250 nm was also obtained. The relative concentrations of DNA-bound and unbound SspCTyr were calculated based on a two-state model of SspCTyr–DNA binding according to the following equation: [θ]₂22 = f_1[θ]₂22 + (1 − f_1)[θ]₂22, where [θ]₂22 is the observed mean residue ellipticity corrected for DNA and buffer contributions, f_1 is the fraction of DNA-bound protein, [θ]₂22 is the mean residue ellipticity of DNA-bound SspCTyr (~18,400 degrees cm² dmol⁻¹), and [θ]₂22 is the mean residue ellipticity of unbound SspCTyr (~4,000 degrees cm² dmol⁻¹). Calculated proportions of DNA-bound and unbound SspCTyr were consistent with changes in the near-UV CD spectrum (300 to 250 nm) of the titrated polynucleotide. Therefore, fractional saturation of the titrated polynucleotide was determined from calculations of bound α/β-type SASP. McGhee-von Hippel binding densities (ω) were expressed as fractional DNA saturation (ω), and ω was determined by the site saturation of SspCTyr with DNA as described (27), and intrinsic binding constants (K) and cooperativity factors (ω) were determined by iterative nonlinear least-squares fitting of the McGhee-von Hippel model to experimental data. Forward titrations of poly(dG)–poly(dC), poly(dA–dT)–poly(dA–dT), and pUC19 with SspCTyr in 5 mM sodium phosphate (pH 7.5) and 40 mM NaCl were performed twice each, from which an experimental error of approximately ±15% for K_0 was estimated.

CD-monitored thermal unfolding/dissociation experiments were performed on pre-equilibrated complexes of α/β-type SASP (5 μM) and DNA (25 μM in base pairs) in 10 mM sodium phosphate (pH 7.5) layered on top of 4 mM of NaCl with stirring. The heating rates were 25 and 50 °C/min, and the CD signal at 222 nm was measured at every 0.5 or 1 °C interval. The midpoint of each transition (defined as T_m) was determined by taking the first derivative of ellipticity with respect to the inverse of the absolute temperature as described (28).

Kinetic experiments were performed by adding DNA to a stirring solution of α/β-type SASP and monitoring the CD signals at 222 nm as a function of time. This procedure entitled a dead time of ~5 s, although no change in the CD signals at 222 nm (aside from the contribution from buffer contributions) was observed during the dead time. Initial rates of SspCTyr binding were determined by performing least-squares linear regression analysis on the linear portion of the curves. Rates were calculated using the same formula outlined above for equilibrium measurements. Binding rate constants (k_b) were calculated according to the following binding reaction: Δ[Spctyr] = k_b[Spctyr]₀[DNA]₀ per s, where Δ[Spctyr] = [Spctyr]₀ − [Spctyr] is the calculated initial SspCTyr–DNA binding rate in nanomolar bound SspCTyr per s, [Spctyr]₀ is the initial concentration of unbound SspCTyr, and [DNA]₀ is the initial concentration of unbound polynucleotide. These experiments were repeated as 4-point titrations, and the average of all data points was used to determine an initial rate. This model is useful for describing the initial binding reaction at low binding densities, but does not account for overlapping binding sites or potential anti-cooperativity at high α/β-type SASP binding densities (29, 30).

CD spectra of SspCTyr–oligonucleotide complexes were obtained from solutions containing 25 μM protein and 145 μM (in base pairs) oligonucleotide in 1.3 mM sodium phosphate (pH 7.5) at 4 °C in a 1-mm path length cuvette. Each solution was allowed to equilibrate at 4 °C for at least 15 h prior to spectrum acquisition, and all spectra were the average of three scans.
RESULTS

\(\alpha/\beta\)-Type SASP Become \(\alpha\)-Helical upon Binding to DNA—
Several studies suggest that \(\alpha/\beta\)-type SASP lack higher order structure in the absence of DNA, but become more compact upon binding to DNA (9–11). However, these studies employed indirect methods and were therefore not able to determine which type or degree of structural changes occur in \(\alpha/\beta\)-type SASP upon binding to DNA. To explore these issues more definitively, we have used CD spectroscopy to study the changes in \(\alpha/\beta\)-type SASP secondary structure upon the binding of these proteins to DNA. As expected, the far-UV CD spectra of a number of \(\alpha/\beta\)-type SASP from a variety of bacterial species (SspC\(^{TYR}\) from \(B.\) subtilis, SASP-A from \(B.\) megaterium, BcfI from \(B.\) cereus, and CbiC from \(C.\) bifermans) were indicative of a largely random coil conformation in the absence of DNA (Fig. 1 and data not shown) (31). The CD spectra of \(\alpha/\beta\)-type SASP are characterized by a large lobe of negative ellipticity at 200 nm and a less intense lobe of positive ellipticity at 190 nm. However, only very minor changes in the CD spectrum of SspC\(^{TYR}\) were seen with poly(dA) upon addition of poly(dT) (Fig. 1 and data not shown). Difference spectra in which the CD spectrum of free DNA is subtracted from the spectrum of the \(\alpha/\beta\)-type SASP-DNA complex (32, 33) are characteristic of \(\alpha\)-helical proteins, showing nearly equal lobes of negative ellipticity at 208 and 222 nm and a large lobe of positive ellipticity at 190 nm (Fig. 1A and data not shown). Ellipticity values (200–240 nm) from difference spectra were used to estimate the amount of secondary structure in DNA-bound \(\alpha/\beta\)-type SASP; these values ranged from 56 to 69% \(\alpha\)-helix, 4 to 8% \(\beta\)-sheet, and 27 to 35% random structure for the four \(\alpha/\beta\)-type SASP tested.

These calculations assume that only minor changes occur in the DNA component of the CD spectra compared with the changes that occur in the protein component (32, 34). These studies suggest that the large changes in the CD spectra of \(\alpha/\beta\)-type SASP upon addition of poly(dG)-poly(dC) are due to \(\alpha/\beta\)-type SASP binding to DNA. To confirm this, the interactions between several \(\alpha/\beta\)-type SASP and poly(dA)-poly(dT) were examined by CD spectroscopy. DNase I protection studies have shown that \(\alpha/\beta\)-type SASP do not bind or bind extremely weakly to poly(dA)-poly(dT) (16). Consistent with these previous findings, the difference spectra of \(\alpha/\beta\)-type SASP/poly(dA)-poly(dT) mixtures showed only minor differences when compared with the spectra of \(\alpha/\beta\)-type SASP in the absence of DNA (Fig. 1B and data not shown). These data support the conclusion that the changes in ellipticity upon mixing \(\alpha/\beta\)-type SASP and poly(dG)-poly(dC) are due to \(\alpha/\beta\)-type SASP-DNA binding, and not some other effect arising from the polyelectrolyte nature of nucleic acids. In addition, no CD spectral changes were seen in SspC\(^{TYR}\) (25 \(\mu\)M) mixed with 145 \(\mu\)M (in base pairs or bases) single-stranded DNA, single-stranded RNA, or double-stranded DNA (data not shown), consistent with previous results showing no interaction between \(\alpha/\beta\)-type SASP and these nucleic acids (16).
α/β-type SASP and DNA has been studied previously (16, 35), little quantitative data relating to the binding constants of these proteins for DNA have been obtained. Thermodynamic parameters for nonspecific protein-nucleic acid interactions are commonly determined by fluorescence quenching in which intrinsic tryptophan residue fluorescence is quenched as the protein binds to a nucleic acid (24, 27). However, α/β-type SASP lack tryptophan residues (7, 21), and attempts to introduce tryptophan residues into α/β-type SASP have resulted in proteins that either have significantly reduced affinity for DNA (36) or exhibit no change in fluorescence upon binding to DNA. Therefore, we sought to exploit the large change in mean residue ellipticity at 222 nm ([θ]222) as α/β-type SASP bind to DNA to extract thermodynamic parameters for the binding interaction between these two molecules.

SspCTyr was used for most of the following experiments because it is the α/β-type SASP that has been characterized in vitro and in vivo most extensively (16, 18, 35). Initial equilibrium titrations were performed as reverse titrations in which progressively more DNA was added to a constant amount of SspCTyr. Reverse titrations of SspCTyr with all polynucleotides displayed isodichroic points at −204.5 nm (Fig. 2 and data not shown), suggesting (although not proving) that SspCTyr-DNA binding can be described by a simple two-state model (31). An isodichroic point at this wavelength is also characteristic of random coil to α-helix peptide transitions (31). Reverse titrations of SspCTyr with poly(dG)–poly(dC) under tight-binding (stoichiometric) conditions were undertaken to determine the SspCTyr-binding site size in base pairs and the [θ]222 value for SspCTyr bound to DNA (Fig. 3). Knowledge of the site size and the [θ]222 values for SspCTyr bound ([θ]222) and not bound ([θ]unbound) to DNA allows calculation of the concentrations of bound and unbound protein in forward titrations under nonstoichiometric conditions. Reverse titrations of SspCTyr (at 5 and 10 μM) with poly(dG)–poly(dC) displayed sharp break points at DNA concentrations corresponding to a site size of ~4 bp (Fig. 3). The same 4-bp site size was determined by a forward titration of poly(dG)–poly(dC) with SspCTyr in which CD signals from both SspCTyr and poly(dG)–poly(dC) were monitored (data not shown). Forward and reverse titrations also gave the same corrected [θ]222 value of approximately −18,400 degrees cm² dmol⁻¹ for SspCTyr bound to poly(dG)–poly(dC) (Fig. 3 and data not shown). Similar analysis also determined a 4-bp DNA binding site size for CbiC from C. bifermentans with poly(dG)–poly(dC) (data not shown). We were unable to directly determine the site sizes for other polynucleotides by CD because stoichiometric binding conditions could not be obtained. However, scanning transmission electron microscopic studies show that each SspCTyr monomer covers 13.9 Å of random sequence linear pUC19 DNA, corresponding to ~4.1 bp of B-form DNA covered per SspCTyr monomer. In addition, electron micrographs of negatively stained, unfixed complexes of SspC and a 392-bp fragment of random sequence DNA show a 28-Å repeating substructure consistent with the 4-bp site size (15). The 4-bp site size is also in good agreement with the range of 4–5 bp determined for the site size of SspCTyr on poly(dG)–poly(dC), poly(dG–dC)–poly(dG–dC), poly(dA–dT)–poly(dA–dT), and pUC19 by DNase I protection assays (16). Based upon all of these data, the binding site sizes of SspCTyr and CbiC under these conditions were assumed to be 4 bp for all polynucleotides.

Quantitative analysis of reverse titrations to extract thermodynamic parameters involves the graphical binding density function analysis of Bujalowski and Lohman (24, 37). Unfortunately, we were unable to perform such an analysis for two reasons: 1) at protein concentrations above 10 μM, there is precipitation at the high protein/DNA ratios employed during reverse titrations; and 2) each titration point had to be obtained from separate solutions due to the slow kinetics of α/β-type SASP-DNA binding (see below), making the binding den-

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3 C. S. Hayes, M. A. Ross, and P. Setlow, unpublished results.

4 J. Setlow, B. Setlow, and P. Setlow, unpublished results.
sity function analysis very time-consuming and prohibitive in terms of polynucleotide usage. Therefore, we used data from forward titrations (in which increasing amounts of SspCTyr were added to a constant amount of DNA) under non-stoichiometric conditions to determine binding parameters for the interaction between SspCTyr and various polynucleotides as described by Kowalczykowski et al. (27). With the exception of poly(dG)poly(dC), SspCTyr-DNA complexes required several minutes to several hours to reach equilibrium. Therefore, each titration point had to be determined from separate solutions that had been allowed to equilibrate for at least 15 h at 21 °C. The concentrations of unbound and DNA-bound SspCTyr for each titration solution were calculated as described under “Experimental Procedures,” and the amount of DNA-bound SspCTyr was used to calculate the fractional saturation of the DNA lattice based on the determined site size of 4 bp. The calculated values of fractional saturation were confirmed by proportional changes in the near-UV CD spectra (250–300 nm) of the titrated DNA lattice (Fig. 4 and data not shown). The maximum change in near-UV ellipticity between free DNA and α/β-type SASP-bound DNA at a given wavelength was typically only 5–6 millidegrees at the polynucleotide concentrations used (Fig. 4 and data not shown). Therefore, we chose to calculate fractional saturation from [θ]222 instead of the near-UV ellipticities because the protein-derived signals had a larger signal to noise ratio (Fig. 4 and data not shown).

The McGhee-von Hippel equation for nonspecific protein-nucleic acid binding (38) was fitted to SspCTyr-polynucleotide forward titration data, and the thermodynamic parameters and were determined from the fits (Table I), where is the intrinsic binding constant of a monomer for an isolated DNA binding site, and is a dimensionless cooperativity factor describing the relative affinity of a ligand for a contiguous isolated binding site (27, 38, 39). and were not reported for α/β-type SASP-pUC19 interactions (Table I) because McGhee-von Hippel isotherms did not fit the data well (data not shown), presumably due to the heterogeneous nucleotide sequence of plasmid DNA. In addition, accurate determination of and for SspCTyr-poly(dG)poly(dC) binding was not possible due to the high affinity of this interaction (Table I). Under identical buffer and salt conditions, SspCTyr bound all polynucleotides with similar affinities, with the exception of poly(dG)poly(dC), which was bound much more tightly (Table I). The order of binding affinity was as follows: poly(dG)poly(dC) > pUC19 > poly(dA-dT)poly(dA-dC) > poly(dA-dT)poly(dA-dC), with binding constants (Kω) ranging from 1.3 × 10^7 M^-1 for poly(dG-dC)poly(dG-dC) to >10^7 M^-1 for poly(dG)poly(dC) (Table I). These data disagree somewhat with previous results obtained by DNase I protection assays (16). In that study, the order of binding affinity was determined to be as follows: poly(dG)poly(dC) > poly(dG-dC)poly(dG-dC) > pUC19 > poly(dA-dT)poly(dA-dT) (16). The differences in observed relative binding affinities are probably due to the higher temperature (37 °C; see “Thermal Stability of α/β-Type SASP-DNA Complexes” below) used in the previous study (16). Forward titrations of linear pUC19 plasmid DNA and poly(dG)poly(dC) with CbiC from C. bifermentans were also performed to examine the differences in binding affinity compared with SspCTyr (Table I). CbiC bound to both polynucleotides with lower affinity than SspCTyr (Table I), which agrees well with data from DNase I protection and gel shift assays using pUC19 plasmid DNA (data not shown).

Although the apparent binding constants (Kω) for SspCTyr interactions with poly(dG-dC)poly(dG-dC) and poly(dA-dT)poly(dA-dT) are similar, there is a significant difference in the binding cooperativities for each polynucleotide. It has been reported previously that the SspCTyr-polysA-dTpoly(dA-dT) interaction is more cooperative than the interaction of SspCTyr with poly(dG-dC)poly(dG-dC) (16). The current analysis confirms and quantifies this finding, with values for SspCTyr-polysA-dTpoly(dA-dT) binding determined to be ~1.8- and 7.5-fold greater than values for SspCTyr-polysC-dCpoly(dG-dC) binding (Table I).

The binding of nonspecific DNA-binding proteins to DNA is typically very sensitive to salt concentration due to the polyelectrolyte effect; this is the entropically favorable release of ordered cations from nucleic acids that drives many nonspecific protein-DNA interactions. To examine the importance of this effect on the α/β-type SASP-DNA interactions, the effect of salt concentration on SspCTyr-polysA-dTpoly(dA-dT) binding...
Interestingly, no significant decrease in the percent change in ellipticity of poly(dA-dT) with increasing amounts of SspC<sup>Tyr</sup> in 5 mM sodium phosphate (pH 7.5) and 40 mM NaCl at 21 °C were acquired as described under "Experimental Procedures." The concentrations of added SspC<sup>Tyr</sup> were 0 (curve a), 3 (curve b), 6 (curve c), 9 (curve d), 12 (curve e), 15 (curve f), and 18 (curve g) μM. B, the percent change in the ellipticity of poly(dA-dT)poly(dA-dT) at 271 nm ([]) and the fractional saturation of poly(dA-dT)poly(dA-dT) calculated from [θ]<sub>222</sub> were plotted as a function of total SspC<sup>Tyr</sup> concentration from a forward titration of poly(dA-dT)poly(dA-dT) in 5 mM sodium phosphate (pH 7.5) and 40 mM NaCl at 21 °C. The concentrations of added SspC<sup>Tyr</sup> were the same as for A. mdeg, millidegrees.

**TABLE I**

Equilibrium binding constants (K<sub>ω</sub>) for the α/β-type SASP interaction with polynucleotides

| α/β-Type SASP | Polynucleotide | [NaCl] | K<sub>ω</sub> | K<sup>α</sup> | K<sup>β</sup> |
|---------------|----------------|--------|-------------|-------------|-------------|
| SspC<sup>Tyr</sup> | Poly(dG) · poly(dC) | 40 | >10<sup>7</sup> | ND | ND |
| | Poly(dG-dC) · poly(dG-dC) | 80 | 4.8 × 10<sup>6</sup> | ND | ND |
| | Poly(dG-dT) · poly(dG-dC) | 40 | 1.3 × 10<sup>5</sup> | 9200 ± 880 | 80 ± 6.0 |
| | Poly(dG-dT) · poly(dG-dT) | 80 | 1.6 × 10<sup>5</sup> | 2100 ± 200 | 75 ± 6.8 |
| | pUC19 (linear) | 40 | 3.2 × 10<sup>5</sup> | ND | ND |
| | Poly(dA-dT) · poly(dG-dC) | 20 | 4.8 × 10<sup>5</sup> | 2000 ± 300 | 240 ± 36 |
| | Poly(dA-dT) · poly(dA-dT) | 40 | 1.4 × 10<sup>5</sup> | 1000 ± 200 | 140 ± 27 |
| | pUC19 (linear) | 80 | 3.8 × 10<sup>4</sup> | 640 ± 170 | 59 ± 14 |

* Intrinsic binding constants for isolated, non-contiguous DNA binding sites were determined as described under “Experimental Procedures.”

* Cooperative factor was determined as described under “Experimental Procedures.”

* Not determined.

* Titration was performed in 10 mM sodium phosphate (pH 7.5) at 21 °C.

was examined at 20, 40, and 80 mM NaCl (Table I). In contrast to many nonspecific protein-nucleic acid binding interactions in which ω is insensitive to salt (39–42), the cooperativity factor decreased as the salt concentration was increased (Table I). Interestingly, no significant decrease in ω was seen in the binding of SspC<sup>Tyr</sup> to poly(dG-dC)poly(dG-dC) when the salt concentration was increased from 0 to 40 mM (Table I). The number of ionic interactions involved in nonspecific protein-DNA interactions can be obtained from the slope of a log(K<sub>ω</sub>) versus log[NaCl] plot (43, 44). The log(K<sub>ω</sub>/log[NaCl] plot for the SspC<sup>Tyr</sup>poly(dA-dT)poly(dA-dT) interaction is linear within this salt concentration range, with a slope equal to −0.85 (data not shown), suggesting the formation of approximately one ionic interaction between each SspC<sup>Tyr</sup> monomer and poly(dA-dT)poly(dA-dT) upon binding (43, 44).

**Thermal Stability of α/β-Type SASP-Polynucleotide Complexes**—Thirty-four α/β-type SASP have been identified at the gene or protein level from 13 different Gram-positive endospore-forming species (7, 22). The DNA-binding properties of 11 of these proteins have been examined in some detail (9, 16).<sup>5</sup> It is becoming clear that despite the high degree of sequence conservation between these proteins, significant differences in DNA-binding affinities exist (16).<sup>5</sup> Because forward titrations are time-consuming and expensive in terms of protein and DNA, we sought to develop a rapid alternative method of determining relative α/β-type SASP-DNA binding affinities. Therefore, the thermal stability of the α/β-type SASP-DNA interaction was determined by monitoring the CD signals at 222 nm of pre-equilibrated α/β-type SASP-DNA complexes as a function of temperature. Each α/β-type SASP-polynucleotide complex dissociated sharply over a characteristic temperature

<sup>5</sup> C. S. Hayes and P. Setlow, unpublished results.
range, and the hierarchy of thermal stabilities for SspC<sup>Tyr</sup>, polynucleotide complexes was as follows: poly(dG)·poly(dC) > pUC19 ≈ poly(dG·dC)·poly(dG·dC) > poly(dG·dT)·poly(dA·dC) > poly(dA·dT)·poly(dA·dT) (Table II).

Quantitative analysis of thermal unfolding/dissociation requires that the process be reversible. Typically, >80% of the original [θ]<sub>222</sub> value was recovered within 1 h after cooling to 20 °C, provided that the solutions were not heated beyond the temperature of complete dissociation (data not shown). In addition, thermal dissociation curves for SspC<sup>Tyr</sup>·poly(dA·dT)·poly(dA·dT) and SspC<sup>Tyr</sup>·poly(dG)·poly(dC) complexes obtained at heating rates of 25 and 50 °C/h were superimposable (data not shown), suggesting that the dissociation reaction is at equilibrium at each point along these thermal dissociation curves. However, dissociation curves for SspC<sup>Tyr</sup>·pUC19 complexes at the two heating rates used were not exactly superimposable, with a <i>T<sub>m</sub></i> value 1 °C lower for the dissociation conducted at 25 °C/h compared with 50 °C/h (data not shown). This minor difference in the <i>T<sub>m</sub></i> value is probably due to increased protein damage to unbound α/β-type SASP that occurs at higher temperatures. All α/β-type SASP from Bacillus species contain a conserved Asn residue within an Asn-Gly sequence, which deamidates rapidly <i>in vitro</i>, and deamidation of this specific Asn residue abolishes the DNA-binding activity of α/β-type SASP (10). Because deamidation of unbound α/β-type SASP occurs so rapidly at high temperatures (10), it is difficult to confirm that each point on the thermal dissociation curves for pUC19 and poly(dG·dC)·poly(dG·dC) represents an equilibrium condition.

The hierarchy of thermal stability varied somewhat from that of the equilibrium binding constants determined in 5 mM sodium phosphate (pH 7.5) and 40 mM NaCl at 21 °C. In general, the thermal stabilities were a reflection of both the intrinsic binding affinity between α/β-type SASP and DNA and the melting temperatures of the polynucleotides used. Each α/β-type SASP-polynucleotide complex underwent dissociation at temperatures lower than or coincident with the DNA melting transition (Table II), indicating that α/β-type SASP do not increase the melting point of DNA. This property of the α/β-type SASP-DNA interaction may account for the unexpectedly low thermal stability of α/β-type SASP·poly(dA·dT)·poly(dA·dT) complexes (Table II) and explains the previously observed low affinity seen in DNase I protection assays that were conducted at 37 °C (16).

Although the <i>T<sub>m</sub></i> values obtained with polynucleotides do not exactly correspond to the equilibrium binding constants determined at 21 °C, thermal dissociations are still useful to quickly determine relative differences in α/β-type SASP binding affinity for a given polynucleotide. For instance, there is a substantial difference in the <i>T<sub>m</sub></i> values for SspC<sup>Tyr</sup> complexes with poly(dG)·poly(dC) versus poly(dG·dC)·poly(dG·dC) (Table II), consistent with the differences in their respective equilibrium binding constants. Therefore, <i>T<sub>m</sub></i> values do reflect the intrinsic binding affinity when comparing DNAs that have similar melting temperatures (Tables I and II). The <i>T<sub>m</sub></i> values determined

![Figure 5: Kinetics of SspC<sup>Tyr</sup>-DNA binding](image)
for SspCTyr and CbiC complexes with poly(dG)-poly(dC) or pUC19 DNA indicate that CbiC-DNA complexes are less stable than the corresponding SspCTyr-DNA complexes (Table II). The difference in thermal stability is consistent with the equilibrium binding constants for poly(dG)-poly(dC) and pUC19 determined for CbiC and SspCTyr (Table I). Similar Tm values for the SspCTyr- and CbiC-poly(dA-dT)-poly(dA-dT) interactions (Table II) probably indicate that the thermal stability of these complexes is primarily a function of this polynucleotide's low melting point. Thus, the relative thermal stabilities of α/β-type SASP-DNA complexes can serve as a rapid quantitative alternative to detailed equilibrium binding studies. Indeed, this relationship has been confirmed using several different mutant forms of SspC that have both lower and higher affinities for DNA than the wild-type protein.5

α/β-Type SASP-Polynucleotide Binding Kinetics—As previously mentioned, the interaction of SspCTyr with most polynucleotides required several minutes to several hours to reach equilibrium. Slow kinetics of α/β-type SASP-DNA complex formation have been reported in previous studies of α/β-type SASP-DNA binding (16) and are not particularly surprising given that substantial changes in both protein and DNA conformation occur upon complex formation. The kinetics of α/β-type SASP-DNA binding were studied by monitoring CD signals at 222 nm as a function of time after the addition of DNA. Polynucleotides (15 μM in base pairs) were added to a stirring solution of SspCTyr (5 μM), and the ellipticity at 222 nm was monitored for 15 min (Fig. 5). The measured ellipticities were corrected for the addition of DNA and used to calculate DNA-bound protein based upon the same equation used in the equilibrium studies. The initial rates of SspCTyr-DNA binding were calculated from least-squares linear regression analysis of the linear portion of the curves. The relative rates of SspCTyr binding to polynucleotides were as follows: poly(dG)-poly(dC) > pUC19 > poly(dG-dT)-poly(dA-dC) > poly(dA-dT)-poly(dA-dT) > poly(dG-dC)-poly(dG-dC) (Fig. 5 and Table III). The binding rates of SspCTyr for poly(dG-dC)-poly(dG-dC) and poly(dA-dT)-poly(dA-dT) were particularly slow (Fig. 5 and Table III), which necessitated the long incubation times used in the equilibrium binding studies outlined above. However, there was a wide range of initial binding rates for pUC19 plasmid DNA and different α/β-type SASP (5 μM), with SASP-β from C. bifermentans reaching equilibrium within 1 min, whereas SASP-A and SASP-C from B. megaterium failed to show any detectable binding within the first 10 min of mixing (data not shown). Based on studies with pUC19 and poly(dG)-poly(dC), the relative rates of α/β-type SASP binding were as follows: SASP-β (C. bifermentans) > SspCTyr > BceI > SASP-α (C. bifermentans) > CbiC > SASP-C > SASP-A (data not shown). This hierarchy of α/β-type SASP-DNA binding rates corresponds to the relative affinities of these proteins for DNA as determined by DNase I protection (9, 16).

Binding rate constants were also determined to compare the binding of SspCTyr to several different polynucleotides. Because accurate CD measurements require micromolar concentrations of macromolecules, we were unable to conduct kinetic experiments under pseudo first-order conditions in which the DNA is in vast excess, as the DNA concentrations required would be prohibitively high. Instead, kinetic binding experiments were conducted using SspCTyr and linear pUC19 plasmid DNA over a range of concentrations, and initial rates of binding were calculated (Table III). For each given concentration of SspCTyr, a 2-fold increase in plasmid DNA concentration resulted in a corresponding 2-fold increase in the initial rate of binding (Table III). For each given concentration of plasmid DNA, a 2-fold increase in SspCTyr concentration resulted in a 4-fold increase in the initial rate of binding (Table III). These data indicate that the binding interaction between SspCTyr and pUC19 is second-order with respect to SspCTyr concentration and therefore is not a simple bimolecular reaction. Instead, the rate-limiting step of binding involves the dimerization of SspCTyr monomers at the DNA binding site. Based upon these results, the binding rate constants were calculated using the initial binding rates according to the following rate equation: Δ[SspC]/Δt = k[SspC]2[plUC19], where [SspC]0 is the concentration of DNA-bound SspCTyr, [SspC]0 is the initial concentration of unbound SspCTyr, [plUC19]0 is the initial concentration of free 8-bp protein-binding sites, and k is the binding rate constant. DNA concentration was expressed in terms of 8-bp binding sites (4 bp for each SspCTyr monomer) to reflect the dimerization of SspCTyr during the binding interaction. The same second-order dependence on SspCTyr concentration was observed with poly(dG-dT)-poly(dA-dC) (Table III) and poly(dA-dT)-poly(dA-dT) (data not shown) and therefore appears to be a general property of the SspCTyr-DNA interaction. This model was supported by other kinetic studies that showed that the initial rate of change in pUC19 conformation as measured by CD at 263 nm increased 4-fold when the added SspCTyr concentration was doubled (data not shown). Additional kinetic studies using SASP-α from C. bifermentans and pUC19 DNA gave similar results (data not shown).

### Table III

| Polynucleotide          | [DNA] µM | [SspCTyr] µM | Initial rate of binding b s⁻¹ | kₐ b s⁻¹ |
|-------------------------|----------|--------------|-----------------------------|----------|
| pUC19 (linear)          | 0.95     | 2.5          | 2.2                         | 38       |
|                         | 1.9      | 2.5          | 4.0                         | 34       |
|                         | 1.9      | 5.0          | 22                          | 46       |
|                         | 1.9      | 10           | 72                          | 73       |
|                         | 3.5      | 5.0          | 34                          | 36       |
|                         | 3.5      | 10           | 140                         | 37       |
|                         | 3.8      | 20           | 330                         | 22       |
|                         | 7.5      | 20           | 1000                        | 34       |
| Poly(dG-dC) - poly(dG-dC) | 1.9  | 5.0          | 1.1                         | 2.2      |
| Poly(dA-dT) - poly(dA-dT) | 1.9  | 5.0          | 14                          | 3.0      |
|                         | 1.9      | 5.0          | 6.2                         | 13       |
|                         | 1.9      | 10           | 25                          | 13       |
| Poly(dG) - poly(dC)     | 1.9  | 5.0          | 240                         | 500      |

a DNA concentration is expressed as micromolar 8-bp binding sites.
b SspCTyr-polynucleotide binding rates were determined as described under “Experimental Procedures,” and rates are reported as nanomolar SspCTyr bound to polynucleotide per s. The binding rate constant (kₐ) was calculated using initial concentrations of SspCTyr and polynucleotides and the initial binding rate as described under “Experimental Procedures.”

c This number represents the mean ± S.D. of the calculated rate constants for the SspCTyr-pUC19 binding interaction.

### Thermodynamics of α/β-Type SASP-DNA Binding

α/β-Type SASP-DNA complexes (Table I) and pUC19 DNA interact in a second-order reaction (data not shown). This hierarchy of α/β-type SASP-DNA binding rates corresponds to the relative affinities of these proteins for DNA as determined by DNase I protection (9, 16).

Because accurate CD measurements require micromolar concentrations of macromolecules, we were unable to conduct kinetic experiments under pseudo first-order conditions in which the DNA is in vast excess, as the DNA concentrations required would be prohibitively high. Instead, kinetic binding experiments were conducted using SspCTyr and linear pUC19 plasmid DNA over a range of concentrations, and initial rates of binding were calculated (Table III). For each given concentration of SspCTyr, a 2-fold increase in plasmid DNA concentration resulted in a corresponding 2-fold increase in the initial rate of binding (Table III). For each given concentration of plasmid DNA, a 2-fold increase in SspCTyr concentration resulted in a 4-fold increase in the initial rate of binding (Table III). These data indicate that the binding interaction between SspCTyr and pUC19 is second-order with respect to SspCTyr concentration and therefore is not a simple bimolecular reaction. Instead, the rate-limiting step of binding involves the dimerization of SspCTyr monomers at the DNA binding site. Based upon these results, the binding rate constants were calculated using the initial binding rates according to the following rate equation: Δ[SspC]/Δt = k[SspC]²[plUC19], where [SspC]₀ is the concentration of DNA-bound SspCTyr, [SspC]₀ is the initial concentration of unbound SspCTyr, [plUC19]₀ is the initial concentration of free 8-bp protein-binding sites, and k is the binding rate constant. DNA concentration was expressed in terms of 8-bp binding sites (4 bp for each SspCTyr monomer) to reflect the dimerization of SspCTyr during the binding interaction. The same second-order dependence on SspCTyr concentration was observed with poly(dG-dT)-poly(dA-dC) (Table III) and poly(dA-dT)-poly(dA-dT) (data not shown) and therefore appears to be a general property of the SspCTyr-DNA interaction. This model was supported by other kinetic studies that showed that the initial rate of change in pUC19 conformation as measured by CD at 263 nm increased 4-fold when the added SspCTyr concentration was doubled (data not shown). Additional kinetic studies using SASP-α from C. bifermentans and pUC19 DNA gave similar results (data not shown).

Interaction of SspCTyr with Oligonucleotides—Although α/β-type SASP and their DNA-binding properties have been studied for several years, there are no high resolution structural data for this novel class of nonspecific DNA-binding proteins. Because all α/β-type SASP are essentially without structure in the absence of DNA, high resolution structural information will be obtained only with a defined, non-degenerate complex of an α/β-type SASP with a double-stranded oligonucleotide. It has been shown previously that SspCTyr can protect a GC-rich oligonucleotide as small as 12 bp from digestion with DNase I (16). More recently, it has been shown by polycrystalline gel shift analysis that SspCTyr can bind to 10-bp GC-rich oligonucleotides. The current study has demonstrated that CD spectroscopy is a very sensitive tool capable of detecting α/β-type SASP-DNA interactions. Therefore, we used CD spectroscopy
to examine the binding of SspC<sup>Tyr</sup> to a variety of oligonucleotides to identify small DNA fragments that still support SspC<sup>Tyr</sup> binding and that may be useful in biophysical characterization of the complex by multidimensional NMR or X-ray crystallography.

Because α/β-type SASP tend to form more stable complexes with GC-rich polynucleotides, we focused on making oligonucleotides that were very GC-rich. Two classes of oligonucleotides were studied. The first class contained self-annealing oligonucleotides of 6, 8, 10, and 12 bp with or without single 3′-dA overhangs. The second class of oligonucleotides was composed of 5–8-bp homo-oligomers of dG and dC, which were annealed to one another. The homo-oligomers also contained single 3′-dA overhangs to prevent strand slippage. SspC<sup>Tyr</sup> bound to oligonucleotides larger than 7 bp, as determined by a significant change in [θ]<sub>222</sub> as measured from difference spectra (Fig. 6). The change in [θ]<sub>222</sub> was greater for homo-oligomers than for self-annealing oligomers of the same length (Fig. 6), consistent with the difference in SspC<sup>Tyr</sup> affinity for poly(dG)poly(dC) and poly(dG-dC)poly(dG-dC). In addition, the degree of CD spectral changes tended to decrease as the size of the oligonucleotide was decreased (Fig. 6). The shortest oligonucleotide that still supported SspC<sup>Tyr</sup> binding was a 6-bp homo-oligonucleotide, 5′-d(G<sub>6</sub>A)5′-d(C<sub>6</sub>A) (Fig. 6). No interaction was detected between SspC<sup>Tyr</sup> and a 5-bp homo-oligonucleotide, 5′-d(G<sub>5</sub>A)5′-d(C<sub>5</sub>A), or a 6-bp self-annealing oligonucleotide, 5′-d(GCGGCA)2, indicating binding constants of <10<sup>5</sup> M<sup>-1</sup> (Fig. 6). Each of the two oligonucleotides that failed to bind to SspC<sup>Tyr</sup> were double-stranded, as determined by near-UV CD spectroscopy and 20% polycrylamide gel electrophoresis (data not shown). Therefore, SspC<sup>Tyr</sup> appears to require a minimum of 6 bp of duplex DNA with single 3′-overhangs to bind productively.

**DISCUSSION**

The data presented in this study clearly demonstrate that the α/β-type SASP lack significant secondary structure when not bound to double-stranded DNA. However, in the DNA-bound state, α/β-type SASP acquire a significant amount of α-helical secondary structure and therefore appear to fold upon the DNA scaffold. To our knowledge, the general phenomenon of nucleic acid binding coupled to folding of a completely disordered protein has at least three precedents in the literature: 1) The N protein from bacteriophage λ is completely disordered, but becomes partially α-helical upon binding to its specific boxB RNA sequence (45); 2) a fragment of the high mobility group chromosomal protein HMG-I is a random coil in the absence of DNA, but adopts a defined conformation within the minor groove of bound DNA (46); and 3) the Phd protein of phage P1 is largely unfolded at 37 °C, but becomes α-helical upon binding to its specific operator sequence (34). In addition, several other site-specific DNA-binding proteins undergo transitions in which disordered domains become ordered and form binding interfaces with specific DNA sites (47). The possible advantages of having an unfolded protein recognize nucleic acids have been outlined recently by Frankel and Smith (48). These include the ability to add other macromolecules to the complex in an ordered fashion and a mechanism by which the cell can monitor the functional (folded versus unfolded) state of the protein ligand (49). These two properties could be important to α/β-type SASP DNA complex formation and α/β-type SASP degradation after spore germination, respectively. First, protein–protein contacts are formed between α/β-type SASP only while bound to DNA (17). The formation of potential protein–protein binding surfaces induced by DNA could direct the further addition of α/β-type SASP molecules to the ends of DNA-bound protein clusters and therefore regulate protein binding. Second, the α/β-type SASP are toxic when overexpressed in *E. coli* (49) and can also inhibit transcription during spore germination and outgrowth (50). Consequently, these proteins must be degraded early in spore germination such that vegetative growth may resume. The unfolded nature of unbound α/β-type SASP ensures that these proteins are very susceptible to germination protease and possibly other proteases.

The binding constants determined for the α/β-type SASP-
DNA interaction are similar to those determined for other nonspecific DNA-binding proteins involved in maintaining general chromatin structure (24, 51). However, during sporulation, the α/β-type SASP accumulate to millimolar concentrations within the spore core, and the total amount of α/β-type SASP within the spore appears to be sufficient to saturate the spore chromosome (4). The SspCTVα binding constants were all determined under relatively low salt conditions (<80 mM) so that the concentrations of DNA-bound and unbound α/β-type SASP could be measured most accurately by CD spectroscopy. However, it is difficult to relate the conditions used in vitro to determine binding constants to the environment within the dormant spore core. It is not clear whether there are free monovalent ions or free water within the dormant spore, and all divalent cations probably exist as chelates with pyridine-2,6-dicarboxylic acid (dipicolinic acid) (3), so the actual binding constant for the interaction in vivo could be substantially higher. However, during spore germination and outgrowth, several changes occur within the spore core, including rehydration, volume expansion, release and re-accumulation of potassium ions, and release of dipicolinic acid plus its chelated cation, volume expansion, release and re-accumulation of potassium ions. Furthermore, the two major α/β-type SASP in B. subtilis (α and β) have a lower affinity for DNA in vitro compared with SspCTVα (16, 54). Therefore, a substantial fraction of DNA-bound α/β-type SASP would dissociate under germination conditions and be quickly cleaved by germinase protease. Rapid degradation of unbound α/β-type SASP leads to further dissociation by mass action until the chromosome is free of α/β-type SASP, as suggested previously (50).

The moderate degree of binding cooperativity seen in the α/β-type SASP-DNA binding interaction has been described previously, but never quantitated (15, 16). A previous study also reported that α/β-type SASP binding to poly(dA-dT)poly(dA-dT) was very cooperative, but that binding to poly(dG-dC)poly(dG-dC) was not (16). This difference in cooperativity has been confirmed and quantitated here; and in addition, α/β-type SASP-DNA binding cooperativity has been shown to depend upon salt concentration. The polynucleotide-specific differences in binding cooperativity are thought to be related to the ability of the DNA to adopt an A-like conformation, as the α/β-type SASP change DNA structure to an A-like conformation in which the pitch of the double helix is not changed significantly (15, 54). GC-rich polynucleotides adopt the A-conformation in solution more readily than do AT-rich polynucleotides. Accordingly, binding cooperativity is thought to be largely due to local changes in DNA structure near the ends of α/β-type SASP clusters that favor binding of additional α/β-type SASP contiguously at that site rather than at other non-contiguous binding sites. However, protein-protein interactions probably also contribute to the binding cooperativity, and the dependence of α upon salt concentration may reflect the importance of ionic protein-protein interactions. Chemical cross-linking studies have shown that the positively charged amino terminus of one DNA-bound α/β-type SASP interacts with an acidic region on another adjacent DNA-bound α/β-type SASP (17). Other studies have demonstrated that positively charged residues near the amino terminus of SspC increase DNA-binding affinity, although these residues do not appear to directly interact with DNA. The decrease in binding cooperativity with increased ionic strength is consistent with the disruption of an ionic protein-protein interaction and, in combination with the aforementioned data, suggests that this protein-protein interaction makes a significant contribution to DNA-binding affinity.

Perhaps the most unexpected finding in the current study is that the initial rate of SspCTVα (and SASP-α from C. bifermens)-polynucleotide binding is second-order with respect to initial unbound protein concentration. The simplest interpretation of these data is that two SspCTVα monomers are required for each productive binding event to occur. A trimolecular binding reaction coupled with the extensive macromolecular rearrangements that occur during α/β-type SASP-DNA binding could account for the very slow binding kinetics. However, the second-order dependence upon protein concentration was not observed in kinetic studies with SASP-β from C. bifermens or SASP-C from B. megaterium (data not shown). This is particularly surprising given the high degree of amino acid sequence conservation between SspCTVα and SASP-C and between SASP-α and SASP-β from C. bifermens (21). These findings suggest that there may be two different mechanisms of α/β-type SASP-DNA binding. Alternatively, it is possible that the binding mechanism involves multiple kinetic intermediates, and the binding of individual α/β-type SASP may be rate-limited at different intermediate steps. It is clear that more detailed kinetic and structural studies are warranted to more clearly define the mechanism of α/β-type SASP-DNA binding.

The ultimate goal of these studies on α/β-type SASP is to obtain a high resolution structure of an α/β-type SASP-polynucleotide complex, and the current study has been very important in facilitating further biological studies aimed at determining such a structure. It has become clear from the polynucleotide studies that the DNA binding site size for SspCTVα (and probably all α/β-type SASP) is 4 bp and that productive DNA binding probably requires at least two SspCTVα monomers. This information is important for designing oligonucleotides for high resolution structural studies, particularly for NMR, where only relatively small complexes are tractable. Based on a DNA binding site size of 4 bp and a requirement for at least two bound α/β-type SASP, the smallest double-stranded oligonucleotide predicted to still support α/β-type SASP binding would be 8 bp in length. However, the smallest oligonucleotide that interacted with SspCTVα was a 6-bp molecule with single 3' overhangs. This is still consistent with the determined site size and postulated requirement for two bound SspCTVα monomers, as the two 3' overhangs increase the overall dimensions of the oligonucleotide to approximately those of a blunt-ended 8-bp duplex. The observation that binding is abruptly lost between the 6- and 5-bp homo-oligonucleotides with single 3' overhangs is also consistent with the binding model, in that the 5-bp oligonucleotide would be predicted to support binding of only one SspCTVα molecule. It should be pointed out that a site size of 4 bp does not necessarily indicate that the protein ligand directly interacts with all 4 bp of the binding site; instead, it is a measure of how many base pairs are occluded by the ligand. The exact nature of the α/β-type SASP-DNA binding interaction will only be known when a high resolution structure is obtained. This should yield important data in general terms, as α/β-type SASP are a unique class of DNA-binding proteins that have no amino acid sequence similarity to any other DNA-
binding proteins (7, 21). Also of interest is the exact molecular nature of the change in DNA conformation induced by α/β-type SASP binding, as this conformational change is the biochemical basis for spore resistance to UV radiation (3, 4). Based upon the SspCTyr binding, as this conformational change is the biochemical microscopy.

Acknowledgments—We thank Richard A. Ando and Kendall L. Knight for helpful suggestions on determining equilibrium binding constants and Margery A. Ross for providing additional pUC19 plasmid DNA. Jane Setlow, Beth Yu Lin, Martha Simon, and Joseph Wall determined the SspCTyr-binding site by scanning transmission electron microscopy.

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