Studies of SpoIIAB mutant proteins elucidate the mechanisms that regulate the developmental transcription factor σ^F in *Bacillus subtilis*

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σ^F , the first compartment-specific sigma factor of sporulation, is regulated by an anti-sigma factor, SpoIIAB (AB) and its antagonist SpoIIAA (AA). AB can bind to σ^F in the presence of ATP or to AA in the presence of ADP; in addition, AB can phosphorylate AA. The ability of AB to switch between its two binding partners regulates σ^F . Early in sporulation, AA activates σ^F by releasing it from its complex with AB. We have previously proposed a reaction scheme for the phosphorylation of AA by AB which accounts for AA's regulatory role. A crucial feature of this scheme is a conformational change in AB that accompanies its switch in binding partner. In the present study, we have studied three AB mutants, all of which have amino-acid replacements in the nucleotide-binding region; AB-E104K (Glu¹⁰⁴ → Lys) and AB-T49K (Thr⁴⁹ → Lys) fail to activate σ^F , and AB-R105A (Arg¹⁰⁵ → Ala) activates it prematurely. We used techniques of

enzymology, surface plasmon resonance and fluorescence spectroscopy to analyse the defects in each mutant. AB-E104K was deficient in binding to AA, AB-T49K was deficient in binding to ADP and AB-R105A bound ADP exceptionally strongly. Although the release of σ^F from all three mutant proteins was impaired, and all three failed to undergo the wild-type conformational change when switching binding partners, the phenotypes of the mutant cells were best accounted for by the properties of the respective AB species in forming complexes with AA and ADP. The behaviour of the mutants enables us to propose convincing mechanisms for the regulation of σ^F in wild-type bacteria.

Key words: *Bacillus subtilis*, differential gene expression, fluorescence spectroscopy, spoIIAB, sporulation, transcription factor.

INTRODUCTION

Sporulation in *Bacillus subtilis* has become a paradigm for the study of differential gene expression in prokaryotes (for reviews see Kroos et al. [1] and Piggot and Losick [2]). In response to nutrient deprivation, an asymmetric septum is formed that divides the *B. subtilis* cell into two compartments: a larger mother cell and a smaller prespore. Differential gene expression is established in these two compartments through the activation of a series of cell-type-specific transcription factors, known as sigma factors. The earliest sigma factor to be activated is the prespore-specific σ^F . σ^F is crucial to the success of sporulation since it is responsible (both directly and indirectly) for the subsequent activation of the remaining sigma factors in both compartments.

The activity of σ^F is regulated by three proteins: SpoIIAB (AB), SpoIIAA (AA) and SpoIIIE. In the presence of ATP, the anti-sigma factor AB can act either to form an inactive complex with σ^F or as a specific protein kinase for AA [3,4]. AB can also form a stable complex with AA in the presence of ADP [5,6]. AB requires the presence of ATP or ADP to interact with either of its protein partners. The activity of σ^F in the cell is regulated by the concentration of dephosphorylated AA [7–10]. Before asymmetric septation, and in the mother cell, AA is predominantly in its phosphorylated form. In this state it is unable to interact with AB, leaving the anti-sigma factor free to inhibit σ^F [5,11]. This situation is reversed in the prespore through the activity of SpoIIIE, a specific protein phosphatase that hydrolyses AA-P (AA-phosphate) to AA [12–14]. This dephosphorylated AA attacks the AB· σ^F complex and induces the release of free σ^F , allowing the transcription factor to bind to the core RNA polymerase

and direct the transcription of the prespore-specific genes [7,9,10,15]. σ^F is unable to re-bind to AB while AB is interacting with AA [16]. Since AA is phosphorylated during its interaction with AB, and cellular concentrations of AA and AB are similar, we have suggested that repeated cycling of AA through its phosphorylated and unphosphorylated forms is required to maintain σ^F activity [17]. *In vitro*, the phosphorylation of AA by AB follows an unusual biphasic time-course, with a moderate pre-steady-state rate giving way to a lower steady-state rate [17]. This reduction in rate after the initial reaction cycle is due to the formation of AA·AB·ADP complexes, which accumulate in the steady-state phase of the reaction [8,10,16,18]. The formation of these complexes, and the consequent slowing of the phosphorylation reaction in the steady state, makes a crucial contribution to the correct regulation of σ^F , since they detain AB in a form in which it cannot interact with σ^F . Changes in the AA protein that prevent the formation of AA·AB·ADP complexes lead to the loss of σ^F activity [16].

Scheme 1(A) shows the phosphorylation pathway of AA by AB, as previously proposed in Clarkson et al. [8]. An important aspect of this scheme is a conformational change in AB, to the form AB^{closed}. This change was proposed to account for the kinetics of the phosphorylation reaction [8,19], since it is required to prevent rapid nucleotide exchange and thus to allow the formation of the long-lived AA·AB·ADP complexes (species 7). Scheme 1(B), which is intended to supplement Scheme 1(A), proposes a reaction scheme for the direct attack of AA on the AB· σ^F ·ATP complex [10]. We have shown recently that in this attack, the induced release of σ^F precedes the phosphorylation of AA [9]. Here too a conformational change may be involved [9,10], but the evidence

Abbreviations used: AA, SpoIIAA; AA-P, SpoIIAA-phosphate; AB, SpoIIAB; SPR, surface plasmon resonance.

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Scheme 1 Interactions of AB with σ^F and AA

(A) Phosphorylation pathway of AA by AB. (B) Induced release of σ^F .

for such a change is less strong than it is for the conformational change shown in Scheme 1(A). Part of the purpose of the present study is to examine whether the attack of AA on the AB · σ^F · ATP complex, with the consequent release of σ^F , does indeed lead to a conformational change in AB (as provisionally shown in Scheme 1B).

The determination of the crystal structure of AB [20], in a complex with ADP and σ^F , demonstrated that this protein adopts the GHKL (gyrase, heat-shock protein 90, kinase, MutL) superfamily fold. This superfamily has a novel nucleotide-binding region with a flexible 'ATP-lid' loop that protrudes above the nucleotide. In many structures, such as that of AB, this lid covers the nucleotide-binding pocket. ATP-lids are often important for the biological activity of a protein, altering their conformation on binding ligands [21,22].

In the present study, we determined the effects of three mutations on the properties of AB and examined the extent to which the results agree with our model of the wild-type regulation of σ^F (as shown in Schemes 1A and 1B). The mutant proteins studied were AB-T49K (Thr⁴⁹ → Lys), AB-E104K (Glu¹⁰⁴ → Lys) and AB-R105A (Arg¹⁰⁵ → Ala). All three mutations lie

Figure 1 Positions of mutations in the structure of AB

The monomers of the AB dimer are coloured light blue and violet, and the ADP molecules are coloured blue. Thr⁴⁹ is coloured red, Glu¹⁰⁴ is coloured purple and Arg¹⁰⁵ is coloured green. This Figure was prepared from the co-ordinates of AB deposited in the Protein Data Bank under the accession code 1L00.

within the nucleotide-binding region of AB (see Figure 1): E104K and R105A are located within the ATP-lid, whereas T49K is adjacent to the nucleotide under the lid. The mutants AB-T49K and AB-E104K have been described previously [15]. AB-R105A was created by site-directed mutagenesis for the present study, but has also been described elsewhere [7]. The techniques used to study the properties of AB were kinetic analysis of the phosphorylation reaction and its inhibition by σ^F , analysis of complex formation by SPR (surface plasmon resonance), and fluorescence spectroscopy to examine the induced release of σ^F and conformational changes in AB.

EXPERIMENTAL

Production of proteins

The synthesis of AB and AA proteins in *Escherichia coli* and their purification *in vitro* were carried out using the methods of Lee et al. [16]. Expression plasmids used for wild-type AA and AB were described by Diederich et al. [6] and those for AB-E104K and AB-T49K by Garsin et al. [15]. Expression plasmids for AB-R105A and the double mutants AB-F97W,E104K, AB-F97W,T49K and AB-F97W,R105A were created in the present study, by the method described previously for the introduction of mutations into AB [8]. To create *spoIIAB-R105A*, the primers

used for the site-directed mutagenesis were R105A-FW, 5'-GAGCTTGAGGCGTCTGGAATG-3', and R105A-BW, 5'-CATTCCAGACGCCTCAAGCTC-3'.

The primers to amplify the entire gene were described previously [8]. To create the mutant genes *spoIIAB-F97W,E104K*, *spoIIAB-F97W,T49K* and *spoIIAB-F97W,R105A*, plasmids containing *spoIIAB-E104K*, *spoIIAB-T49K* and *spoIIAB-R105A* were used as templates for the site-directed mutagenesis, together with the primers described previously for the introduction of the AB-F97W mutation [8]. σ^F proteins were prepared by the methods described in Clarkson et al. [9]. An expression plasmid for σ^F -W46L,W190L was created in the present study by the method described previously for the introduction of mutations into σ^F [9]. In this case, the plasmid containing *spoIIAC-W46L* was used as a template. The primers used for the site-directed mutagenesis (which introduced the W190L mutation) were W190L-FW, 5'-ACTCAGAAGAAAAATTGTTTGACAAAAT-3', and W190L-BW, 5'-GCAATTTTGTCAAACAATTTTCTTCTG-3'.

All proteins were prepared in a buffer containing 50 mM Tris/HCl (pH 7.5), 50 mM KCl, 1 mM dithiothreitol and 0.5 mM $MgCl_2$. Protein-concentration measurements were carried out by the Bradford method with Bio-Rad protein assay solution. To calibrate this method, a standard curve was produced with protein concentrations standardized from quantitative amino-acid analysis. Samples for quantitative amino-acid analysis were run on an ABI 420A derivatizer/analyser (Applied Biosystems) following hydrolysis for 24 h at 110 °C in 5.7 M HCl.

Replacement of wild-type AB and σ^F in *B. subtilis*

The replacement of wild-type *spoIIAB* with *spoIIAB-R105A* and the replacement of wild-type *spoIIAC* with *spoIIAC-W46L,W190L* in cells of *B. subtilis* was carried out by the method described previously for the introduction of the AB-F97W mutation [8]. The mutation was introduced into a pMLK83 plasmid containing the entire *spoIIA* locus [8]. The primers used for the mutagenesis were those described above for the introduction of these mutations into the expression plasmids. A *B. subtilis* strain was created (named 168ED-1) in which the entire *spoIIA* locus was deleted from *B. subtilis* strain 168ED, and a *lacZ* reporter gene, under the control of the *spoIIQ* promoter, was integrated into the chromosome. The pMLK83 plasmid was transformed into strain 168ED-1 and integrated into the *amyE* locus by double crossover. As controls, a pMLK83 plasmid containing the wild-type *spoIIA* locus and parental pMLK83 were also transformed into 168ED-1. The resulting strains were induced to sporulate, and the β -galactosidase activity was compared with that in a Spo^+ strain in which a *lacZ* reporter had been integrated into strain 168ED. The controls showed that the activity of σ^F was not significantly affected by the deletion of *spoIIA* from its wild-type position and its re-integration into the *amyE* locus.

Incorporation of ^{32}P from $[\gamma\text{-}^{32}P]ATP$ into AA

The kinetics of phosphorylation of AA by AB were followed by measuring the uptake of radioactivity from $[\gamma\text{-}^{32}P]ATP$ into the protein over time. The method used was described previously [16]. Reactions were stopped by adding 20 % trichloroacetic acid together with 1 mg/ml BSA. The precipitated protein was collected and washed on nitrocellulose filters, and radioactivity was estimated by liquid-scintillation counting.

As a result of the impurity of the $[\gamma\text{-}^{32}P]ATP$ used, it was not possible to determine directly the moles of phosphate incorporated from the total counts in a known concentration of ATP. To deter-

mine this value, we ran phosphorylation reactions of a known quantity of AA to completion. The known AA concentration and the total counts incorporated were used to calculate the counts per mol of phosphorylated AA. This value was used to calibrate the observed counts incorporated in subsequent time-course experiments carried out on the same day.

SPR and native-PAGE

To separate the proteins, 10 % native-PAGE gels were used, as described previously [6]. $AB \cdot \sigma^F$ complexes were formed with 4–8 μM AB, 12 μM σ^F and 5–30 μM nucleotides, and $AB \cdot AA$ complexes were formed with 5–10 μM AB, 20 μM AA (wild-type or AA-S58A) and 5–1000 μM nucleotides. The dissociation constants (K_d) for AB proteins with either AA or σ^F were determined by SPR, by the method described previously [17]. Values obtained were from an average of at least three experiments. Either AA or σ^F was immobilized on the Sensorchip and AB was used as the analyte.

Fluorescence spectroscopy

Fluorescence spectroscopy was performed as described previously [8,9]. An excitation wavelength of 290 nm was chosen to excite tryptophan fluorescence. An emission wavelength of 355 nm was used to measure fluorescence from a tryptophan probe in AB and 345 nm was used to measure fluorescence from a tryptophan probe in σ^F . Slit widths were set to 7.5 nm for both the excitation and emission wavelengths. All experiments were performed at 25 °C. Inner filter effects were not significant at the nucleotide concentrations used. The data shown have been corrected for dilution effects and for additional fluorescence from reagents added during the time course. In studies of $AB \cdot \sigma^F$ interactions, the minor effect of adding nucleotide to fluorescent σ^F was subtracted from the data shown. Data shown are from individual experiments. However, the experiments were repeated several times, and similar results were obtained in all cases.

RESULTS

Phenotypes of *B. subtilis* cells containing *spoIIAB* mutations

The mutations AB-T49K and AB-E104K have been shown previously to cause a severe block in σ^F activation during sporulation [15]. Cells harbouring these mutants were arrested in sporulation at the stage of polar septation, and had barely detectable levels of σ^F activity even 5 h after the induction of sporulation.

The phenotype of *B. subtilis* cells containing the AB-R105A mutation has also been described previously [7]. In the present study, we assayed σ^F activity in cells containing the AB-R105A mutation. Similar results were obtained to those described by Ho et al. [7], with the maximum level of σ^F activity (2–3 h after the induction of sporulation) being approx. 6 times that of the wild-type (results not shown). However, in our experiments, substantial σ^F activity was detected even at the induction of sporulation. The activity of σ^F in the mutant 1 h after induction was similar to the maximum level observed in the wild-type 4 h after induction. Measurement of attenuation of cultures of cells containing AB-R105A showed that lysis began to occur 3 h after the induction of sporulation, whereas wild-type cells remained alive for at least 6 h (results not shown).

Kinetics of the phosphorylation reaction

The phosphorylation of AA by wild-type AB has unusual reaction kinetics that are important for the correct activation of σ^F

Figure 2 Time course of the phosphorylation of AA by wild-type AB (◆), AB-E104K (▲), AB-T49K (●) and AB-R105A (■)

The inset shows the reaction catalysed by AB-T49K and has the same dimensions on the axes as the main figure

[8,16,17,19]. The kinetics of phosphorylation reactions catalysed by $0.5 \mu\text{M}$ AB-E104K, AB-T49K, AB-R105A or wild-type AB were assessed by measuring the incorporation of ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into $40 \mu\text{M}$ AA. Figure 2 shows the reaction catalysed by wild-type AB compared with that by AB-E104K, AB-T49K and AB-R105A. As described previously, the wild-type reaction is biphasic, with a moderate pre-steady-state rate giving way to a lower steady-state rate. Magnin et al. [17] reported that the change in rate occurred after 1 mol of AA had been phosphorylated per AB dimer. However, in the present study, it was found that the change in rate occurred after 2 mol of AA had been phosphorylated. Our previous conclusion turned out to be the result of errors in protein concentration measurement (which we have now corrected by quantitative amino-acid analysis; see the Experimental section) and impurities in the labelled ATP. To correct for this latter problem, we ran reactions to completion using a known concentration of AA. The incorporated radioactivity was then calibrated with the amount of phosphorylated AA (see the Experimental section). After correcting for these errors, we found that the pre-steady-state rate and the steady-state rate of the reaction with wild-type AB were approx. $1 \times 10^{-2} \text{ s}^{-1}$ and $2 \times 10^{-3} \text{ s}^{-1}$ respectively.

The time courses of reactions catalysed by AB-E104K and AB-T49K were linear with time until the AA was exhausted (Figure 2). The rate of reaction catalysed by AB-E104K (approx. $7 \times 10^{-3} \text{ s}^{-1}$) was lower than the pre-steady-state of the wild-type, whereas the rate of the reaction catalysed by AB-T49K was much higher (approx. $8 \times 10^{-2} \text{ s}^{-1}$).

The reaction catalysed by AB-R105A showed biphasic kinetics, with the change in reaction rate occurring after 2 mol of AA had been phosphorylated per AB dimer (Figure 2). The pre-steady-state rate (approx. $6 \times 10^{-2} \text{ s}^{-1}$) was higher than that with wild-type AB (although since the pre-steady state was very brief, our estimate of its rate is subject to considerable error). The steady-state rate (approx. $3 \times 10^{-4} \text{ s}^{-1}$) was much lower than with the wild-type, to the extent that continued phosphorylation was barely detectable.

Table 1 K_m and K_d values for wild-type AB, AB-E104K, AB-T49K and AB-R105A

		Wild-type AB	AB-E104K	AB-T49K	AB-R105A
K_m	AA	$\ll 0.5 \mu\text{M}$	$4\text{--}10 \mu\text{M}$	$0.75 \mu\text{M}$	–
	ATP	$2.2 \mu\text{M}$	$1.4 \mu\text{M}$	$20.5 \mu\text{M}$	–
K_d	AA, ADP	47 nM	141 nM	$> 10\,000 \text{ nM}$	14 nM
	AA-S58A, ATP	83 nM	$> 10\,000 \text{ nM}$	$> 10\,000 \text{ nM}$	226 nM
	AA-S58A, ADP	734 nM	$> 10\,000 \text{ nM}$	$> 10\,000 \text{ nM}$	17 nM
	σ^F , ATP	68 nM	74 nM	107 nM	56 nM
	σ^F , ADP	179 nM	124 nM	$> 10\,000 \text{ nM}$	46 nM

Our provisional conclusion from these results is that AB-E104K and AB-T49K are unable to form the long-lived complex with AA and ADP (species 7 in Scheme 1A) that is responsible for slowing the phosphorylation reaction, and that AB-R105A forms a complex whose lifetime is even longer than that of the wild-type $\text{AA} \cdot \text{AB} \cdot \text{ADP}$.

Affinity for AA, σ^F and nucleotides

To try to identify particular changes in the AB protein that might be responsible for the observed changes in the phosphorylation kinetics and in the activation of σ^F , we next examined the ability of AB-E104K, AB-T49K and AB-R105A to bind to individual ligands. Preliminary studies with native-PAGE to visualize complexes of the AB mutants with either AA or σ^F indicated that AB-E104K was defective in binding AA, that AB-T49K was defective in binding both AA and σ^F and that AB-R105A was not defective in binding either protein (results not shown). The findings of these preliminary studies were checked by SPR. Table 1 shows the K_d values for protein–protein complex formation of wild-type AB, AB-E104K, AB-T49K and AB-R105A with wild-type AA, AA-S58A and σ^F (AA-S58A is a non-phosphorylatable mutant of AA, which was used to examine complex formation with AA in the presence of ATP). Examples of SPR sensorgrams, showing the association and dissociation of the complexes, are given in the Supplementary Material (available at <http://www.BiochemJ.org/bj/384/bj3840169add.htm>).

Table 1 also shows the K_m values for AA and ATP, in reactions catalysed by wild-type AB (pre-steady-state), AB-E104K and AB-T49K. K_m values were derived from a double-reciprocal Lineweaver–Burk plot of the rate of phosphorylation at various substrate concentrations. AA concentrations were varied from 0.5 to $4 \mu\text{M}$ and ATP concentrations were varied from 0.5 to $8 \mu\text{M}$. It was not possible to derive accurate K_m values for the reaction catalysed by AB-R105A as the pre-steady-state phase was too rapid and too brief to obtain accurate data. For the wild-type reaction, this analysis revealed only that the K_m for AA must be much less than the lowest concentration used ($0.5 \mu\text{M}$), since the reaction rates at this concentration and above were very similar.

The results in Table 1 show that the interaction of AB-E104K with AA was considerably weaker than that of wild-type AB. In the presence of ATP, both the K_m for AA and the K_d value for AA-S58A were higher by well over an order of magnitude. The K_d value for wild-type AA in the presence of ADP was also higher, but was less significantly affected by the mutation. The K_d values for the interaction of AB-E104K with σ^F were not significantly different from those of wild-type AB, suggesting that this mutant protein interacts with nucleotides and σ^F in a similar way to the wild-type. Similarly, published results by Garsin et al. [23] showed that E104K does not interfere with nucleotide binding. The results of Garsin et al. [23] also suggest that AB-E104K has a higher K_m for AA than wild-type AB, a difference which

the authors attributed to a defect in the capacity of AB-E104K to bind to AA. However, Garsin et al. [15] showed no defect in the binding of AB-E104K to AA in the presence of ADP.

AB-T49K interacted more weakly with AA, σ^F and nucleotides than wild-type AB. The K_m values of AB-T49K for both AA and ATP were substantially higher than those of wild-type AB, and the K_d values for complex formation with both AA and σ^F in the presence of either nucleotide were significantly weaker. These results suggest that the altered affinities of AB-T49K are predominantly due to weak nucleotide binding. Nucleotide is required for AB to bind to either AA or σ^F , and therefore a defect in nucleotide binding would result in a weaker apparent affinity for either protein. The interaction least significantly affected by the AB-T49K mutation was its binding to σ^F in the presence of ATP. Garsin et al. [15] showed some years ago that the interaction of AA in the presence of ADP with AB-T49K is substantially weaker than that with wild-type AB.

AB-R105A interacted more strongly than wild-type AB with both AA and σ^F in the presence of ADP, but its interactions in the presence of ATP were either unaffected (as with the interaction with σ^F) or slightly weaker (as in the case of AA-S58A). These results suggest that AB-R105A binds ADP much more tightly than the wild-type AB, but that the strength of its binding of ATP is not much different from that of the wild-type.

These results, when taken together with those reported in Figure 2, support our contention that the steady state of the AA phosphorylation reaction involves the formation of AA·AB·ADP complexes, and that the rate-limiting step in the steady state of the wild-type reaction is the dissociation of these complexes [8,16,18]. AB-E104K and AB-T49K are both impaired in AA·AB·ADP complex formation. Therefore for these mutants, the dissociation of these complexes (if indeed such complexes are formed) is not rate-limiting in phosphorylation reactions; consequently they have linear, rather than biphasic, kinetics. In contrast, AB-R105A forms complexes with AA and ADP that have a lower tendency to dissociate than the corresponding wild-type complexes, and consequently it has a lower phosphorylation rate in the steady-state phase.

Inhibition by σ^F

In our next series of studies, we examined if σ^F was able to inhibit phosphorylation reactions of AA by AB. The wild-type reaction was studied over a range of protein concentrations of AA and σ^F , with 0.5 μ M AB used to catalyse the phosphorylation of 5–40 μ M AA in the presence of 5–10 μ M σ^F . As found previously [16], σ^F did not inhibit this reaction (results not shown). In contrast, reactions catalysed by AB-E104K and AB-T49K were inhibited strongly by σ^F . At 0.5 μ M AB and 40 μ M AA, 5 μ M σ^F inhibited these reactions by approx. 98% (Figure 3A). At these protein concentrations, no inhibition of the AB-R105A reaction was observed, but at lower ratios of AA to σ^F , partial inhibition occurred (Figure 3B). These results have interesting implications for the induced release of σ^F by AA in cells carrying these mutant proteins: in the wild-type, σ^F does not interfere with the phosphorylation of AA, and, by the same token, AA can disrupt the σ^F ·AB complex. In the mutants, in contrast, σ^F inhibits the phosphorylation of AA (particularly strongly in mutant cells with AB-E104K or AB-T49K), and so it may be that AA is unable to disrupt the complex between σ^F and the mutant proteins. This possibility is examined below.

σ^F ·AB complex dissociation by AA

To examine directly the dissociation of the σ^F ·AB complex by AA, we employed fluorescence spectroscopy, which enabled us

Figure 3 Inhibition by σ^F of the phosphorylation of AA

(A) Reactions catalysed by AB-E104K and AB-T49K. (B) Reactions catalysed by AB-R105A. AA (40 μ M) was phosphorylated by 0.5 μ M AB-E104K (triangles), AB-T49K (circles) and AB-R105A (squares). Closed symbols show reactions in the absence of σ^F and open symbols show reactions in the presence of 5 μ M σ^F . In (B), 5 μ M AA was phosphorylated by 0.5 μ M AB-R105A in the absence ($\times$) and presence (+) of 5 μ M σ^F .

to follow the amount of σ^F bound to AB in real time. As a reporter of σ^F ·AB interactions, we used a mutant of σ^F , W46L, which has been shown to have a reproducible fluorescence response on binding to AB, but is indistinguishable from the wild-type protein in respect of binding to AB and activity *in vivo* [9]. In these experiments (shown in Figure 4), the fluorescence intensity was recorded every 1 s throughout the time course. At the start of the experiments, 1 μ M AB (wild-type or mutant) was incubated together with 1.3 μ M σ^F -W46L, in the absence of nucleotide (whose presence is essential for the formation of σ^F ·AB complexes). The fluorescence intensity of this mixture was taken as a baseline value from which subsequent percentage reductions in fluorescence intensity were calculated. After 1 min, 100 μ M ATP was added to promote the formation of σ^F -W46L·AB complexes, which leads to a reduction in the fluorescence intensity of approx. 10%. This reduction in fluorescence intensity was found to be similar with the mutant and wild-type AB proteins.

After a further 5 min, AA was added to the solution. The blue lines in Figure 4 demonstrate the effect of adding AA to wild-type AB· σ^F -W46L complexes, and show that these complexes were rapidly and completely dissociated. We have shown previously that this dissociation is much faster than the spontaneous release of σ^F -W46L from a complex with wild-type AB, indicating that the interaction with AA actively dissociates the complex [9]. σ^F -W46L remained free until all the AA was phosphorylated by the free AB; thereafter AB re-bound to σ^F -W46L and the

Figure 4 Changes in fluorescence intensity from a fluorescent probe in σ^F

The fluorescence intensities were recorded in real time during the formation of σ^F -W46L · AB complexes and the dissociation of this complex on the addition of AA. σ^F -W46L (1.3 μ M) and AB (1 μ M) were incubated together at zero time, and a baseline value was determined (see the Experimental section). After 1 min, 100 μ M ATP was added, followed after a further 5 min by SpoIIAA. **(A)** A reaction with wild-type AB (blue line) is compared with reactions with AB-T49K (red line) and AB-E104K (purple line). AA (5 μ M) was added to the reaction with wild-type AB, and 10 μ M AA was added to the reactions with AB-T49K and AB-E104K. **(B)** Comparison of results from wild-type AB reacting with 5 μ M AA (blue line) with those from AB-R105A reacting with 2.5 μ M AA (dark green line), 5 μ M AA (light green line) and 10 μ M AA (brown line). The black points show a simulation of the dissociation derived from the off-rate of the AB-R105A · σ^F complex (see text for details).

fluorescence returned to the level that is characteristic of the AB · σ^F -W46L complexes.

The purple and red lines in Figure 4(A) show the effect of adding 10 μ M AA to complexes formed with AB-E104K and AB-T49K. The results show that, on the addition of AA, only approx. 15% of the complexes dissociated, even though the concentration of AA was 2-fold higher than that in the experiment with wild-type AB. We attribute the relative ineffectiveness of AA in dissociating the complexes to the low affinity of AB-E104K and AB-T49K for AA in the presence of ATP (Table 1).

As shown in Figure 4(B), the pattern of σ^F dissociation from AB-R105A was very different from either the wild-type AB or

the other two mutants. Complete dissociation was not observed until the concentration of AA was raised to 5 μ M, twice the level necessary with the wild-type. Furthermore, the rate of dissociation of the σ^F complex with AB-R105A was much lower than that with wild-type AB (see the blue line in Figure 4B). We compared the rates of dissociation with the rate of spontaneous dissociation of the complexes, i.e. the off-rate of AB-R105A · σ^F complexes in the absence of AA as measured by SPR ($13 \times 10^{-3} \text{ s}^{-1}$). The black points in Figure 4(B) show a simulation of AB-R105A · σ^F complex dissociation, derived from this off-rate and the protein concentrations used in the experiments. (The on-rate in this simulation was set to a very low value ($2 \text{ M}^{-1} \cdot \text{s}^{-1}$) so as to mimic a condition in which re-binding is prevented.) The results show that the experimental curves are similar to the simulation. This demonstrates that at these protein concentrations (which are similar to cellular levels), the addition of AA does not increase the off-rate from the complex. Following dissociation, σ^F · AB-R105A complexes re-formed extremely slowly. Reactions with 1 μ M AB-R105A and 5 μ M AA continued over longer time courses, and showed that re-binding occurred at a rate of approx. 10% per h (results not shown). This extremely low rate of re-binding corresponds to the extremely low steady-state rate of phosphorylation observed for this mutant (see Figure 2).

We may interpret the AB-R105A curves as follows. The dissociation of AB-R105A from σ^F occurs relatively slowly. However, when a dimer of AB-R105A does dissociate from σ^F , it will rapidly phosphorylate two molecules of AA and subsequently form complexes with two further molecules of AA and ADP. This accounts for the observation that 2.5 μ M AA is sufficient to release only just over half the σ^F , since each dimer of AB-R105A will consume four molecules of AA. Since dissociation of ADP from AB-R105A is extremely slow, AB-R105A · AA · ADP complexes formed in this process have exceptionally long lifetimes. σ^F cannot re-bind to AA · AB · ADP complexes [16], and therefore re-association of σ^F with AB is also extremely slow.

Changes in the ATP-lid on interaction with AA and σ^F

In previous studies [8], a fluorescence probe in the ATP-lid region of AB was used to detect changes in the state of the protein. This probe was created by replacing a phenylalanine residue at position 97 of AB with a tryptophan residue. The resulting mutant protein, AB-F97W, showed distinctive changes in its fluorescence intensity when bound to ADP, ATP and AA, but was indistinguishable from the wild-type in its activity *in vivo* during sporulation and in its interactions with AA and σ^F . We have now introduced this mutation into AB-E104K, AB-T49K and AB-R105A. The resulting double-mutant proteins (AB-F97W,E104K, AB-F97W,T49K and AB-F97W,R105A respectively) had a similar fluorescence intensity when excited at 290 nm to that of AB-F97W. The introduction of the F97W mutation made no observable difference to interactions of these proteins with AA and σ^F either in complex formation, as observed by native-PAGE, or during phosphorylation reactions (results not shown).

To study the interactions of these fluorescent versions of AB with σ^F , we made a double mutant of σ^F (W46L,W190L) to remove both the tryptophan residues from this protein, so that we were able to follow fluorescence intensity changes from AB alone. The effects of the double mutation in the cell was checked by replacing the wild-type σ^F gene with the W46L,W190L mutant gene in the genome of *B. subtilis* and monitoring σ^F activity during sporulation. In addition, complexes between σ^F -W46L,W190L and wild-type AB were studied by native-PAGE and SPR.

Figure 5 Changes in fluorescence intensity in AB proteins on binding σ^F and on phosphorylating AA

In all experiments, the proteins were incubated together at zero time, and a baseline value was determined (see the Experimental section). After 1 min, 100 μM ATP was added. (A) Fluorescence changes of 1 μM AB proteins on binding to 1.3 μM σ^F -W46L,W190L. (B) Fluorescence changes during phosphorylation reactions of 7.5 μM AA by 1 μM AB proteins. Blue line, AB-F97W; purple line, AB-F97W,E104K; red line, AB-F97W,T49K; green line, AB-F97W,R105A.

σ^F -W46L,W190L was indistinguishable from the wild-type protein by all these criteria (results not shown).

Figure 5(A) shows the fluorescence intensity changes of AB-F97W (blue line), AB-F97W,E104K (purple line), AB-F97W,T49K (red line) and AB-F97W,R105A (green line) on binding σ^F . At the start of these experiments, 1 μM AB proteins was incubated together with 1.3 μM σ^F -W46L,W190L, giving a fluorescence intensity which we take as the baseline for the experiment. Since the interaction between σ^F and AB requires the presence of a nucleotide, no interaction occurred until, after 1 min, 100 μM ATP was added. The results show that the fluorescence intensity change on binding σ^F was similar for all four proteins, suggesting that, on binding to σ^F , the conformation of the mutant proteins is similar to that of the wild-type.

In the final series of experiments, we used the same fluorescent derivatives of the wild-type and mutant AB proteins (AB-F97W, AB-F97W,E104K, AB-F97W,T49K and AB-F97W,R105A) to phosphorylate AA. Figure 5(B) shows the fluorescence intensity

changes during this reaction. At the start of these experiments, 1 μM AB proteins was incubated together with 7.5 μM AA, and, after 1 min, 100 μM ATP was added. The wild-type mimic AB-F97W (blue line) displayed a large decrease in fluorescence intensity over the first 2 min of the phosphorylation reaction. We have shown previously that this decrease occurs during the approach of the reaction to steady state and that it may be attributed to a conformational change in AB during the phosphorylation of AA [8]. On prolonged incubation, after all the AA in the mixture was phosphorylated, the fluorescence returned to the level characteristic of AB-F97W when bound to ATP alone (results not shown). The fluorescence changes occurring in AB-F97W,E104K and AB-F97W,T49K under the same conditions were much less conspicuous (purple and red lines in Figure 5B). On the addition of ATP to a mixture also containing AA, both mutant proteins briefly displayed a reduction in fluorescence intensity of approx. 8–10%. Following completion of the phosphorylation reaction, the fluorescence intensity of these mutant proteins returned to the level that is characteristic of these proteins when bound to ATP alone [8]. The fluorescence intensity of AB-F97W,T49K returned to this level more rapidly than AB-F97W,E104K, reflecting the difference in the reaction rates of these two mutants (Figure 2). AB-F97W,R105A (Figure 5, green line) also displayed a reduction in fluorescence intensity of approx. 10% after the addition of ATP, but in contrast with the other two mutant proteins, the fluorescence intensity remained at this level (Figure 5B). Experiments over longer time periods showed that AB-F97W,R105A returned to the level characteristic of ATP binding alone extremely slowly, as would be expected from its very low rate of phosphorylation of AA.

As is clear from Figure 5(B), all three mutant proteins showed a much smaller reduction in fluorescence intensity than the wild-type mimic AB-F97W during the phosphorylation of AA (AA concentrations higher than 7.5 μM did not substantially increase the change in fluorescence intensity displayed by these mutants; results not shown). The simplest explanation for this result is that the large reduction in fluorescence intensity in AB-F97W is due primarily to a conformational change in that protein in interacting with AA, and that AB-F97W,E104K, AB-F97W,T49K and AB-F97W,R105A do not undergo this transition.

A comparison of Figures 5(A) and 5(B) also shows that the three mutant proteins demonstrated a similar (small) reduction in fluorescence intensity on binding to σ^F and during the interaction with AA, whereas wild-type AB showed a much larger reduction in fluorescence intensity when interacting with AA than when binding to σ^F . We infer that the release of σ^F that is induced when AA attacks the wild-type $\sigma^F \cdot \text{AB} \cdot \text{ATP}$ complex is accompanied by a change in the conformation of wild-type AB. In contrast, the results with the mutants suggest that, to the extent that partner-switching occurs, it does not elicit a conformational change.

DISCUSSION

Scheme 2(A) is a composite of Schemes 1(A) and 1(B). We hope to demonstrate that Scheme 2(A) is likely to represent correctly the pathways by which wild-type AB interacts with its partner proteins AA and σ^F . The remainder of this paper, which will use the results from the mutants to illuminate the interactions of wild-type AB, will treat AB-E104K and AB-T49K together, since their similarities, both in their behaviour *in vitro* and in their effects on the phenotype of the cell, are much more conspicuous than their differences. We shall show that the interactions that the three mutant AB proteins make with AA and σ^F can be described by

Scheme 2 Interactions of σ^F and AA with either wild-type or mutant AB proteins

Schemes for the interactions of wild-type AB (A), AB-E104K and AB-T49K (B), and AB-R105A (C). The phosphorylation pathway is shown in normal text and the interactions with σ^F are shown in outline.

introducing simple modifications to Scheme 2(A), thus lending support to that scheme as representing the wild-type.

The time-course of phosphorylation of AA by wild-type AB is strikingly biphasic [16,17] (see also Figure 2). We have shown previously that this time course is due to the formation of long-lived AA·AB·ADP complexes, requiring a conformational change in AB that converts it into a form that binds ADP very strongly [8]. The change in conformation, to the form designated AB^{closed} in the part of Scheme 2(A) in normal type, accompanies the binding of AA in the step leading from species 2 to species 3, and has been attributed to a change in the ATP-lid [8]. A con-

formational change in AB on binding AA was proposed in earlier work in this laboratory [19].

AB-E104K and AB-T49K are impaired in the binding of AA (Table 1), and show a linear time-course of phosphorylation (Figure 2). Moreover, the reduction in fluorescence of the AB proteins on binding to AA (which we take to be a direct reflection of a change in conformation) is much smaller for these two mutant proteins than it is for wild-type AB (Figure 5B). We conclude that they probably fail to undergo the conformational change in the ATP-lid; consequently, they are unable to form significant quantities of species 7 (Scheme 2A), and, in addition, the slow step of the steady-state wild-type reaction (from species 5 to species 6) is eliminated.

The difference in fluorescence behaviour between wild-type AB on the one hand and AB-E104K and AB-T49K on the other hand (Figure 5) is relevant also to our understanding of the release of σ^F from σ^F ·AB complexes. The simplest conclusion from the results shown in Figure 5 is that wild-type AB undergoes a conformational change in the ATP-lid when it switches partners from σ^F to AA. We note that in the mutants we have studied in which conformational changes in the ATP-lid seem not to occur, the release of σ^F is impaired. However, our results do not exclude the mechanism suggested by Campbell et al. [20], in which steric clashes between AA and σ^F induce the release of σ^F . In addition, we do not rule out the possibility that more than one conformational change occurs during the reactions shown in Scheme 2(A). In particular, since SpoIIAB is dimeric, these reactions may involve changes in the interactions between the subunits.

We therefore propose that AB-E104K and AB-T49K will follow the scheme shown in Scheme 2(B), which involves no conformational changes in AB. This scheme is consistent with the fact that phosphorylation catalysed by AB-E104K and AB-T49K is inhibited by σ^F (Figure 3A). With these mutant proteins, the affinity of σ^F for AB·ATP is much the same as it is with wild-type AB (Table 1), so that when σ^F is present, it will bind to the mutant AB·ATP, forming species 8. However, the extent of conversion of species 8 into species 9 (Scheme 2A) will be negligible with AB-E104K and AB-T49K, both because a conformational change in these proteins does not occur, and because the binding of AA is too weak to drive the formation of species 9. Species 8 will therefore accumulate, thus diminishing the quantity of AB available to phosphorylate AA, and the phosphorylation reaction will be inhibited.

In cells carrying AB-E104K or AB-T49K, the defect in induced release prevents AA at the concentrations in the cell (approx. 2:1 AA/AB dimer [17]) from inducing the release of significant quantities of σ^F . This feature, together with the defect in the formation of a complex with AA in the presence of ADP, accounts for the severe block in σ^F activation, with the result that σ^F is unavailable to form RNA polymerase holoenzyme.

The situation with AB-R105A is rather different. Like the other two mutant proteins, it fails to undergo a conformational change on binding AA (Figure 5B), and yet the time-course of phosphorylation of AA is biphasic, with the steady-state rate being extremely low (Figure 2). These results can be explained by supposing that this mutant protein is permanently in a state similar to that assumed by wild-type AB when its ATP-lid is closed. In this state, the initial phosphorylation catalysed by an enzyme molecule bound to ATP is fast; however, the rate of subsequent (steady-state) phosphorylation is limited by the very slow exchange of ADP trapped inside the lid for ATP.

If AB-R105A resembles AB-E104K and AB-T49K in its failure to undergo a conformational change on binding AA, how can we explain its behaviour when σ^F is present during the

phosphorylation of AA, which is very different from that of the other two mutant enzymes (Figure 3)? Unlike the other two mutants, AB-R105A has at least as high an affinity for AA as wild-type AB has (Table 1). Consequently, when the ratio of AA to σ^F is sufficiently high, mass action will drive a significant conversion of species **8** into species **9**. It follows that the phosphorylation reaction will be inhibited only if the AA/ σ^F ratio is relatively low, as shown in Figure 3(B). Scheme 2(C), which takes these points into account, illustrates the reaction scheme that we propose for AB-R105A.

The results also allow us to account for the premature and excessive activation of σ^F in cells with the AB-R105A mutation. After a rapid initial round of phosphorylation, AB-R105A is trapped in exceptionally long-lived AA·AB·ADP complexes (Figure 2) and is unable to return to inhibit σ^F . Dephosphorylation of a little AA-P will suffice to maintain σ^F in the free form, and σ^F will be activated even at the low levels of SpoIIE activity that are found before asymmetric septation. Later in sporulation, σ^F will become permanently activated in both compartments. The excessive σ^F activity eventually leads to cell lysis.

The results from all three of the AB mutants demonstrate the importance of both the induced release of σ^F and the subsequent interaction of AB·ADP with AA in the regulation of σ^F activity in the wild-type. By means of the induced-release mechanism, dephosphorylated AA rapidly and effectively disrupts σ^F ·AB complexes. However, for correct regulation of σ^F activity, it is important that, following induced release, AB interacts appropriately with AA. If the interaction with AA is too brief, as it is with AB-E104K and AB-T49K, σ^F is released for too short a time to be effective in transcription. If, in contrast, the interaction is too protracted, as it is with AB-R105A, σ^F becomes activated too early and lysis results. The present results suggest that if AB is deficient in the induced release of σ^F , but also differs in its interaction with AA, it is the latter effect that is the more important in determining the cell's phenotype (see also Lee et al. [16]).

The mutations that we have studied have been described previously [7,15,23]. Garsin et al. [23] found that AB-E104K, and, to a lesser extent, AB-T49K, were blocked in the phosphorylation of AA. Our conclusions are different, perhaps because Garsin et al. did not carry out a full kinetic analysis of phosphorylation. However, we agree with the finding of Garsin et al. [15] that AB-T49K is defective in binding to AA in the presence of ADP, and believe that this defect is important in determining the phenotype of AB-T49K in the cell. Ho et al. [7] reported that AB-R105A was also defective in the phosphorylation of AA. Our results show that the initial cycle of phosphorylation is not blocked, but the rate of reaction thereafter is extremely low. As already explained, we believe that the formation of exceptionally stable AB-R105A·AA·ADP complexes accounts for the premature and excessive activation of σ^F noted by Ho et al. [7].

The three mutations studied in the present paper are all within the nucleotide-binding region of AB [20], which appears to be a critical region for the function of this protein. Elucidation of the crystal structure of AB [20] allows us to speculate about how these particular mutations may induce the changes in the properties of AB observed (see Figure 1). Glu¹⁰⁴ is located at the top of the ATP-lid of AB, a region in which AA is predicted to bind. The replacement of this residue with a lysine residue is likely to disrupt the binding of AA by direct steric hindrance. Thr⁴⁹ is located underneath the ATP-lid, and the insertion of a lysine residue here is likely to clash with the lid, disrupting its structure and weakening its interaction with the nucleotide. Arg¹⁰⁵ is also located in the ATP-lid, and the side-chain points towards

the position that would be occupied by the γ -phosphate of a bound ATP molecule. The removal of this positively charged residue is likely to disrupt the structure of the lid and may allow the lid to approach the bulk of the AB molecule more closely. This effect would slow down lid opening and nucleotide exchange.

These studies have revealed how the interactions of wild-type AB have been optimized during evolution for the correct regulation of σ^F . They have demonstrated the mechanisms by which premature activation of σ^F is prevented, and those by which it is efficiently activated in the prespore. These attributes enable AB to act as an on/off switch during the initiation of differential gene expression.

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