The effect of Mg$^{2+}$ on the binding of the *Streptococcus pneumoniae* single-stranded DNA binding (SSB) proteins, SsbA and SsbB, to various dT$_n$ oligomers was examined by polyacrylamide gel electrophoresis. The results were then compared with those that were obtained with the well characterized SSB protein from *Escherichia coli*, SsbEc. In the absence of Mg$^{2+}$, the results indicated that the SsbEc protein was able to bind to the dT$_n$ oligomers in the SSB$_{35}$ mode, with only two of the four subunits of the tetramer interacting with the dT$_n$ oligomers. In the presence of Mg$^{2+}$, however, the results indicated that the SsbEc protein was bound to the dT$_n$ oligomers in the SSB$_{65}$ mode, with all four subunits of the tetramer interacting with the dT$_n$ oligomers. The SsbA protein behaved similarly to the SsbEc protein under all conditions, indicating that it undergoes Mg$^{2+}$-dependent changes in its DNA binding modes that are analogous to those of the SsbEc protein. The SsbB protein, in contrast, appeared to bind to the dT$_n$ oligomers in an SSB$_{65}$-like mode in either the presence or the absence of Mg$^{2+}$, suggesting that it may not exhibit the pronounced negative intrasubunit cooperativity in the absence of Mg$^{2+}$ that is required for the formation of the SSB$_{35}$ mode. Additional experiments with a chimeric SsbA/B protein indicated that the structural determinants that govern the transitions between the different DNA binding modes may be contained within the N-terminal domains of the SSB proteins.

We have recently amplified the ssbA and ssbB genes from *S. pneumoniae* genomic DNA, developed efficient expression systems, and purified the SsbA and SsbB proteins to apparent homogeneity (1, 6). In our initial investigations, we found that the SsbA and SsbB proteins, like the SsbEc protein, formed stable homotetramers in solution (7). However, although the ssDNA binding properties of the SsbA protein appeared to be similar to those of the SsbEc protein, the ssDNA binding characteristics of the SsbB protein were quite different. For example, although the SsbB protein was able to bind to the shorter oligomer dT$_{50}$ with an affinity similar to that of the SsbEc and SsbA proteins, our results indicated that two SsbEc or SsbA tetramers were able to bind to the longer oligomer dT$_{75}$, whereas only a single SsbB tetramer was able to bind to this ssDNA. The apparent differences in the stoichiometries of binding to dT$_{75}$ and other longer oligomers indicated that the SsbB protein interacts with ssDNA in a manner different from that of the SsbEc and SsbA proteins (7).

The ssDNA binding properties of the SsbEc protein have been shown to exhibit a complex dependence on solution conditions (2). In general, at lower DNA binding densities and higher monovalent or divalent salt concentrations, the SsbEc protein binds in either the SSB$_{65}$ or the SSB$_{35}$ mode in which all four subunits of the tetramer interact with ssDNA, occluding ~56 or 65 nucleotides of ssDNA/tetramer, respectively (since the physical distinction between the SSB$_{65}$ and SSB$_{35}$ modes is not clear, they will be referred to collectively here as the SSB$_{65}$ mode). At higher DNA binding densities and lower salt concentrations, however, the SsbB protein can bind in the SSB$_{65}$ mode in which only two subunits of the tetramer interact with ssDNA, occluding ~35 nucleotides of ssDNA/tetramer (2).

Our initial analysis of the ssDNA binding properties of the SsbA and SsbB proteins was based on polyacrylamide gel shift assays that were carried out using the standard gel electrophoresis running buffer, TBE (Tris borate (pH 8.5)/EDTA) (7). However, Ssb proteins have been found to act as accessory factors for a variety of enzymes involved in DNA metabolism, many of which are dependent on Mg$^{2+}$ for activity. We have therefore examined the effect of Mg$^{2+}$ on the ssDNA binding properties of the SsbA and SsbB proteins. The ssDNA binding reactions were carried out in a typical enzyme reaction buffer (25 mM Tris acetate (pH 7.5)/MgCl$_2$) with various concentrations of magnesium acetate, and the resulting complexes were analyzed by electrophoresis in polyacrylamide gels that contained the same buffer and magnesium acetate concentrations as in the reaction solutions. The results of this analysis have led to an explanation for the previously observed differences in the ssDNA binding properties of the SsbA and SsbB proteins. In addition, a new chimeric SSB protein was prepared as a first step toward identifying the structural basis for the differences in the ssDNA binding properties of the SsbA and SsbB proteins.
**Streptococcus pneumoniae SsbA and SsbB Proteins**

**EXPERIMENTAL PROCEDURES**

**Materials**—*S. pneumoniae* SsbA protein (1) and SsbB protein (6) were prepared as described. *E. coli* SSB protein was from Promega. dT

Polycrylamide Gel Electrophoresis Mobility Shift Assays—The ssDNA binding reaction solutions contained 25 mM Tris acetate (pH 7.5), 5% glycerol, 1 mM dithiothreitol, and the concentrations of magnesium acetate, dTn, and SSB protein given in the legends for Figs. 2–8 and 10. The reaction solutions were incubated at 37 °C for 15 min. Aliquots (20 μl) were removed from each reaction solution and added to 2 μl of gel loading solution (0.25% bromphenol blue, 40% sucrose). The aliquots were analyzed by electrophoresis on 5% native polyacrylamide gels using a system consisting of 25 mM Tris acetate (pH 7.5) and the same concentration of magnesium acetate as in the reaction solutions. Bands corresponding to unbound and SSB-bound dTn oligomers were visualized by autoradiography (these reactions contained 32P-end-labeled dTn oligomers), and bands corresponding to unbound and dTn-bound SSB protein were visualized by G-250 BioSafe Coomassie Brilliant Blue staining (these reactions contained only unlabeled dTn oligomers).

**RESULTS**

**Binding Modes of the SsbEc Protein**

In the initial set of experiments, the complexes that were formed between the SsbEc protein and the oligomers dT10 and dTn were examined to establish whether polycrylamide gel electrophoresis could be used to analyze the binding modes of SSB proteins. The binding reactions were carried out in a reaction buffer consisting of 25 mM Tris acetate (pH 7.5) and either 0 or 10 mM magnesium acetate, and the resulting complexes were analyzed by electrophoresis in polycrylamide gels that contained the same buffer and magnesium acetate concentrations as in the reaction solutions.

**Forward Titrations with dT75**—A set of forward titration experiments was carried out in which a fixed concentration of 32P-end-labeled dT75 was incubated with increasing concentrations of SsbEc protein. The complexes that were resolved on the polycrylamide gels were visualized by autoradiography to monitor the changes in the mobility of the dT75 that occur when it binds to SsbEc protein. When increasing concentrations of SsbEc protein were added to dT75, in the absence of Mg2+, an initial complex with a gel mobility lower than that of unbound dT75 was formed at the lower protein concentrations (Fig. 2). A further increase in the concentration of SsbEc protein resulted in the disappearance of this initial complex and the appearance of a second complex in the presence of Mg2+ (7). These results indicated that only a single tetramer of SsbEc protein was able to bind to dT75 at the higher protein concentrations in the absence of Mg2+.

A different result was obtained when increasing concentrations of SsbEc protein were added to dT75 in the presence of Mg2+ (10 mM) (Fig. 2). In this case, an initial complex was again formed at the lower protein concentrations. However, in contrast to the results that were obtained in the absence of Mg2+, there was no indication of the formation of the second complex in the presence of Mg2+, even at the highest concentration of SsbEc protein examined (Fig. 2). These results indicated that only a single tetramer of SsbEc protein was able to bind to dT75 in the presence of Mg2+.

These results suggested that in the absence of Mg2+, the SsbEc protein was able to bind to dT75 in the previously described SSB10 mode, with two of the four subunits of each of the two bound tetramers interacting with the dT75 (occluding 35 nucleotides/tetramer), whereas in the presence of Mg2+, the SsbEc protein was binding to dT75 in the SSB65 mode, with all four subunits of a single tetramer interacting with the dT75 (occluding 65 nucleotides) (2).
Reverse Titrations with dT35—A complementary set of reverse titration experiments was carried out using the oligomer, dT35, which is only long enough to bind to two of the four subunits of the SsbEc tetramer (2). In these reactions, a fixed concentration of SsbEc protein was incubated with increasing concentrations of dT35. The complexes that were resolved on the polyacrylamide gels were visualized by protein staining to monitor the changes in the mobility of the SsbEc protein that occur when it binds to dT35.

When the SsbEc protein was incubated with increasing concentrations of dT35, in the absence of Mg2+, a new band with a gel mobility greater than that of the free SsbEc protein was formed (Fig. 3). A parallel reverse titration experiment that was carried out using 32P-end-labeled dT35 confirmed that the new band corresponded to an SsbEc-(dT35)2 complex (gel not shown). The concentration of dT35 that was required to convert the free SsbEc protein to the complex corresponded to approximately one dT35 molecule/tetramer of SsbEc protein. These results indicated that in the absence of Mg2+, the SsbEc tetramer was able to bind a single dT35 molecule (through two of the four subunits of the tetramer) to form an SsbEc-dT35 complex (the additional negative charges contributed by the phosphoryl groups of the second dT35 presumably increase the mobility of the doubly liganded complex relative to that of the singly liganded complex).

The results of the reverse titrations were consistent with those from the forward titrations and indicated that the SsbEc protein was able to bind to the dT75 oligomers in the SSB35 mode (with two of four subunits interacting with dT75) in the absence of Mg2+ and in the SSB65 mode (with all four subunits interacting with dT75) in the presence of Mg2+ (10 mM). These results are consistent with previous fluorescence studies of the SsbEc protein (see “Discussion”) and indicated that the Mg2+-polyacrylamide gel electrophoresis method described here could be used to analyze the binding modes of the SsbA and SsbB proteins.

Binding Modes of the SsbA Protein

Forward and reverse titration experiments analogous to those described above for the SsbEc protein were used to analyze the DNA binding modes of the SsbA protein.

Forward Titrations with dT75—When increasing concentrations of SsbA protein were added to 32P-end-labeled dT75 in the absence of Mg2+, an initial complex with a gel mobility lower than that of unbound dT75 was formed at the lower protein concentrations (Fig. 4). A further increase in the concentration of SsbA protein resulted in a decrease in this initial complex and the appearance of a second complex of even lower gel mobility (Fig. 4). These results were similar to those that were obtained for the SsbEc protein (Fig. 2) and to those that were obtained previously for the SsbA protein using the TBE buffer system (7) and indicated that both the SsbA and SsbB proteins were able to bind to dT75 in the absence of Mg2+.

When increasing concentrations of SsbA protein were added to dT75 in the presence of Mg2+, an initial complex was again formed at the lower protein concentrations (Fig. 4). However, in contrast to the results that were obtained in the absence of Mg2+, a further increase in the concentration of dT75 resulted in the disappearance of the first complex and the appearance of a new band with even greater gel mobility (Fig. 3). A parallel reverse titration experiment that was carried out using end-labeled dT35 confirmed that both the first and the second bands corresponded to SsbEc-(dT35)2 complexes and that the second complex contained approximately twice as much dT35 as the first (gel not shown). These results indicated that in the presence of Mg2+, the SsbEc tetramer was able to bind two dT35 molecules (through all four subunits of the tetramer) to form an SsbEc-(dT35)2 complex (the additional negative charges contributed by the phosphoryl groups of the second dT35 presumably increase the mobility of the doubly liganded complex relative to that of the singly liganded complex).

Reverse Titrations with dT35—When the SsbA protein was incubated with increasing concentrations of dT35 in the absence of Mg2+, a single complex with a gel mobility greater than that of the free SsbA protein was formed (Fig. 3). A parallel reverse titration experiment that was carried out using end-labeled dT35 confirmed that the new band corresponded to an SsbA-dT35 complex (the additional negative charges contributed by the phosphoryl groups of the second dT35 presumably increase the mobility of the doubly liganded complex relative to that of the singly liganded complex).

The results of the reverse titrations were consistent with those from the forward titrations and indicated that the SsbA protein was able to bind to the dT35 oligomers in the SSB35 mode (with two of four subunits interacting with dT35) in the absence of Mg2+ and in the SSB65 mode (with all four subunits interacting with dT35) in the presence of Mg2+ (10 mM).
was formed (Fig. 5). These results were similar to those that were obtained with the SsbEc protein (Fig. 3) and indicated that in the absence of Mg\(^2+\), the SsbA tetramer was able to readily bind a single dT\(_{35}\) molecule to form an SsbA-dT\(_{35}\) complex (the slight increase in the apparent mobility of this complex at higher dT\(_{35}\) concentrations suggests that a second dT\(_{35}\) may bind weakly to the SsbA protein under these conditions).

When the SsbA protein was incubated with increasing concentrations of dT\(_{35}\) in the presence of Mg\(^2+\), an initial complex was formed at the same concentration of dT\(_{35}\) that was observed for the formation of the SsbA-dT\(_{35}\) complex in the absence of Mg\(^2+\) (Fig. 5). However, in contrast to the results that were obtained in the absence of Mg\(^2+\), a further increase in the concentration of dT\(_{35}\) resulted in the disappearance of the first complex and the appearance of a new complex with even greater gel mobility (Fig. 5). These results were again similar to those that were obtained with the SsbEc protein (Fig. 3) and indicated that in the presence of Mg\(^2+\), the SsbA tetramer was able to bind two dT\(_{35}\) molecules to form an SsbA-(dT\(_{35}\))\(_2\) complex.

The similarity of the results that were obtained in the forward and reverse titration experiments, both in the absence and in the presence of Mg\(^2+\), indicated that the SsbA and SsbEc proteins undergo similar Mg\(^2+\)-dependent changes in the modes in which they bind to ssDNA.

**Binding Modes of the SsbB Protein**

Forward and reverse titration experiments were also carried out to analyze the DNA binding modes of the SsbB protein.

**Forward Titrations with dT\(_{75}\)**—When increasing concentrations of SsbB protein were added to dT\(_{75}\) in the absence of Mg\(^2+\), a complex with a gel mobility lower than that of unbound dT\(_{75}\) was formed at the lower protein concentrations (Fig. 6). However, in contrast to the results that were obtained with the SsbA and SsbEc proteins, there was no indication of the formation of a second complex with dT\(_{75}\) in the absence of Mg\(^2+\), even at the highest concentrations of SsbB protein examined. These results indicated that only one tetramer of SsbB protein was able to bind to dT\(_{75}\) in the presence of Mg\(^2+\) (7).

A similar result was obtained when increasing concentrations of SsbB protein were added to dT\(_{75}\) in the presence of Mg\(^2+\) (Fig. 6). In this case, an initial complex was formed at the lower protein concentrations, but there was no indication of the formation of a second complex with dT\(_{75}\) by the SsbB protein, even at the highest concentrations of SsbB protein examined. These results indicated that only one tetramer of SsbB protein was able to bind to dT\(_{75}\) in the presence of Mg\(^2+\).

**Reverse Titrations with dT\(_{35}\)**—When the SsbB protein was incubated with increasing concentrations of dT\(_{35}\) in the absence of Mg\(^2+\), a new band with a gel mobility greater than that of the free SsbB protein was formed (Fig. 7). However, in contrast to the results that were obtained with the SsbEc and SsbA proteins in the absence of Mg\(^2+\), a further increase in the concentration of dT\(_{35}\) resulted in the disappearance of the first band and the appearance of a new band with even greater gel mobility (Fig. 7). A parallel reverse titration experiment that was carried out using end-labeled dT\(_{35}\) demonstrated that both the first and the second bands corresponded to SsbB-(dT\(_{35}\))\(_2\) complexes (gel not shown). These results suggested that in the absence of Mg\(^2+\), the SsbB tetramer was able to bind two molecules of dT\(_{35}\) (through all four subunits of the tetramer) to form an SsbB-(dT\(_{35}\))\(_2\) complex.

A similar result was obtained when the SsbB protein was incubated with increasing concentrations of dT\(_{35}\) in the presence of Mg\(^2+\) (Fig. 7). However, in this case, the first and second complexes appeared concurrently (rather than sequentially) at the lower dT\(_{35}\) concentrations. A further increase in the dT\(_{35}\) concentration resulted in the disappearance of the first complex and an increase in the intensity of the second complex. A parallel reverse titration experiment that was carried out using end-labeled dT\(_{35}\) confirmed that both bands corresponded to SsbB-(dT\(_{35}\))\(_2\) complexes (gel not shown). These results suggested that in the presence of Mg\(^2+\), the SsbB tetramer was able to bind two dT\(_{35}\) molecules (through all four subunits of the tetramer), perhaps with positive intrasubunit cooperativity, to form an SsbB-(dT\(_{35}\))\(_2\) complex.

The results from both the forward and the reverse titrations indicated that the SsbB protein differed from the SsbEc and SsbA proteins in that it appeared to be able to bind to the dT\(_{n}\) oligomers with all four subunits of the tetramer in either the presence or the absence of Mg\(^2+\).

**Dependence of DNA Binding Modes on Mg\(^2+\) Concentration**

To more precisely define the Mg\(^2+\) dependence of the DNA binding modes of the SsbEc, SsbA, and SsbB proteins, the binding of these pro-
The dependence of the binding of the SsbEc protein to dT75 on Mg2+ concentration is shown in Fig. 8. In the absence of Mg2+, the dT75 was almost completely converted into the second complex with only a small amount of the first complex apparent. This was consistent with the results in Fig. 2 and indicated that two SsbEc tetramers were able to bind to dT75 under these conditions. At 1 mM Mg2+, the band corresponding to the second complex was fainter, and there was a smear leading down to the position where the first complex was found. This indicated that the second complex was able to form under these conditions but was less stable than in the absence of Mg2+. At 2.5 mM Mg2+, the dT75 appeared mainly as a band at the position of the first complex with an upward smear, suggesting that some dissociation of the second complex had occurred during electrophoresis. At 5 mM Mg2+, the dT75 appeared as a discrete band at the position corresponding to the first complex, with no indication of the formation of the second complex. This result was essentially identical to that observed at 10 mM Mg2+ and indicated that the first complex was the dominant complex for the SsbEc protein at the higher Mg2+ concentrations.

The dependence of the binding of the SsbA/B protein to dT75 on Mg2+ concentration is shown in Fig. 10. When increasing concentrations of SsbA/B protein were added to dT75, the dT75 was largely converted into the second complex, although a band corresponding to the first complex was also visible. This was consistent with the results in Fig. 4 and indicated that two SsbA/B tetramers were able to bind to dT75 under these conditions. At 1 mM Mg2+, the dT75 appeared as a discrete band at the position of the first complex, with no indication of the formation of the second complex. This result was essentially identical to those that were obtained at the higher Mg2+ concentrations and indicated that the first complex was the dominant complex for the SsbA/B protein in the presence of Mg2+.

The dependence of the binding of the SsbB protein to dT75 on Mg2+ concentration is shown in Fig. 6. The SsbB protein formed only a single complex with dT75 over the entire range of Mg2+ concentrations examined. This indicated that the first complex was the dominant complex for the SsbB protein both in the presence and in the absence of Mg2+.

**Binding Modes of a Chimeric SsbA/B Protein**

Proteolysis studies have shown that the SsbEc protein consists of an N-terminal domain that contains the subunit tetramerization and ssDNA binding sites and a C-terminal domain that is terminated by an acidic tail (2). A comparison of the primary sequences reveals that the N-terminal regions of the SsbA and SsbB proteins (amino acids 1–106) are highly similar to the corresponding region of the SsbEc protein (amino acids 1–116) (Fig. 1). However, the sequence comparison indicates that the C-terminal region of the SsbB protein is significantly shorter than that of either the SsbEc or the SsbA protein (Fig. 1). This suggested that the shorter C-terminal domain may be responsible for differences between the ssDNA binding properties of the SsbB protein and those of the SsbA and SsbEc proteins. To explore this idea, a chimeric SSB protein was constructed in which the C-terminal domain of the SsbB protein (amino acids 105–131) was joined to the N-terminal regions of the SsbA and SsbEc proteins (amino acids 1–106). The resulting chimeric SsbA/B protein (15,039 Da/monomer) was similar in size to the native SsbB protein (14,926 Da/monomer) and significantly smaller than the native SsbA protein (17,350 Da/monomer) (Fig. 9). The binding of the SsbA/B protein to dT75 is shown in Fig. 10.

When increasing concentrations of SsbA/B protein were added to 32P-end-labeled dT75 in the presence of Mg2+, a complex with a gel mobility lower than that of unbound dT75 was formed (Fig. 10). The mobility of this complex was greater than that of the complex formed with the SsbB protein and essentially identical to that of the complex formed with the SsbEc protein (Fig. 10). This result was consistent with the similar sizes of the SsbA/B and SsbB proteins and indicated that the complex corresponded to the binding of a single SsbA/B tetramer to the dT75. There was no indication of the formation of a second complex under these conditions, even at the highest concentration of SsbA/B protein examined (Fig. 10). These results were similar to those that were obtained with the SsbA and SsbB proteins and indicated that a single tetramer of SsbA/B protein was able to bind to dT75 in the presence of Mg2+ (Fig. 10).

When increasing concentrations of SsbA/B protein were added to...
Streptococcus pneumoniae SsbA and SsbB Proteins

FIGURE 8. Dependence of the DNA binding modes of the SSB proteins on Mg2+-concentration. The reaction solutions contained 25 mM Tris acetate (pH 7.5), 0.07 μM dT75 (oligomer concentration), the indicated concentrations of magnesium acetate, and 0.3 μM SsbEc protein, SsbA protein, or SsbB protein (tetramer concentration). The reactions were analyzed by polyacrylamide gel electrophoresis using a running buffer consisting of Tris acetate (pH 7.5) and the concentrations of magnesium acetate that were included in the individual reactions (the gels shown in the figure are composites consisting of lanes taken from separate gels that were run at the indicated concentrations of magnesium acetate). The bands corresponding to unbound dT75 (ssDNA) and various (SSB)n-dT75 complexes (I and II) were visualized by autoradiography (the lane designated M corresponds to dT75 in the absence of SSB protein).

dT75 in the absence of Mg2+, an initial complex with a gel mobility lower than that of unbound dT75 was formed at the lower protein concentrations (Fig. 10). The mobility of this initial complex was again greater than that of the complex formed under these conditions with the SsbA protein but essentially identical to that of the complex formed with the SsbB protein (Fig. 10). This indicated that the initial complex corresponded to the binding of a single SsbA/B tetramer to the dT75. A further increase in the concentration of SsbA/B protein resulted in a diminishment in this initial complex and the appearance of a second complex of even lower gel mobility (Fig. 10). This result indicated that two tetramers of SsbA/B protein were able to bind to dT75 in the absence of Mg2+. Under the same conditions, a second complex was also observed with the SsbA protein, whereas there was no indication of the formation of a second complex with the SsbB protein (the increased gel mobility of the second SsbA/B complex relative to the second SsbA complex was presumably due to the smaller size of the SsbA/B protein) (Fig. 10). The band corresponding to the second SsbA/B complex did appear to be fainter than that for the second SsbA complex, suggesting that the second SsbA/B complex may be less stable than the second SsbA complex under these conditions. Nevertheless, these results indicated that the replacement of the C-terminal domain of the native SsbA protein with the shorter C-terminal domain from the SsbB protein does not fundamentally alter the binding of the protein to dT75.

DISCUSSION

The results presented here demonstrate that Mg2+-dependent changes in the DNA binding modes of the SsbEc protein can be monitored by Mg2+-polyacrylamide gel electrophoresis. The experimental strategy consisted of forward titration reactions in which increasing concentrations of SsbEc protein were added to a fixed concentration of dT75 and reverse titration reactions in which increasing concentrations of dT35 were added to a fixed concentration of SsbEc protein. In the absence of Mg2+, the forward titration results indicated that two SsbEc tetramers were able to bind to dT75, implying that the SsbEc tetramers were binding in the SSB35 mode under these conditions with only two of the four subunits of each tetramer interacting with the dT75. This conclusion was supported by the reverse titration results, which indicated that only one dT35 was able to bind to the SsbEc tetramer in the absence of Mg2+, suggesting that only two of the four subunits of the SsbEc tetramer were able to bind to ssDNA under these conditions. In the presence of Mg2+, in contrast, the forward titration results indicated that only one SsbEc tetramer was able to bind to dT75, suggesting that the SsbEc tetramer was binding in the SSB65 mode under these conditions with all four subunits of the tetramer interacting with the dT75. This conclusion was also supported by the reverse titration results, which indicated that two molecules of dT35 were able to bind to the SsbEc tetramer in the presence of Mg2+, suggesting that all four subunits of the tetramer were able to bind to ssDNA under these conditions. These results are consistent with previous fluorescence studies, which have shown that the SSB35 mode of the SsbEc protein is generally favored in the absence of or at low concentrations of Mg2+, whereas the SSB65 mode is favored at higher Mg2+ concentrations (2).

The SsbA protein behaved similarly to the SsbEc protein in the forward and reverse titration experiments, both in the presence and in the absence of Mg2+. This indicates that the SsbA protein undergoes Mg2+-dependent transitions between an SSB35-like binding mode and an SSB65-like binding mode that are analogous to those of the SsbEc protein. In contrast, the results with the SsbB protein indicated that one tetramer of SsbB protein was able to bind to dT75 in the forward titrations and that two dT35 molecules were able to bind to the SsbB tetramer in the reverse titrations, both in the presence and in the absence of Mg2+. These results suggested that the SsbB protein differed from the SsbEc and SsbA proteins in that it appeared to bind to the dT oligomers in an SSB65-like mode, even in the absence of Mg2+.

It has been proposed that the ability of the SsbEc protein to bind to ssDNA in the SSB35 mode at low salt concentrations is due to a high degree of negative intrasubunit cooperativity among the DNA binding sites of the SsbEc tetramer (2, 7). For example, it has been shown by fluorescence analysis that when the SsbEc protein is titrated with increasing concentrations of dT35 at low salt concentrations, one molecule of dT35 binds to two subunits of the tetramer with high affinity, but the binding of a second molecule of dT35 to the remaining two subunits is greatly reduced (9). At higher salt concentrations, however, the SsbEc protein is able to readily bind two molecules of dT35, indicating that the negative intrasubunit cooperativity is reduced or eliminated under these conditions (9). Although the molecular basis for the negative intrasubunit cooperativity is not known, it has been suggested that it is due at least in part to electrostatic repulsions between the phosphoryl groups of the segments of ssDNA that are bound in the first and second subunits and the segments of ssDNA bound in the third and fourth subunits of the SsbEc tetramer and that the relief of negative intrasubunit cooperativity that is observed at higher salt concentrations may be due to a reduc-
tion in this electrophoretic separation which results from the binding of cations to the ssDNA (2, 9).

The reverse titration gel shift results reported here, which indicated that the SsbEc protein was able to bind one molecule of dT₃₅ in the absence of Mg²⁺ and two molecules of dT₃₅ in the presence of Mg²⁺, are consistent with the fluorescence results described above. Thus, the results that suggested that the SsbB protein was able to bind two molecules of dT₃₅ in either the presence or the absence of Mg²⁺ indicate that the SsbB protein does not exhibit the pronounced negative intrasubunit cooperativity at low salt concentrations that has been found for the SsbEc protein. An absence of negative intrasubunit cooperativity and the resulting stabilization of an SSB₆₅-like binding mode would also account for the results that suggested only a single SsbB tetramer was able to bind to dT₇₅ in either the absence or the presence of Mg²⁺. In either case, the binding of an SsbB tetramer to dT₇₅ in an SSB₆₅-like mode would prevent a second SsbB tetramer from binding to that dT₇₅ molecule.

Our new results provide an explanation for our previously observed differences in the apparent stoichiometries for the binding of the SsbB protein and the SsbEc and SsbA proteins to various dTₙ oligomers (7). Since our original analysis was carried out using the standard gel electrophoresis buffer, TBE, which contains no Mg²⁺, it is likely that the SsbA and SsbEc proteins were able to bind to the dTₙ oligomers in an SSB₆₅ mode, whereas the SsbB protein was restricted to binding to the oligomers in an SSB₆₅-like mode. The new results indicate that the observation that the binding properties of the SsbB protein and those of the SsbEc and SsbA proteins are more similar in the presence of Mg²⁺ in that all three proteins appear to bind to dTₙ oligomers in an SSB₆₅-like mode under these conditions. However, the reverse titration experiments indicated that even in the presence of Mg²⁺, there are differences between the SsbB protein and the SsbA and SsbEc proteins in terms of the intrasubunit cooperativity for ssDNA binding. Although the presence of Mg²⁺ appears to reduce the negative intrasubunit cooperativity for ssDNA binding by the SsbEc and SsbA proteins, the SsbB protein may actually bind ssDNA with positive intrasubunit cooperativity in the presence of Mg²⁺.

The most obvious structural difference between the SsbB protein and the SsbA and SsbEc proteins is in the length and composition of the C-terminal domains of these proteins. It is conceivable that the differences in the C-terminal domains could affect the degree of intrasubunit cooperativity and the relative stabilities of the different binding modes of the various SSB proteins. However, our results indicated that the chimeric SsbA/B protein (in which the entire C-terminal domain of the SsbA protein was replaced with the shorter C-terminal domain of the SsbB protein) undergoes Mg²⁺-dependent transitions between an SSB₆₅-like binding mode and an SSB₆₅-like binding mode that are similar to those of the native SsbA protein. This suggests that the apparent inability of the SsbB protein to readily bind in an SSB₆₅-like mode in the absence of Mg²⁺ is not due simply to the shorter length of the C-terminal domain of the SsbB protein. It remains possible that the C-terminal SsbB domain may interact differently with the N-terminal domain of the SsbB protein than it does with the N-terminal domain of the chimeric SsbA/B protein and that this interaction modifies the ssDNA binding properties of the SsbB protein. However, the similarity of the DNA binding properties of the SsbA and SsbA/B proteins suggests that the primary structural determinants that govern the stability of the DNA binding modes may be contained within the N-terminal domains of the various SSB proteins.

It has been suggested that the different DNA binding modes of the SsbEc protein may be used selectively for different functions in the cell (2). The finding that the SsbA protein exhibits Mg²⁺-dependent changes in its binding modes that are similar to those of the SsbEc protein provides further support for the proposal that the SsbA protein may be the S. pneumoniae analog of the SsbEc protein, a general purpose SSB involved in routine DNA functions. Although the SsbB protein is strongly induced during natural transformation in S. pneumoniae, the specific role of the SsbB protein in this process is not known. However, it is conceivable that the apparent preferential formation of the SSB₆₅-like binding mode by the SsbB protein may reflect an adaptation that enhances the ability of the SsbB protein to function during natural transformation in S. pneumoniae.
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