JOURNAL OF BACTERIOLOGY, Dec. 1996, p. 6730–6735 Vol. 178, No. 23
0021-9193/96/$04.00+0

Protein Conformational Change and Nucleotide Binding Involved in Regulation of σ^F in *Bacillus subtilis*

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Received 5 July 1996/Accepted 23 September 1996

We have studied the ability of three mutant forms of SpoIIAA, containing amino acid substitutions at the site of phosphorylation (serine 58), to interact with SpoIIAB. Native gel analysis revealed that SpoIIAAS58A could form a complex with SpoIIAB in the presence of ADP and more strongly in the presence of ATP. SpoIIAAS58N did not form a complex with SpoIIAB in the presence of ADP but displayed some interaction with SpoIIAB in the presence of ATP. SpoIIAAS58D was unable to form a complex with SpoIIAB in the presence of either ADP or ATP. Corresponding differences were found in the behavior of the three mutant proteins when studied by gel permeation with high-performance liquid chromatography and limited proteolysis. SpoIIAAS58A behaved like the wild-type SpoIIAA, SpoIIAAS58D like SpoIIAA-P, and SpoIIAAS58N in a way that was intermediate between the behaviors of SpoIIAA and SpoIIAA-P. Limited proteolysis was also used to show that on binding of ADP or ATP SpoIIAB undergoes a shift in conformation. The affinity of SpoIIAB for ADP and ATP was determined by limited proteolysis in the presence of a wide range of nucleotide concentrations. The results indicated that SpoIIAB has approximately equal affinity for ADP and for ATP.

The gram-positive soil bacterium *Bacillus subtilis* provides a classic example of prokaryotic development in that it is able to sporulate when starved of nutrients (for a review, see reference 12). Early in sporulation the cell forms two different compartments, the forespore and the mother cell. The two compartments undergo differential gene expression, both contributing to the development of the forespore into a highly resistant endospore. Studies of sporulation at a molecular level can contribute toward a general understanding of how cells divide asymmetrically (18, 35).

Differential gene expression during sporulation is achieved by the activation of compartment-specific sigma factors, which bind to core RNA polymerase to direct transcription of certain genes. Early in sporulation correct activation in the forespore of one of these sigma factors, σ^F, is crucial. σ^F not only directs gene expression in the forespore but also indirectly activates mother cell-specific transcription (17, 19, 22, 23).

σ^F is encoded by the promoter-distal gene of *spoIIA*, *spoIIAC*. Genetic experiments have shown that the other two *spoIIA* genes, *spoIIAA* and *spoIIAB*, are involved in the regulation of σ^F activity, with *spoIIAB* negatively regulating σ^F and *spoIIAA* negatively regulating *spoIIAB* (33). Although the σ^F protein is present in the preseptational cell, σ^F activity is released in the forespore only upon completion of septation. In the preseptational cell and the mother cell, σ^F is inhibited by SpoIIAB, an anti-sigma factor, while the release of σ^F activity in the forespore is dependent on SpoIIAA, an anti-anti-sigma factor (2, 8). In addition SpoIIAB is a serine protein kinase that phosphorylates SpoIIAA (27). Subsequent investigations have sought to define the mechanism by which these three proteins interact in controlling σ^F activity.

The specificity of binding of SpoIIAB depends on adenine nucleotides (2). In the presence of ADP, SpoIIAB and SpoIIAA form a complex, while the presence of ATP allows SpoIIAB both to phosphorylate SpoIIAA (8, 27) and to form a complex with σ^F (2). It was previously suggested that reduced ATP levels in the forespore could promote the formation of SpoIIAB-SpoIIAA complexes and the release of σ^F activity, whereas in the preseptational and mother cells, with increased levels of ATP, SpoIIAB could bind instead to σ^F. Close examination of this partner-switching model (2) would require an analysis of the response of SpoIIAA and SpoIIAB to adenosine nucleotides. However, investigations of the binding interactions of the two proteins are hampered by the fact that the phosphorylation of wild-type SpoIIAA by SpoIIAB in the presence of ATP is extremely rapid (8, 27). We have therefore turned to mutant SpoIIAA proteins for such binding studies.

In experiments using site-directed mutagenesis of SpoIIAA at serine 58, which was recently confirmed as the site of phosphorylation (29), the serine was replaced with alternative amino acids (8). When serine was replaced with alanine, the mutant protein formed complexes with SpoIIAB in the presence of ADP or ATP; in the corresponding strain σ^F activity was released prematurely. When serine was replaced with aspartate (which partially resembles the larger, charged phosphoserine) the mutant SpoIIAA mimicked SpoIIAA-P in failing to form complexes with SpoIIAB in the presence of either of the two nucleotides; the phenotype of the mutant strain displayed a block in σ^F activity. These results led to the conclusion that the phosphorylation state of SpoIIAA was crucial in regulating σ^F activity (see also reference 2). In support of this conclusion, it has been shown that phosphorylation of SpoIIAA results in a large conformational change in the protein that correlates with its inability to bind SpoIIAB (24). In addition, it has been demonstrated that unphosphorylated SpoIIAA not only binds efficiently to free SpoIIAB but can also reverse SpoIIAB-mediated inhibition of σ^F activity by first disrupting the SpoIIAB-σ^F complex and then binding the released SpoIIAB (10).

Another gene involved in the regulation of σ^F is *spoIIE* (26), the product of which has recently been identified as a phosphatase specific for SpoIIAA-P (9). Located in the septum, this protein has also been shown to be involved in the correct formation of the asymmetric septum (4, 15). After

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septation SpoIIE is thought to convert inactive preseptational SpoIIAA-P, segregated to the forespore on septation, to SpoIIAA, which is then capable of participating in forespore-specific inhibition of SpoIIAB (3, 15).

Here we present results of in vitro experiments that have been used to study properties of SpoIIAA and SpoIIAB. We have measured interactions of SpoIIAB with SpoIIAA and three mutant forms of SpoIIAA in the presence of ADP and ATP and have shown that SpoIIAB has a higher affinity for two of the mutant SpoIIAA proteins when ATP is present rather than ADP. In addition the differing behaviors of the mutant forms of SpoIIAA have been correlated with differences in their structural conformation. We further show that SpoIIAB undergoes a change in conformation upon nucleotide binding and that the affinity of SpoIIAB for ADP and ATP is approximately equal.

MATERIALS AND METHODS

Plasmids and bacterial strains. Plasmids for expression of the wild-type *spoIIAA* and *spoIIAB* genes, pEAA and pEAB, along with plasmids for expression of mutant *spoIIAAS58A* and *spoIIAAS58D* genes, were isolated from *Escherichia coli* DH5α as described previously (8, 27). In addition the mutant *spoIIAAS58N* gene derived from *B. subtilis spo-562* (14) was inserted into a pET-3 plasmid carried in *E. coli* DH5α, and the plasmid was isolated as described above. *E. coli* BL21 (DE3) cells were transformed (31) with the plasmids mentioned above.

Overproduction and purification of SpoIIA proteins. Transformants were grown and induced as previously described (8). All proteins were purified as previously described (8). Cell extracts were subjected to anion exchange by fast-protein liquid chromatography (FPLC). The column fractions which were enriched in SpoIIA proteins were subjected to gel filtration over Sephadex G-50. The fractions containing purified protein were concentrated by ultracentrifugation with the help of Centricon-10 concentrators (Amicon) and stored in 50% glycerol at −20°C.

PAGE. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) the standard buffer system was employed with appropriate modifications and solutions (20, 31). Stacking and 15% separating gels were used. Samples were run at 30 mA (constant current). Coomassie brilliant blue staining-destaining was used to visualize protein bands. For nondenaturing (native) PAGE, 12 and 18% gels containing 200 mM Tris-HCl (pH 8.5)–20% glycerol were used. Samples were run overnight (4°C) at 3 mA (constant voltage)/5-ml gel. Staining-destaining was carried out as described above.

Analysis of protein-protein interactions on native PAGE. SpoIIA protein interactions were analyzed on 12% native gels. Proteins (1 to 5 μg) were used in reaction mixtures of 30 μl, which contained 3 μl of phosphorylation-binding buffer (500 mM Tris-HCl [pH 7.6], 500 mM KCl, 100 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM EDTA). Relevant nucleotides (ADP or ATP) were included in the reaction mixtures at a final concentration of 100 μM, and the running buffer included the same nucleotide at a concentration of 100 μM. Reactions were done on ice and samples were run as described earlier after the addition of 5 μl of native sample buffer (50% glycerol, 0.01% bromophenol blue).

HPLC. A Shodex KW-802.5 fitted to a Beckman 165 detector and Beckman dual channel recorder was used. The column was equilibrated in high-performance liquid chromatography (HPLC) buffer (100 mM Tris-HCl [pH 7.5], 200 μM $MgSO_4$, 0.5 mM dithiothreitol) and developed in the same buffer at a flow rate of 0.5 ml/min. The apparent molecular masses of SpoIIA proteins were derived from their elution times by the use of five standards (Sigma): aprotinin, 6.5 kDa; cytochrome *c*, 12.4 kDa; carbonic anhydrase, 29 kDa; ovalbumin, 45 kDa; and bovine serum albumin, 66 kDa for the monomer and 132 kDa for the dimer.

Limited proteolysis. (i) Proteolysis of SpoIIAA, SpoIIAA-P, and SpoIIAA mutant proteins. Protein (50 μg) was mixed with 5 μg of trypsin in 120 μl of 100 mM Tris-HCl (pH 8). After incubation at 37°C for 50 min the mixture was subjected to electrophoresis by Tricine-SDS-PAGE (32): a 35-ml urea-SDS slab gel was prepared (separating gel: 18.5% acrylamide [46.5% (wt/vol) acrylamide–3% (wt/vol) *N*,*N*′-methylene-bisacrylamide stock], 33.3% 3 M Tris-HCl [pH 8.45, containing 0.3% SDS], 8 M urea; stacking gel: 3.84% acrylamide, 25% 3 M Tris-HCl [pH 8.45, containing 0.3% SDS]). Cathode buffer was 100 mM Tris–100 mM Tricine–0.1% SDS (pH 8.25). Anode buffer was 200 mM Tris-HCl (pH 8.9). After electrophoresis the peptides were fixed in glutaraldehyde (36). After fixation, the gels were washed in H_2O (2 times for 10 min) and stained-destained as described earlier. Controls lacking protease were treated in the same manner. A second digestion was carried out in which trypsin was replaced with 0.5 μg of proteinase K. The incubation time here was 60 min at 37°C.

(ii) Proteolysis of SpoIIAB. Protein (12 μg) was incubated in 30 μl of 100 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$ (where stated) containing 0.04 μg of trypsin. After incubation at 37°C for 5 min, samples were frozen. In some samples 1 mM ADP or 1 mM ATP was included. All samples were thawed on ice, loaded onto 18% nondenaturing gels at 4°C, and run overnight at 3 mA.

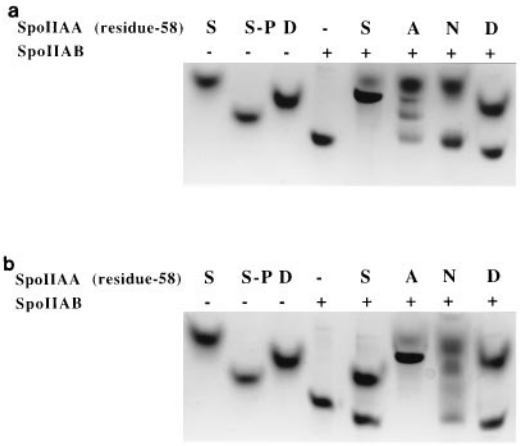

FIG. 1. (a) Nondenaturing PAGE gel run in buffer containing ADP. The first four lanes are markers (SpoIIAAS58A and SpoIIAAS58N run at the same position as SpoIIAA). The remaining lanes contain samples from mixtures of incubations carried out in the presence of ADP. S, SpoIIAA; S-P, SpoIIAA-P; A, SpoIIAAS58A; N, SpoIIAAS58N; D, SpoIIAAS58D; B, SpoIIAB. (b) Nondenaturing PAGE gel run in buffer containing ATP. Lane contents are as described for panel a except that incubation mixtures contained ATP instead of ADP.

Measurement of K_{ADP} and K_{ATP} values of SpoIIAB. SpoIIAB was digested with trypsin over a wide range of ADP or ATP concentrations from 1 nM up to 10 mM. For both ADP and ATP binding assays four different trypsin concentrations were used in the assay (4.5, 6.2, 7.9, and 10 ng of enzyme per assay). At one of these enzyme concentrations (7.9 ng per assay) the two series of ADP and ATP digestions were repeated in the absence of $MgCl_2$. Samples were run overnight at 4°C on 18% nondenaturing gels; gels were stained with Coomassie blue, destained, and dried on transparent cellophane. The area quantification mode was used on the gel-scanning facility of a DU-650 Beckman Spectrophotometer to quantify the amount of undigested SpoIIAB remaining in each assay on the gels. Protease resistance of 100% was represented by the fraction of SpoIIAB present in the control lane lacking trypsin; 0% resistance was represented by the fraction present in the control lane lacking nucleotide. K_{ADP} and K_{ATP} values were obtained by plotting $\log_{10}$ nucleotide concentration against the fraction of SpoIIAB remaining undigested and determining the nucleotide concentration at which 0.5 of the total SpoIIAB had been digested.

RESULTS

Interaction of SpoIIAB with SpoIIAA and mutant forms of SpoIIAA. In the presence of ADP, SpoIIAA or SpoIIAAS58A can form a complex with SpoIIAB (8); in the presence of ATP SpoIIAA is phosphorylated by SpoIIAB, but SpoIIAAS58A, which lacks the serine at the site of phosphorylation (residue 58), again forms a complex with SpoIIAB (8). Replacement of the serine 58 with aspartate generates a mutant protein, SpoIIAAS58D, that resembles SpoIIAA-P in both its running position on native PAGE and its inability to interact with SpoIIAB in the presence of ADP or ATP (8).

We have carried out experiments to extend these results and to study in addition the interaction of SpoIIAB with a third mutant protein, SpoIIAAS58N, which has asparagine at residue 58.

The three mutant proteins (SpoIIAAS58A, SpoIIAAS58D, and SpoIIAAS58N) were compared to SpoIIAA and SpoIIAA-P by analysis on native PAGE, in respect of their interactions with SpoIIAB in the presence of ADP or ATP. Nucleotide was present not only in the reaction mixture but also during electrophoresis (i.e., in the running buffer). SpoIIAAS58D failed to interact with SpoIIAB in the presence of ADP or ATP (Fig. 1). However, the SpoIIAAS58A mutant protein formed a complex with SpoIIAB in the presence of ATP (Fig. 1b). The

TABLE 1. Elution times, apparent logarithmic molecular masses, and apparent molecular masses of the SpoIIA proteins used in HPLC experiments[a]

Protein	Elution time (min)	Log. apparent mol mass (kDa)	Apparent mol mass (kDa)
SpoIIAA	18.26	4.20	15.8
SpoIIAAS58A	18.34	4.19	15.5
SpoIIAAS58N	18.02	4.23	17.0
SpoIIAAS58D	17.42	4.30	20.1
SpoIIAA-P	16.87	4.37	23.5

[a] Elution time was used to calculate the apparent logarithmic (Log.) molecular masses of samples after the Shodex column was calibrated with five standard proteins as described in Materials and Methods.

similar complex formed between the two proteins in the presence of ADP was clearly weaker, since the gel showed some of the SpoIIAAS58A and SpoIIAB in noncomplexed form (Fig. 1a). In the presence of ADP, any complex formed between SpoIIAAS58N and SpoIIAB did not survive electrophoresis, but when ATP was provided the two interacted to form a weak complex (represented by smearing between the individual running positions of the two proteins) (Fig. 1).

We can describe the behavior of SpoIIAAS58N in the presence of ATP as intermediate between the behaviors of the two earlier-characterized mutant proteins SpoIIAAS58A (which complexes efficiently with SpoIIAB in the presence of ATP) and SpoIIAAS58D (which forms no complexes with SpoIIAB). We can also say that the presence of ATP appears to permit stronger interaction between SpoIIAB and SpoIIAAS58A or SpoIIAAS58N than ADP does.

Correlation of protein interactions with protein conformation. Like SpoIIAAS58D, SpoIIAA-P fails to interact with SpoIIAB in the presence of nucleotides (2, 8, 24). It has recently been shown that this lack of interaction accompanies a change in conformation experienced by SpoIIAA on phosphorylation (24). In preliminary studies of the conformation of the three SpoIIAA mutant proteins we subjected them to gel permeation on HPLC. In this system proteins that are approximately spherical show relative mobilities that depend on the logarithm of their molecular weight, but proteins that are far from spherical can deviate sharply from this pattern (16).

By calibrating the gel filtration column with five standard (globular) proteins and then using the same column to measure the elution times of SpoIIAA, SpoIIAA-P, and the three mutant SpoIIAA proteins, we derived apparent molecular masses for these SpoIIAA derivatives. The results (Table 1) show that SpoIIAAS58A has an apparent molecular mass close to that of the wild-type SpoIIAA and SpoIIAAS58D a much higher apparent molecular mass, nearer to that of SpoIIAA-P. Again the behavior of SpoIIAAS58N was intermediate.

We also used limited proteolysis to study the conformation of the SpoIIAA mutant proteins. SpoIIAA and SpoIIAA-P were digested alongside the mutant forms to allow comparisons to be made. Tryptic digests of SpoIIAA and SpoIIAA-P demonstrated that the phosphorylated form was much more resistant to proteolysis (Fig. 2) (see also reference 24). After 1 h exposure to trypsin much of the SpoIIAA-P remained undigested and the digested portion had given rise to a ladder of peptide bands. Under the same conditions, most of the SpoIIAA was digested and a very low-molecular-mass peptide smear was evident. The SpoIIAAS58A mutant protein behaved much like SpoIIAA, and the SpoIIAAS58D mutant protein behaved much like SpoIIAA-P. The digestion profile of SpoIIAAS58N was intermediate between those of SpoIIAA-P and SpoIIAA (Fig. 2). A similar relative insensitivity of SpoIIAA-P and the corresponding pattern of digestion of the mutant proteins was evident on proteolysis with proteinase K (results not shown).

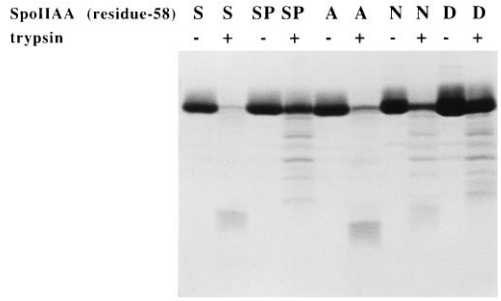

FIG. 2. SDS-PAGE gel of SpoIIAA, SpoIIAA-P, and SpoIIAA mutant proteins after limited proteolysis by trypsin. Each protein digest is accompanied by a control from which protease was absent. S, SpoIIAA; SP, SpoIIAA-P; A, SpoIIAAS58A; N, SpoIIAAS58N; D, SpoIIAAS58D.

SpoIIAB undergoes similar conformational changes upon binding ADP or ATP. In the absence of nucleotide (ADP or ATP) SpoIIAB does not interact with SpoIIAA (2, 8). However, SpoIIAB has the ability to bind ATP and phosphorylate SpoIIAA (27) or to bind ADP and form an ADP-containing complex with SpoIIAA (2, 8, 24). It therefore seemed possible that SpoIIAB on binding either ADP or ATP may undergo a conformational change that permits interaction with SpoIIAA.

The conformation of SpoIIAB in the presence and absence of nucleotide was investigated by digestion with trypsin, with native PAGE being used to display the digestion products. These digestions included Mg^{2+} ions, which are known to be needed for nucleotide binding by kinases. A control assay in which Mg^{2+} ions were omitted but nucleotides were included was also performed.

Proteolysis of SpoIIAB in the absence of ADP, or in the presence of ADP but the absence of Mg^{2+} ions, resulted in similar patterns of digestion (Fig. 3). In the presence of ADP and Mg^{2+} ions SpoIIAB displayed an increased resistance to trypsin compared with the controls (Fig. 3); this suggested that on binding ADP SpoIIAB underwent a shift in conformation that led to some sites becoming unavailable for trypsin cleavage. Identical results were obtained when ADP was replaced by ATP (Fig. 3). In another experiment we showed that Mg^{2+} ions alone failed to protect SpoIIAB from digestion (results not shown).

SpoIIAB has approximately equal affinity for ADP and ATP. SpoIIAB is a serine protein kinase with some sequence similarity to the family of bacterial histidine protein kinases (11,

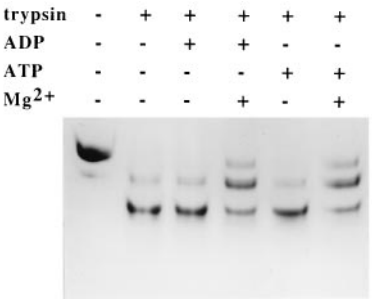

FIG. 3. Proteolysis of SpoIIAB by trypsin displayed on nondenaturing PAGE. Each lane contains SpoIIAB with the additions shown.

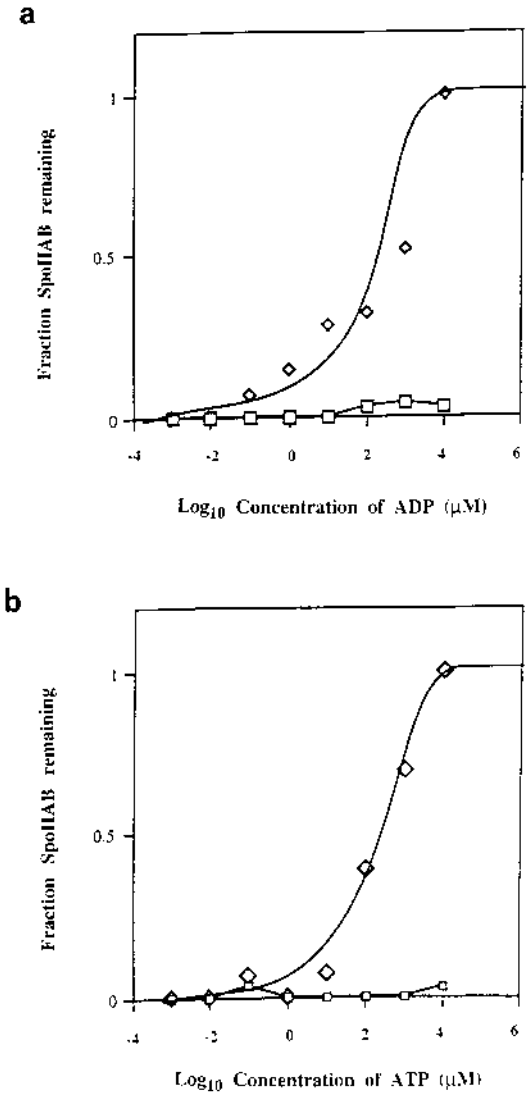

FIG. 4. (a) Plot of $\log_{10}$ ADP concentration against fraction of SpoIIAB remaining after digestion using 10 mM Mg^{2+} and 7.9 ng of trypsin per assay (diamonds). Included is a second line (squares) which represents a control in which SpoIIAB is digested in the same way but in the absence of Mg^{2+}. From this plot a K_{ADP} value of 200 μM was calculated for SpoIIAB. (b) Plot of $\log_{10}$ ATP concentration against fraction of SpoIIAB remaining after digestion. Conditions and symbols are as described for panel a. From this plot a K_{ATP} value of 220 μM was calculated for SpoIIAB.

27). It was therefore no surprise to find that SpoIIAB could interact with ADP and ATP, but the relative affinity of SpoIIAB for ADP and ATP was unknown. Exploiting the fact that at low concentrations of trypsin the binding of nucleotide protects SpoIIAB from digestion, we studied ADP and ATP interactions with the protein. SpoIIAB was digested with four different trypsin concentrations over a wide range of ADP and ATP concentrations. The data for nucleotide dependence of protease resistance can be fitted to an equation for ligand binding (21): protease-resistant fraction (fraction maximal binding) = $[\text{ligand}]^2/(K_{\text{ligand}}{}^2 + [\text{ligand}]^2)$. K_{ligand} is the ADP or ATP concentration at half-maximal protection from proteolysis (half-maximal binding).

By plotting the nucleotide concentration against the fraction of SpoIIAB remaining after proteolysis (Fig. 4) we derived an apparent K_{ADP} of 181 μM and an apparent K_{ATP} of 174 μM (Table 2). Controls lacking Mg^{2+} ions showed no obvious protease resistance over the range of nucleotide concentrations, again indicating that the protease resistance reflected ADP (ATP) binding by SpoIIAB.

DISCUSSION

SpoIIAB and SpoIIAA control the activity of σ^F (26, 33). It has been suggested that ADP promotes SpoIIAB-SpoIIAA complex formation, leaving σ^F activity switched on in the forespore; ATP promotes phosphorylation of SpoIIAA by SpoIIAB, which prevents SpoIIAA from interacting with SpoIIAB and allows SpoIIAB to complex with σ^F, thereby switching off the sigma factor's activity in the preseptational and mother cells (2, 8, 24, 27). However, the extent to which different ratios of ATP/ADP in the preseptational cell, mother cell, and forespore contribute to the regulation of σ^F activity is unknown. SpoIIE, a membrane protein located in the septum of sporulating cells capable of dephosphorylating SpoIIAA-P (3, 4, 5, 9, 15), may be the dominant factor in the asymmetric activation of σ^F. What is known is that ADP and ATP are necessary for interactions to take place between the products of the *spoIIA* operon (8), unlike the components of the homologous regulatory system for the stress response sigma factor, σ^B (1).

Here we studied the interaction between SpoIIAA and SpoIIAB in the presence of ADP and ATP, using three SpoIIAA mutants that could not be phosphorylated. Previous studies showed that SpoIIAAS58A formed complexes with SpoIIAB in the presence of either ADP or ATP; on the other hand, SpoIIAAS58D was unable to form complexes with SpoIIAB in the presence of either nucleotide, mimicking the behavior of SpoIIAA-P, which also fails to form complexes with SpoIIAB (2, 8, 24). In the present work a third mutant form of SpoIIAA was included, SpoIIAAS58N. The strain of *B. subtilis* carrying this third mutation, *spo-562* (14), was shown by *lacZ* fusion studies to exhibit weak postseptational σ^F activity; this indicated that SpoIIAAS58N was able to form a complex with SpoIIAB, albeit weakly (6). Native PAGE studies demonstrated that SpoIIAAS58N displayed some interaction with SpoIIAB in the presence of ATP but not in the presence of ADP (Fig. 1). In a similar way SpoIIAAS58A interacted much more strongly with SpoIIAB in the presence of ATP than in the presence of ADP. As reported previously (8), the third mutant, SpoIIAAS58D, showed no ability to interact with SpoIIAB. It was apparent from these experiments that SpoIIAB showed a preference for ATP over ADP in forming complexes with SpoIIAAS58N and SpoIIAAS58A.

Recent results from this laboratory have demonstrated that SpoIIAA and SpoIIAA-P differ in conformation, which presumably accounts for the inability of SpoIIAA-P to form a complex with SpoIIAB (24). To confirm this result we studied the conformations of the three mutant forms of SpoIIAA. Results of HPLC gel permeation and limited proteolysis ex-

TABLE 2. K_{ADP} and K_{ATP} values for SpoIIAB calculated with four different concentrations of trypsin

$K_{\text{nucleotide}}$	Value (μM) with the following trypsin concn (ng/assay)				Avg (μM)
	4.5	6.2	7.9	10	
K_{ADP}	68	254	200	204	181
K_{ATP}	89	147	220	240	174

periments showed that the ability of the three mutants to interact with SpoIIAB was related to their structural conformation. SpoIIAAS58A cannot be phosphorylated but can interact with SpoIIAB in the presence of ATP or (like SpoIIAA) in the presence of ADP. As judged by limited proteolysis and by its behavior on HPLC, this mutant protein showed a conformation similar to SpoIIAA. SpoIIAAS58D, which like SpoIIAA-P cannot form a complex with SpoIIAB, behaved like SpoIIAA-P on limited proteolysis and was eluted in gel permeation on HPLC close to the position of SpoIIAA-P. In SpoIIAAS58N, the polar amino acid asparagine at residue 58 is uncharged (like alanine) but is almost as big as aspartate. Correspondingly, SpoIIAS58N had an elution time in gel permeation on HPLC between those of SpoIIAAS58A and SpoIIAAS58D; it also behaved in digestion in a way which was roughly intermediate between the behaviors of SpoIIAAS58A (and SpoIIAA) and SpoIIAAS58D (and SpoIIAA-P).

Susceptibility to proteolysis is a protein property that can be used indirectly to study ligand binding (7, 21, 30, 34). We exploited the change in conformation apparent on nucleotide binding by using limited proteolysis to estimate SpoIIAB's affinity for ADP and ATP. The K_{ADP} and K_{ATP} values obtained were very similar. This result ruled out the possibility that SpoIIAB interacted more strongly with SpoIIAAS58A and SpoIIAAS58N in the presence of ATP rather than ADP because of an inherent greater affinity for ATP. Other possible explanations for the preference exhibited by SpoIIAB for ATP in interacting with these proteins can be suggested. First, SpoIIAB may undergo a change of conformation when incubated with ATP that is slightly different from that with ADP, in a way that is not apparent on proteolysis of the two protected forms of SpoIIAB. Such a subtle difference may allow SpoIIAB to interact better with these two mutant proteins when itself bound to ATP (10). Secondly, the conformational changes on binding ADP or ATP may be identical, and the reason SpoIIAB binds more efficiently with SpoIIAAS58A and SpoIIAAS58N in the presence of ATP may be that the SpoIIAB and the mutant SpoIIAA protein cooperate in binding ATP. It has recently been shown in our laboratory that SpoIIAA can bind to ATP but not ADP (28). Finally, in binding between SpoIIAB and one of the these two mutant SpoIIAA proteins, the presence of ATP rather than ADP in the reaction pocket may chemically allow more stable complex formation. (A similar reason may account for the observed preference for ATP over ADP that is apparent in σ^F-SpoIIAB complexes [10, 25]).

As far as the interaction between SpoIIAB and the wild-type SpoIIAA is concerned, these results suggest that the apparent preference for ADP as the ligand that mediates this interaction (2) is not an inherent feature of the binding properties of the proteins. Instead, ADP permits wild-type SpoIIAA and SpoIIAB to interact without any covalent change in either molecule. This interaction contrasts with that between SpoIIAA and ATP which, in the presence of SpoIIAB, react rapidly and completely (8, 27) to yield a product, SpoIIAA-P, that cannot bind to SpoIIAB (24). Thus, in terms of the interaction between SpoIIAA and SpoIIAB, the principal distinction between ATP and ADP is that only the former can act as a phosphorylating agent.

Our findings here and elsewhere concord with the recently described models of the regulation of σ^F activity (9, 13). In the preseptational cell and the mother cell SpoIIAB becomes active by changing its tertiary structure through binding adenosine nucleotides; with high ratios of ATP/ADP SpoIIAA is phosphorylated rapidly by SpoIIAB-ATP-Mg^{2+}, rendering it inactive as an anti-anti-sigma factor. On completion of SpoIIAA phosphorylation SpoIIAB forms an ATP-dependent complex with σ^F, thereby inactivating it. After septation SpoIIE is present in the septum and can hydrolyze SpoIIAA-P in the forespore back to active SpoIIAA. Given the increased number of unphosphorylated SpoIIAA molecules and the relative depletion of ATP in the forespore, enough SpoIIAB-σ^F-ATP complexes are now replaced by SpoIIAB-SpoIIAA-ADP complexes to switch on σ^F (10, 24, 25). Thus the phosphorylation state of SpoIIAA in the forespore determines if σ^F is activated. Only when SpoIIAB is forced to interact with SpoIIAA by using ADP instead of ATP is σ^F activity set free to trigger transcription of genes required early in the sporulation process.

ACKNOWLEDGMENTS

We thank D. A. Harris for making HPLC facilities available to us, J. Errington for providing gel-scanning facilities and R. Daniel for his assistance with this procedure, N. J. Edwards for technical assistance, and J. Errington and R. Losick for commenting on the manuscript.

This research was supported by the Medical Research Council and the Biotechnology and Biological Sciences Research Council; from the latter, M.L. also received a Research Studentship.

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