A Role for Asp\textsuperscript{75} in Domain Interactions in the \textit{Bacillus subtilis} Response Regulator Spo0A\textsuperscript{*}

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Spo0A is a two-domain response regulator required for sporulation initiation in \textit{Bacillus subtilis}. Studies on response regulators have focused on the activity of each domain, but very little is known about the mechanism by which the regulatory domain inhibits the activator domain. In this study, we created a single amino acid substitution in the regulatory domain, D75S, which resulted in a dramatic decrease in sporulation \textit{in vivo}. 

\textit{In vitro} studies with the purified Spo0AD75S protein demonstrated that phosphorylation and DNA binding were comparable with wild type Spo0A. However, the mutant was unable to stimulate transcription by $\sigma^P$-RNA polymerase from the Spo0A-dependent spoIIG operon promoter. We suggest that the amino acid Asp\textsuperscript{75} and/or the region within which it resides, the $\alpha$-$\beta$-$\alpha$ loop, are involved in the inhibitory interaction between the regulatory and activator domains of Spo0A.

Sporulation is a developmental process that is activated in \textit{Bacillus} once the culture reaches high density and nutrients become limiting (1). Sporulation involves the creation of two new cells, the forespore and the mother cell, each with its own pathway of gene expression (2). Extensive regulation ensures that all avenues of nutrient acquisition have been exhausted prior to entry into sporulation. Initiation of sporulation is absolutely dependent on the product of the \textit{spo0A} gene, Spo0A (3, 4). Activation of Spo0A is required for transcription of the \textit{spoIIG} operon, which encodes $\sigma^P$ (a mother cell-specific $\sigma$ factor) and the \textit{spoIJA} operon, which encodes $\sigma^F$ (a forespore-specific $\sigma$ factor) (5–7).

Spo0A is a two-domain response regulator (8–10). These proteins are generally composed of an N-terminal regulatory domain and a C-terminal activator domain that carries out the function of the protein. The two domains are joined by a flexible hinge region called the Q linker. These response regulators are activated by phosphorylation of their regulatory domain (11, 12). The regulatory domains have a structure similar to that of the single domain response regulators CheY (13, 14) and Spo0F (15, 16) including the key conserved amino acids known to be important for signal propagation in CheY (reviewed in Refs. 14 and 17).

Spo0A can function as a transcription activator (18–21) or repressor (22, 23), depending on the position of its DNA binding sites (0A boxes) relative to the +1 start site of transcription. In its capacity as an activator, the C-terminal domain of Spo0A interacts with either the vegetative $\sigma$ factor, $\sigma^V$, or the alternate $\sigma$ factor, $\sigma^J$, depending on the promoter (24–27). Phosphorylated Spo0A (Spo0A−P)\textsuperscript{1} increases the rate of transcription initiation by participating in DNA strand separation by RNA polymerase (19, 20, 28). Since the isolated C-terminal domain is capable of stimulating transcription by $\sigma^V$-RNA polymerase \textit{in vitro} (29), it is hypothesized that the function of the regulatory domain is to inhibit the activator domain, and phosphorylation relieves this inhibition.

An unresolved question is why phosphorylation is needed for activation. Mutations in the N-terminal domain of Spo0A have begun to define regions that are involved in protein activation. For example, the sof mutations, which restore sporulation in a $\Delta$spoOF strain, are located in regions involved in the phosphorylation reaction and Spo0A-RNA polymerase interactions (30, 31). A second class of mutants, the sad mutations that are deletions ranging between 1 and 20 amino acids around residue 75 in the N-terminal domain, render Spo0A constitutively active (32). In this study, we focused on the region defined by the sad mutations and examined the effect of an amino acid substitution at position 75. We report that, rather than resulting in a constitutively active protein as had been found when Asp\textsuperscript{75} was deleted, the D75S substitution decreased the transcription activation properties of Spo0A. Phosphorylation and DNA binding by the mutant protein were within normal levels, but transcription stimulation of \textit{spoIIG} operon promoter \textit{in vitro} and sporulation \textit{in vivo} were drastically reduced.

**EXPERIMENTAL PROCEDURES**

\textit{Synthesis and Cloning of Spo0AD75S—}The D75S change was created by site-directed mutagenesis using a two-step PCR. The template DNA was pKk0A, which contains the entire \textit{spo0A} coding sequence (29). The initial round of PCR (30 cycles of the following: 95 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min) created a 121-bp fragment (P1) internal to the \textit{spo0A} gene, which encompassed the unique \textit{Xba}I site and converted the codon for Asp\textsuperscript{75} (GAT) to Ser\textsuperscript{75} (TCT), thereby mutating a unique BglII site. The primers used were (upstream) 5'-AAAAGATCCCGATGTGCTCG-3' and (downstream) 5'-ATGACATTCGGCTGTTTTTTCAGAGATGATT-3'. The second round of PCR (10 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min) with 42 bp upstream of the internal \textit{Xba}I restriction site and (downstream) D75S2 (5'-GCATATGCAGATTCGGCTTGGTTTTTTCAGAGATGATT-3'), which bound 42 bp upstream of the internal \textit{Xba}I site and converted the \textit{Xba}I site to an unique \textit{Xba}I site.

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\textsuperscript{1}The abbreviations used are: Spo0A−P, phosphorylated Spo0A; Spo0B−P, phosphorylated Spo0B; Spo0AD75S, phosphorylated Spo0AD75S mutant; Spo0AN−P, phosphorylated regulatory domain of Spo0A; PCR, polymerase chain reaction; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay.

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3'-end of spo0A). The 600-bp product was digested with XbaI and EcoRI, cloned into pGem-T0A that had been digested with XbaI and EcoRI, and transformed into Escherichia coli DH5α. The plasmid pGem-T0A contained the wild type spo0A gene cloned into the PCR cloning vector pGem-T. Transformants were identified by screening the plasmids for resistance to digoxigenin. The plasmid with the correct insert, which was sequenced in its entirety, was designated pGem-TD75S.

The XbaI/EcoRI fragment from pGem-TD75S was cloned into pJH1408, digested with XbaI and EcoRI, and transformed into E. coli DH5α. The plasmid pJH1408, which replicates in E. coli and integrates in Bacillus subtilis, contains a truncated spo0A gene and upstream sequence cloned into pJH101 (33). Positive clones were identified by monitoring the resistance of the plasmid to digestion by BglII. This clone was designated pJH1408:D75S. Transformation of pJH1408:D75S into B. subtilis JH642 (30) to obtain Campbell type transformants was carried out essentially as described (34). Transformants were selected on L agar plus 5 μg/ml chloramphenicol and screened by PCR using primers that amplified only the full-length copy of the spo0A gene. The 5′ primer was GAATTCCTTGGGGAAGAAAAGCTGGG (the GTG is the spo0A start codon), and the 3′ primer was 0A-4 (31), which bound downstream of the spo0A transcription start. The resultant 860-bp PCR products were digested with BglII. A positive transformant, which had a PCR product that did not digest with BglII, was designated GBS1002 and used for subsequent studies. A transformant whose PCR product did digest with BglII was used as a wild type control (GBS1001). A summary of all plasmids and strains is given in Table I.

**Table I**

| Plasmid or strain | Description | Source |
|-------------------|-------------|--------|
| pJH1408           | Ball/EcoRI fragment containing the truncated spo0A gene cloned into pJH101 | Ref. 9 |
| pJH1408:D75S      | pJH1408 with the D75S mutation | This study |
| pMC0AD75S         | Full-length spo0AD75S cloned into pET16b | This study |
| JH642             | Wild-type B. subtilis, trpC2, phi-1 | Ref. 30 |
| GBS1001           | JH642 with pJH1408 integrated | This study |
| GBS1002           | JH642 with pJH1408:D75S integrated | This study |
| MCA0AD75S         | E. coli expression host BL21(DE3)/pLYS5 | This study |

μM Spob, 0.2 mM ATP, 25 μM GTP, and 25 μCi of [γ-32P]ATP (7000 Ci/mmol; ICN Biochemicals) in 1× transcription buffer in a final volume of 20 μL. Reactions were incubated at 25 °C for 1.5 h and then separated by electrophoresis through a 15% SDS-PAGE. The labeled proteins were detected and quantitated as described above.

**DNase I Footprint and Electrophoretic Mobility Shift Assays—**DNase I footprint analysis was carried out essentially as described previously (20). The indicated concentrations of Spo0A–P or Spo0AD75S/P spo0AD75S–P were incubated with approximately 1.5 × 10^6 cpm of end-labeled BamHI/PvuII fragment from pUC119gtrpA for 5 min at 37 °C in 1× transcription buffer (20) in a final volume of 20 μL. DNA samples were separated on an 8% polyacrylamide sequencing gel containing 7 M urea by electrophoresis at 45 watts for 4 h. The gels were dried and exposed to x-ray film (Kodak XAR) overnight at −80 °C. To determine the nucleotide positions relative to the +1 transcription start site, the end-labeled DNA fragment was digested with Asel (−43) or AluI (−27) to produce size markers and electrophoresed in lanes adjacent to the footprint reactions.

Electrophoretic mobility shift assays (EMSAs) were performed essentially as described previously (35). RNA polymerase (40 nM) and unphosphorylated or phosphorylated Spo0A or Spo0AD75S (concentrations indicated in the legend to Fig. 4) were incubated with approximately 1 × 10^4 cpm of the BamHI/HindIII fragment from pUC119gtrpA, which had been end-labeled with [γ-32P]ATP, for 5 min at 37 °C in 1× gel shift buffer (38) in a final volume of 10 μL. To form the initiated complexes shown in Fig. 4B, the initiation nucleotides ATP and GTP (0.4 mM final concentration) were included in the reaction mixtures. The reactions were stopped by the addition of 3 μL of stop buffer (38) and were immediately loaded onto a 5% nondenaturing gel containing 2% glycerol. Samples were separated at 28 mA for 2.5-3 h, dried, and exposed to x-ray film overnight at −80 °C.

**RESULTS**

An Amino Acid Substitution in the spo0A Regulatory Domain Inhibits Sporulation—Spo0A is a good model for the study of two-domain response regulators because the reduction in percentage sporulation provides a simple method of identifying mutations in spo0A that affect protein function. We are interested in how changes in the regulatory domain lead to Spo0A activation. Ireton et al. (32) reported that the deletion of Asp75 in Spo0A renders the protein constitutively active. Since the deletion might have caused a general structural change, a substitution mutation, D75S, was created by site-directed mutagenesis. The D75S mutation was subsequently cloned into the integrative vector pJH1408 that contains a truncated copy of the spo0A gene as well as the promoter and sequences upstream of the promoter (33), creating pJH1408:D75S. The plasmids pJH1408 and pJH1408:D75S were transformed into B. subtilis 642. Transformants arising from a single cross-over were selected by resistance to chloramphenicol. The transformants were screened for the absence or presence of the D75S mutation, the full-length copy of the gene (designated strains GBS1001 and GBS1002, respectively). A description of the plasmids and strains is given in Table I.

The percentage sporulation for JH642, GBS1001, and GBS1002 was determined by comparing the number of chloroform-resistant spores with the number of viable cells in the culture (Table II). GBS1001 and GBS1002 reached approxi-
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**TABLE II**

| Strain   | Cell count/cell | Spore count/spore | Sporulation/strain |
|----------|-----------------|-------------------|-------------------|
| JH642    | $1.4 \times 10^6$ | $8.7 \times 10^6$ | 62.1              |
| GBS1001  | $5.2 \times 10^6$ | $3.2 \times 10^6$ | 60.6              |
| GBS1002  | $4.4 \times 10^6$ | $1.5 \times 10^6$ | 0.033             |

...mately the same cell density as wild type JH642, although the growth rate of GBS1002 was slightly reduced. However, GBS1002 was severely impaired in its ability to sporulate, yielding only 0.033% spores compared with 62.1 and 60.6% for JH642 and GBS1001, respectively. This result suggested that the D75S mutation severely affected Spo0A function.

**Spo0AD75S Is Phosphorylated by the Phosphorelay**—One possible consequence of a mutation within the N-terminal domain of Spo0A is a disruption of the structure of the aspartate pocket, resulting in the inability of the protein to be phosphorylated by the phosphoprotein phosphotransferase, Spo0B. We expressed and purified Spo0AD75S as described previously (31) and compared the level of phosphorylation with that of wild type Spo0A using an *in vitro* phosphorylation assay (Fig. 1). The rate of phosphorylation was determined by incubating Spo0A or Spo0AD75S with the phosphorelay and [$\gamma$-$^{32}$P]ATP. At the times indicated in Fig. 1A, the proteins were analyzed by SDS-PAGE, and the level of phosphorylation was quantitated. Both proteins were phosphorylated at the same rate by the phosphorelay, which indicated that the D75S mutation had not affected the ability of Spo0B to transfer phosphate to Spo0A. To ensure that the proteins were phosphorylated to the same final level, three concentrations of either Spo0A or Spo0AD75S were incubated with the phosphorelay components, KinA, Spo0F, Spo0B, and [$\gamma$-$^{32}$P]ATP for 90 min and separated by SDS-PAGE. The level of phosphorylation was determined by PhosphorImager analysis (Fig. 1B). The same concentration of either wild type Spo0A or Spo0AD75S exhibited a similar level of phosphorylation, which suggested that the reduced sporulation *in vivo* was not due to a lack of production of Spo0AD75S—P.

**Spo0AD75S Does Not Stimulate Transcription Initiation**—The lack of sporulation suggested that Spo0AD75S did not activate transcription of the stage II operon promoters for spoIIG, spoIIE, and spoIIA. We tested the ability of unphosphorylated and phosphorylated Spo0AD75S to stimulate transcription *in vitro* from the Spo0A-dependent $\sigma^A$ promoter of the spoIIA operon (36). Previous studies have shown that phosphorylation of wild type Spo0A by the phosphorelay dramatically enhances its ability to stimulate transcription *in vitro* (18, 19). Increasing concentrations of Spo0AD75S failed to stimulate transcription even at the highest concentrations tested (Fig. 2). Spo0AD75S—P did stimulate transcription, however, only to the level of unphosphorylated Spo0A.

**Spo0AD75S—P Binds to the spoIIA Promoter**—One potential cause for the reduction in transcription stimulation by Spo0AD75S would be inappropriate binding or lack of binding of the protein to the promoter. We used DNase I footprint analysis to test the binding of the unphosphorylated and phosphorylated forms of Spo0A or Spo0AD75S to the end-labeled spoIIA promoter fragment. After partial digestion by DNase I, the DNA fragments were separated by electrophoresis through a sequencing gel and detected by autoradiography (Fig. 3). The results agreed with previous findings that Spo0A bound weakly to the site 1 $\sigma A$ box (lanes 2–5), while Spo0A–P bound strongly to both site 1 and 2 boxes (lanes 11–14) (18, 19). Spo0AD75S did not bind to the promoter (lanes 6–9). Spo0AD75S—P did bind to the promoter (lanes 15–18) but with a slightly lower affinity than Spo0A–P (compare Spo0A–P and Spo0AD75S–P protein concentrations of 100 and 200 nM; lanes 11 and 12 and lanes 15 and 16, respectively). However, at the protein concentration at which Spo0A–P demonstrated significant transcription stimulation (400 nM in Fig. 3), Spo0AD75S–P protected both the site 1 and site 2 $\sigma A$ boxes (lane 17), indicating that the mutation did not lower transcription stimulation by reducing binding. Furthermore, phosphorylation of Spo0AD75S enhanced binding of the protein to the promoter in the same manner as Spo0A–P.

Another possibility for lack of transcription stimulation by Spo0AD75S would be an effect on the binding of RNA polym...
erase. We used EMSAs to examine the formation of ternary complexes of end-labeled promoter DNA, RNA polymerase, and Spo0A or Spo0AD75S in the absence or presence of initiation nucleotides. The resulting complexes were separated by electrophoresis through a nondenaturing polyacrylamide gel and detected by autoradiography (Fig. 4). As seen previously, RNA polymerase bound to the spoIIG promoter DNA, forming two complexes (Fig. 4A, lanes 1, complexes I and II) (38). The addition of Spo0A (lanes 2–4) or Spo0A–P (lanes 5–7) resulted in the formation of complex I and small amounts of complex II (Spo0A reactions only). Incubation of Spo0AD75S–P and RNA polymerase with the spoIIG promoter resulted in the formation of complexes I and II, indicating that the mutant protein bound to the promoter in a manner similar to Spo0A. However, in the Spo0AD75S–P reactions, a novel complex, with slower mobility than complex I, was also formed. We have termed this complex IV, since the initiated complex was previously designated complex III (38). This result suggested that while Spo0AD75S–P and RNA polymerase bound to the promoter, at least some of the complexes had a conformation different from Spo0A–P-RNA polymerase-promoter complexes.

In the assays shown in Fig. 4B, complexes were formed in the presence of the initiation nucleotides ATP and GTP, which permit RNA polymerase to initiate and transcribe an 11-mer RNA. RNA polymerase alone was unable to form initiated complexes but still bound to the DNA (lane 1). As the concentration of Spo0A increased small amounts of the initiated complex were formed (lanes 2–4, complex III). The addition of
Spo0A–P to RNA polymerase and ATP and GTP resulted in the formation of large amounts of initiated complex (lanes 5–7). Spo0AD75S was unable to stimulate initiation (lanes 8–10), while Spo0AD75S–P (lanes 11–13) did stimulate initiation but only to approximately the same level as Spo0A. The relative amounts of initiated complex formed in all of the reactions corresponded to the levels of transcription stimulation shown in Fig. 2.

**DISCUSSION**

The phosphorylated regulatory domain of Spo0A (Spo0AN–P) has recently been crystallized (39). The overall fold of the domain is the doubly wound (α-β)-barrel structure, typical of single domain response regulators, such as CheY and Spo0F. In comparison with CheY, Spo0AN–P has differences in conformation, particularly in the β3-α3 and β4-α4 loops. These loops are located at the top of the barrel structure adjacent to the phosphorylation site, and the altered conformation is probably due to the presence of the phosphate group. A potential site for interaction between the regulatory and activator domains is the α3-β4 loop located at the bottom of the barrel structure. The positive activation sad mutations (32) deleted all or part of this loop, thus possibly removing the inhibitory portion of the regulatory domain.

In this study, we focused on a mutation in the α3-β4 loop of the Spo0A regulatory domain, which we inferred would be involved in activation. The substitution D75S inhibited sporulation and our evidence suggests that this was due to the loss of the transcription activation properties of Spo0A. Spo0AD75S was able to be phosphorylated, and Spo0AD75S–P bound to the spoIIG promoter and protected both site 1 and site 2 0A boxes like Spo0A–P. However, phosphorylation did not increase the ability of Spo0AD75S to stimulate transcription.

There are two general models to explain how phosphorylation of the N terminus activates Spo0A. In one model, the structure of the N terminus and the Q linker dictate the spatial relationship between the regulator and activator domains. In the unphosphorylated state, this relationship blocks the availability of the σ interaction region. Phosphorylation induces an alteration in the shape of the N terminus (39) that affects the orientation of the linker region, which in turn alters the relative positions of the N- and C-terminal domains. This model is similar to that proposed for NarL based on crystallization data (40, 41). Phosphorylation of NarL is proposed to induce rotation of the regulator domain around the axis of α6, exposing the DNA binding site. The difficulty with the application of this model to Spo0A is that the sequence of the Q linker suggests more flexibility than would be needed to have activation based on a purely structural mechanism. From the crystal structure of the N terminus of Spo0A (39), the loop containing D75S is unlikely to be involved in interactions within the N terminus, so the D75S change would be predicted to be neutral as far as N-terminal shape. This prediction was supported by our observation that the N terminus was phosphorylated normally (Fig. 1) and thus should have retained its three-dimensional structure.

The second model for N terminus inhibition of the C terminus is that specific amino acid side chains interact to hold the two domains together, blocking availability of the σ interaction sites (Fig. 5). Phosphorylation induces a shape change that alters the positions of the interacting residues in the N terminus and prevents their interaction with the C terminus. At the same time, the alteration in the N terminus might promote interactions between residues in other regions of the N or C terminus to increase efficiency of dimer formation or interaction with RNA polymerase (Fig. 5A). Structural analyses of other two domain response regulators have predicted sites of interaction between residues in other regions of the N or C terminus (39, 42). The stabilization of these interactions is likely to be necessary for dimer formation.

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**Fig. 3. Model of the activation of Spo0A by phosphorylation.** A, in the unphosphorylated state, the N-terminal domain (shaded rectangle) domain inhibits the activity of the C-terminal domain (striped rectangle) by direct residue-residue interaction (represented as two dotted lines). These interactions are potentially between the region containing the α3-β4 loop, within which Asp75 resides, with an overall charge of +1 (white oval with +1 in the N-terminal domain), and an unknown region in the C-terminal domain with a presumed negative charge (white oval with two minus signs in the output domain). Upon phosphorylation of the N-terminal domain (white box with P) by Spo0B–P, a conformation change occurs (gray oval) that breaks the interaction with the C-terminal domain. Phosphorylation promotes new interactions with other Spo0A–P molecules and RNA polymerase (not represented). B, the substitution mutation D75S (represented as a black triangle in the N-terminal domain) increases the overall charge of the α3-β4 loop to +2 (white oval with +2 in the N-terminal domain) without altering the shape of the N terminus. This alteration in charge might promote a tighter interaction between regions of the N terminus and C terminus (represented as four dotted lines). Upon phosphorylation by Spo0B–P, the N-terminal domain undergoes a conformation change (shaded oval), but the interactions with the receiver domain are not completely disrupted (represented as two dotted lines). Therefore, Spo0AD75S–P cannot interact properly with RNA polymerase or other Spo0AD75S–P molecules, resulting in a complex that is different from that formed by wild type Spo0A–P RNA polymerase and less efficient at stimulating transcription.

We interpret the effects of the D75S mutation as supporting specific side chain interactions as the mechanism for holding the Spo0A N terminus in place, inhibiting the C terminus (Fig. 5B). A likely explanation of the effects of the D75S mutation is that the negative charge of Asp75 in the wild type protein weakens the interaction between the N- and C-terminal domains by ionic repulsion. Replacing the Asp with Ser stabilizes the interaction of other residues either in the α3-β4 loop or in other regions. The stabilized protein is thus more difficult to disrupt and cannot be activated by the shape change induced by phosphorylation. Consequently, post-phosphorylation protein–protein interactions, such as those with RNA polymerase or other molecules of Spo0A–P, are prevented. The effect of the Asp residue in wild type Spo0A is to increase the sensitivity of the protein to the phosphorylated state. This suggests that the interaction between the N- and C-terminal domains is likely to be hydrophobic rather than ionic, since it was stabilized by decreasing the charge.

While Spo0AD75S–P did not stimulate transcription effectively, it did bind to the 0A boxes better than Spo0A and did form complexes with RNA polymerase. This combination of phenotypes indicates that phosphorylation induces several changes, some of which still occurred in the Spo0AD75S form of
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the protein. Other studies of the regulatory domains of response regulators have suggested that phosphorylation mediates interactions with other proteins through residues on the sides of the structure (e.g. CheY (17, 43–45), OmpR (46, 47), SpoF (48–50), FixJ (51), and VirG (52)). Our studies of other mutations, such as sof114, in the N terminus of Spo0A have shown similar effects (31). Phosphorylation of the N terminus can be viewed as causing a switch in "partner binding" that has been well documented in the phosphotransferase sugar uptake system (53, 54) and in other cases of regulation in Bacillus such as the SpoIIAA/SpoIIB system (55, 56).

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