Evidence in Support of a Docking Model for the Release of the Transcription Factor $\sigma^F$ from the Antisigma Factor SpoIIAB in Bacillus subtilis*

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Cell-specific activation of the transcription factor $\sigma^F$ during the process of sporulation in Bacillus subtilis is governed by an antisigma factor SpoIIAB and an anti-antisigma factor SpoIIAA. SpoIIAB, which exists as a dimer, binds to $\sigma^F$ in a complex of stoichiometry $\sigma^F$-SpoIIAB$_2$. Escape from the complex is mediated by SpoIIAA, which reacts with the complex to cause the release of free $\sigma^F$. Previous evidence indicated that Arg-20 in SpoIIAB is a contact site for both $\sigma^F$ and SpoIIAA and that contact with $\sigma^F$ is mediated by Arg-20 on only one of the two subunits in the $\sigma^F$-SpoIIAB$_2$ complex. Here we report the construction of heterodimers of SpoIIAB in which one subunit is wild type and the other subunit is a mutant for Arg-20. We show that the dissociation constant for the binding of $\sigma^F$ to the heterodimer was similar to that for the wild type, a finding consistent with the idea that $\sigma^F$ contacts Arg-20 on only one of the two subunits. Although SpoIIAA was highly effective in causing the release of $\sigma^F$ from the wild type homodimer, the anti-antisigma factor had little effect on the release of $\sigma^F$ from the heterodimer. This finding is consistent with a model in which SpoIIAA docks on the $\sigma^F$-SpoIIAB$_2$ complex, making contact with the subunit in which Arg-20 is not in contact with $\sigma^F$. SpoIIAB is both an anti-$\sigma^F$ factor and a protein kinase that phosphorylates and thereby inactivates SpoIIAA. We show that SpoIIAA effectively displaces $\sigma^F$ from a complex of $\sigma^F$ with a mutant (SpoIIAB$_{R105A}$) that is impaired in the kinase function of SpoIIAB. This result shows that SpoIIAA-mediated displacement of $\sigma^F$ from SpoIIAB does not require concomitant phosphorylation of SpoIIAA.

Sporulation in the bacterium Bacillus subtilis is an attractive system in which to study the problem of cell-specific gene transcription (1, 2). Sporulation involves the formation of an asymmetrically positioned septum that partitions the developing cell or sporangium into a forespore (the smaller cell) and a mother cell. The transcription factor $\sigma^F$ is activated in a cell-specific manner that limits its activity to the forespore compartment of the sporangium (3). The activity of $\sigma^F$ is governed by a pathway involving the proteins SpoIIAB, SpoIIAA, and SpoIE (3–10). SpoIIAB (henceforth abbreviated AB) is an anti-$\sigma^F$ factor that binds to $\sigma^F$, trapping it in an inactive complex. AB is responsible for holding $\sigma^F$ inactive prior to the formation of the polar septum and in the mother cell after polar division (9–11). The $\sigma^F$ factor escapes from the complex with AB in the forespore in a process that is mediated by the anti-antisigma factor SpoIIAA (henceforth abbreviated AA; see Refs. 12 and 13). The activity of AA is, in turn, controlled by phosphorylation at Ser-58 through the opposing activities of SpoIE, a phosphatase, and AB itself, which is both a serine protein kinase and an antisigma factor (6, 10, 14).

Here we are concerned with the mechanism by which AA mediates the release of $\sigma^F$ from the complex with AB. AB is a dimer and contains an adenosine nucleotide binding pocket that can hold either ATP or ADP (9). The binding of AB to $\sigma^F$ is dependent on the presence of nucleotide in the pocket, with ATP being more effective than ADP (15). The complex is asymmetric, having a stoichiometry of one molecule of $\sigma^F$ per dimer of AB ($\sigma^F$-AB$_2$) (16). Previous work indicated that AA reacts with the $\sigma^F$-AB-AB complex to induce the release of $\sigma^F$ from its antagonist (12, 17). An attractive model for the mechanism of this displacement reaction arose from the x-ray crystallographic studies, which revealed a potential docking site for AA on one of the two AB subunits (18). According to this model, AA would dock on the complex, making contact with one molecule of AB and sterically displacing $\sigma^F$ from the other molecule. Previous work (13) had identified Arg-20 of AB as a contact site for both $\sigma^F$ and AA. A key feature of the docking model is that Arg-20 on one AB subunit is in contact with $\sigma^F$, whereas Arg-20 on the other subunit is exposed to solvent and is free to make contact with AA. A second residue of importance in the docking model is Glu-104 of AB, which is a contact site for AA but not for $\sigma^F$. Substitutions at Glu-104 impaired the capacity of free AB to phosphorylate AA and prevent AA from causing the release of $\sigma^F$ from the $\sigma^F$-AB-AB complex (17). Here we describe the use of heterodimeric mutant forms of the AB to investigate further the docking model and other aspects of the reaction of AA with the $\sigma^F$-AB-AB complex.

EXPERIMENTAL PROCEDURES

Construction of spoIIAB Expression Plasmids—Wild type and mutant forms of spoIIAB were expressed in Escherichia coli strain BL21(DE3)pLysS (Novagen) using an isopropyl-1-thio-β-D-galactopyranoside-inducible phage T7 RNA polymerase system. Three kinds of expression plasmids were constructed for the purification of AB proteins as follows.

An expression plasmid for the purification of untagged AB in the experiment of Fig. 1 was made by amplifying the wild type gene from pDAG14 (13, 16) by PCR using oligonucleotides MH07 and MH08, which contained the restriction sites EcoRI and HindIII, respectively. The PCR product was digested with these enzymes and ligated to the expression plasmid pET-29a (Novagen), which had been similarly enzyme-treated, to create pMH13. Insertion into pET-29a joined spoIIAB

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to the coding sequence for an S-tag. cDNA from E. coli strain BL21(DE3)/pLyS58 (Novagen) was used to create pMHE1.

Expression plasmids for the purification of His6-AB proteins were made by amplifying wild type and mutant forms of spoIAB from pDAG14 (wild type spoIAB gene), pH84 (spoIAB-E104K), pHM88 (spoIAB-R20E), and pHM86 (spoIAB-R105A) by PCR using oligonucleotides MH04 and MH11, which contained restriction sites for BamHI and XhoI, respectively. The PCR products were digested with these enzymes and ligated to the expression plasmid pSETA (Invitrogen) that had been digested with these same enzymes to create plasmid pMH1 (His6-spoIAB), pH8 (His6-spoIAB-E104K), pHM51 (His6-spoIAB-R20E), and pHM54 (His6-spoIAB-R105A). This fused the spoIAB gene downstream and in-frame to six histidine codons in the vector. The plasmids contained a HindIII site 4 bases away from the end of the EcoRI site, which was subsequently used for cloning the second mutant copy of spoIAB gene between restriction sites HindIII and EcoRI. The PCR product was digested with BamHI and EcoRI and ligated to the expression plasmid pOEX-2TK (Amersham Biosciences) that had also been treated with BamHI and EcoRI to create pMH21. This fused spoIAB in-frame to the coding sequence for GST in the vector. By using pHM51 (the expression plasmid for producing His6−ABR20E−) as a template, a DNA sequence downstream encoding ABR105A was created by site-directed mutagenesis using appropriately designed oligonucleotides MH18 and MH86, pDAG14, and the Quick-Change Site-directed mutagenesis kit (Stratagene).

**Construction of spoIAC Expression Plasmids—** Strain LDE7 used for the production of αF in the affinity chromatography experiment of Fig. 3 was described previously (9). The spoIAC expression plasmid used for the production of αF was constructed from chromosomal DNA from B. subtilis PTY79 using the primers, 5′-atgtagctgctgtgaggtaag-3′ and 5′-atgaattccatccgtatgatccat-3′. The PCR fragment was digested with BamHI and EcoRI and ligated into the BamHI/EcoRI-digested vector pGEX-2T (Amersham Biosciences) to generate pMF13 in which the αF-coding sequence was fused to the coding sequence for GST. pMF14 was then transformed into DHBio to create pMHE2.

**Protein Purification—** E. coli strains used for the production of AB and αF were grown at 37 °C in 1 liter of LB containing 75 μg/ml ampicillin and 25 μg/ml chloramphenicol until the culture reached an A600 of 0.4. In the case of strains used for producing AB heterodimer and αF, culture was allowed to grow to an A600 of 0.4 for 5 h prior to induction with 0.4 mM isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. The cells were harvested 3 h later by centrifugation. Cell pellets were suspended in the appropriate binding buffer and disrupted by sonication. After sonication, the insoluble material and the unlysed cells were removed by centrifugation, and the supernatant fluid was collected.

Four kinds of purification procedures were used for purifying AB and αF proteins. For purification of the untagged AB, cell pellets from strain MHE31, which produces S-tagged AB, were suspended in buffer A (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100). The supernatant fluid was transferred to a column (S-protein-agarose for 1 h at 4 °C. After washing with 6 column volumes of buffer A, the S-protein-agarose bound with S-tagged AB was suspended in buffer B (20 mM Tris-HCl, pH 8.4, 150 mM NaCl, 2.5 mM CaCl2). 25 units of biotinylated thrombin were added to the column and stored in 15% glycerol at −80 °C.

Cell pellets from strain MHE21, which produced AB−ABR20E heterodimer, were suspended in buffer C plus 5 mM imidazole. The supernatant fluid was loaded onto Ni2+-NTA-agarose for 1 h at 4 °C. After washing with 6 column volumes of buffer C plus 20 mM imidazole, proteins bound to the column were eluted with buffer C plus 200 mM imidazole. Elution fractions containing the purified proteins were dialyzed into storage buffer and stored in 15% glycerol at −80 °C.

Cell pellets from strain MHE02-produced GST-tagged αF were suspended in 1× PBS, and αF protein was released from the GST tag by cleaving the column with 50 units of thrombin in 1× PBS.

**Affinity Chromatography—** A mutant spoIAB encoding ABR105A was created by site-directed mutagenesis using appropriately designed oligonucleotides MH85 and MH86, pDAG14, and the Quick-Change Site-directed mutagenesis kit (Stratagene).

**Testing Subunit Exchange—** Equimolar amounts (10 μM each) of AB and His6-AB were mixed and incubated at 4 °C for 3 h. The mixture was diluted with buffer C plus 5 mM imidazole to 50 μM. 25 μL of resin (bed volume) was added to the 500-μL mixture, and the sample was rotated on a rotator at 4 °C for another hour. The resin was collected by centrifugation (4000 rpm), washed with 5 column volumes of buffer C plus 20 mM imidazole, and suspended in 25 μL of 2× SDS sample buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol). Eluates from the resin were analyzed by SDS-PAGE.

**Surface Plasmon Resonance—** Kinetic and equilibrium constants of the protein-protein interactions were measured by surface plasmon resonance with a BIAcore 3000 instrument (Amersham Biosciences). To prepare the sensor chip, 80 μl of anti-His antibodies (concentration 50 μg/ml) diluted in 10 mM acetate, pH 4.5, were immobilized on the chip surface at a flow rate of 30 μl/min for 8 min. The flow was stopped and the material near the chip surface was removed by injecting 10 m M HCl at a flow rate of 10 μl/min for 2 min. A sample of solution containing His6-AB, His6−ABR20E, or His6−ABR105A heterodimer dilution in HBS buffer was applied to one flow cell of the CM5 sensor chip already coupled with anti-His antibodies. The analyte αF was dialyzed into binding buffer (100 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 0.5 mM dithiothreitol, and 0.5% AETP) and injected over the sensor surface at a flow rate of 30 μl/min for a total of 60 μl. Concentrations of the analyte αF applied ranged from 20 to 200 nM. After the interaction, 50 μl of 20 mM HCl was injected to regenerate the surface. Moreover, no specific interactions were observed when analyte αF was injected over a flow cell with immobilized anti-His antibody alone. Apparent kinetic constants (k on and k off) were obtained by use of the BIAevaluation software (Amersham Biosciences).

**Production of 125I-Methionine-labeled αF—** Radio labeling of αF using strain MHE5 was as described by Alper et al. (15) with the following modification. The culture (1 L) was grown in the presence of rifampicin for 2.5 h before labeling. Unlabeled cells were harvested before the addition of [125I]methionine, and 1 ml of the cultures was pelleted and lysed in 500 μl of buffer that contained 50 mM Tris-HCl, pH 8, 100 mM NaCl, 0.1 mM dithiothreitol, and 0.5% X-100.

**Affinity Chromatography—** Mutant and wild type forms of AB were mixed with Ni2+-NTA-agarose (30 μl of resin) for 1 h at room temperature in buffer C. The resin was collected by centrifugation (4000 rpm). 100 μl of the radiolabeled αF was added to the resin, and the mixture (original volume of 150 μl) was diluted to a volume of 500 μl with buffer.
Docking Model for Release of $\sigma^F$

C. The column support bed was formed with ~5 µl of glass beads (200–300 µm; Sigma, catalog number G-1277), and the column was filled with buffer C. The column bed was formed by adding the resin mixture and allowing it to settle while the column flowed by gravity. The column was then washed 5–10 times with 5 column volumes of buffer C. The column was eluted with 50 µl of 1% SDS, buffer C, 8 µM non-radioactive $\sigma^F$, or 8 µM AA purified as described previously (13). Photometric Focusing—RL2220 (thr::spoIIQ-lacZ,erm spoIE::kan) was transformed with chromosomal DNA from MHB10 and selected for spectinomycin resistance to create KC365. Cells were induced to sporulate by the resuspension method (25). At indicated times after the initiation of sporulation, 1-ml samples were harvested. For isoelectric focusing (IEF) cells were resuspended in lysis buffer (10 mM Tris-HCl, pH 7.5, 50 mM MgCl$_2$, 10 mM EDTA, 0.5 mg/ml phenylmethylsulfonyl fluoride, 0.5 mg/ml lysozyme, 0.1 mg/ml DNase I) and incubated at 37 °C for 10 min. Lysates were mixed 1:1 volume with 2× IEF sample buffer (8 M urea, 2.6% (v/v) ampholytes, pH 5–6 (Pharmalyte, Amersham Biosciences), 2% Triton X-100, 1% 2-mercaptoethanol, 0.04% bromphenol blue) and loaded onto a 5% polyacrylamide IEF slab gel containing 8% urea and 2.6% (v/v) ampholytes, pH 5–6 (Pharmalyte, Amersham Biosciences). The gel was run at 200 V for 30 min followed by 300 V for 2.5 h with 10 mM phosphoric acid as the anolyte and 20 mM NaOH as the catholyte. Approximately equal numbers of cells were loaded for each sample as determined by A$_{600}$ at the time of harvesting. The protein was electroblotted 20 V overnight (transfer buffer was 25 mM Tris, 193 mM glycine, 20% methanol to Immobilon-P membrane (Millipore) and then incubated with affinity-purified polyclonal anti-SpoI1A antibodies raised in rabbits against SpoI1A purified as described previously (13). Donkey anti-rabbit antibodies labeled with iodine-125 (Amersham Biosciences) were used for detection on Biomax MR film (Eastman Kodak Co.).

**Kinetic Measurements of Kinase Activity**—Phosphorylation reactions were carried out at 21 °C in 50-µl reaction volumes containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM MgCl$_2$, 1 mM dithiothreitol, 1 mM ATP plus 5 µCi of [γ-32P]ATP (6000 Ci/mmol), 25 mM AB. The AA concentration was varied from 62.5 nM to 2 µM. Reactions were stopped by the addition of 850 µl of 10% (v/v) trichloroacetic acid before the rate of phosphorylation reached a maximum. The protein was precipitated by the addition of bovine serum albumin to a final concentration of 1 mg/ml. The pellets were washed three times with 1 ml of 10% trichloroacetic acid and dissolved in 1 ml of Tris base. The final sample was counted in liquid scintillation counter after adding 7 ml of scintillation fluor (Opti-Fluor, Packard Instrument Co.). The time 0 blank was subtracted from each value.

$\sigma^F$-Directed $\beta$-Galactosidase Synthesis—pMH68 containing the spoI1A::R105A mutation was linearized with restriction enzymes StuI and XcmI, and introduced by double recombination into the spoI1A operon of competent cells of MHB26 (thr::spoIIQ-lacZ,erm) by transformation and selection for spectinomycin resistance, creating strain MHB10. Cell pellets from MHB10 were collected at each time point from the beginning of sporulation (time 0) in resuspension medium and treated with 10 µl of 20 mg/ml lysozyme at 30 °C for 15 min. $\beta$-Galactosidase activity of the sample was determined according to Harwood and Cutting (25).

**RESULTS**

**AB Dimer Subunits Do Not Exchange**—Our strategy for studying the contribution of each of the two subunits of AB-AB to its interactions with $\sigma^F$ and AA was to construct heterodimers of the antisigma factor in which one of its two subunits was a mutant for a contact site. The feasibility of such a strategy rested on the premise that AB-AB is stable and hence that heteromeric dimers would not undergo subunit exchange with each other. Subunit exchange would defeat the purpose of constructing heterodimers as it would generate a mixture of homodimers and heterodimers. To investigate whether AB-AB would undergo subunit exchange, we separately purified homodimers of AB-AB that had been tagged with an extension of six histidine residues at its NH$_2$ terminus (His$_8$-AB-His$_8$-AB) and homodimers of the unmodified, wild type protein. Next, we prepared an equimolar mixture of His$_8$-AB-His$_8$-AB and AB-AB and incubated the mixture at 4 °C for 3 h. Finally, we recovered the histidine-tagged protein from the mixture using Ni$^{2+}$-NTA-agarose. When analyzed by SDS-PAGE, material that had adhered to the resin was found to contain only His$_8$-tagged AB (Fig. 1, lane 11; which could be distinguished from unmodified AB by its larger size). Control experiments carried out in parallel showed that His$_8$-tagged AB bound to the resin, whereas unmodified AB did not (Fig. 1, lanes 4 and 8). Conversely, little or no His$_8$-tagged AB was present in the flow-through when either His$_8$-AB alone (lane 3) or a mixture of His$_8$-AB and unmodified AB (lane 10) was applied to the resin. On the other hand, unmodified AB was present in the flow-through when either the untagged protein alone (lane 7) or the mixture of tagged and untagged proteins (lane 10) were applied to the resin. We conclude that little or no His$_8$-AB-AB heterodimers had formed during the incubation period and hence that the dimer does not undergo a significant level of subunit exchange.

**Purification of an AB Heterodimer—Arg-20 of AB was identified previously as a probable contact site both for $\sigma^F$ and AA in that a loss of side chain substitution (R20A) or a replacement with a negatively charged side chain (R20E) impaired binding to either partner protein in vitro and caused high levels of $\sigma^F$ activity in vivo (13). The assignment of Arg-20 as a contact site for $\sigma^F$ was confirmed by the recent determination of the crystal structure of the $\sigma^F$-AB-AB complex (18). The crystal structure revealed that Arg-20 on one of the two AB subunits in the complex is in contact with $\sigma^F$, whereas Arg-20 on the other subunit is exposed to solvent and hence would potentially be available as a docking site for AA. This interpretation predicts that heterodimeric AB in which one subunit is wild type and the other harbors the R20E substitution would be unimpaired in binding to $\sigma^F$. (We used R20E rather than R20A because the effect of the former substitution is more severe than that of the latter; see Refs. 13 and 17.) To create the heterodimer, we co-expressed the genes for the mutant and wild type protein in *E. coli*. The genes were arranged in tandem in a construct in which their transcription was under the control of a promoter recognized by the phage T7 RNA polymerase. The gene for the wild type protein in the construct was preceded by the coding sequence for GST as well as the coding sequence for the cleavage site for the protease thrombin, whereas the gene for the mutant protein was preceded by six histidine codons. Thus, *E. coli* cells harboring the construct were expected to produce GST-AB and His$_8$-tagged AB$^{\text{R20E}}$.

Expression of the construct was expected to generate three kinds of dimers: GST-AB homodimers, His$_8$-AB$^{\text{R20E}}$ homodimers, and GST-AB-His$_8$-AB$^{\text{R20E}}$ heterodimers. A lysate was prepared from cells of *E. coli* harboring the construct, and the following procedure was used to separate the heterodimers from the homodimers and other proteins in the lysates. First,
we used Ni²⁺-NTA-agarose to purify His₈-AB homodimers and GST-AB-His₈-ABR₂₀E heterodimers from the lysate. Lane 1 of Fig. 2 shows that both GST- and His₈-tagged proteins had adhered to the column. Second, a glutathione-Sepharose (GST) resin was used to separate GST-AB-His₈-ABR₂₀E heterodimers from the His₈-AB homodimers. The purified heterodimers (Fig. 2, lane 2) were treated with thrombin to remove the GST tag, resulting in purified AB-His₈-ABR₂₀E heterodimers. Finally, glutathione-Sepharose was used to remove the GST tag that had been released by thrombin treatment and residual GST-AB-His₈-ABR₂₀E heterodimers that had not undergone proteolytic cleavage from the AB-His₈-ABR₂₀E heterodimers. The purified AB-His₈-ABR₂₀E heterodimers were recovered in the flow-through fraction (Fig. 2, lane 3).

By using similar procedures we also constructed and purified wild type homodimers of His₈-tagged AB and mutant homodimers of His₈-tagged ABR₂₀E. For simplicity the two His₈-tagged homodimers are henceforth referred to as AB-AB and ABR₂₀E-ABR₂₀E, and the His₈-tagged heterodimer is called AB-ABR₂₀E.

Kinetic and Equilibrium Constants for the Interaction of σF with SpoIIAB Homodimers and Heterodimers—We used surface plasmon resonance to determine the equilibrium dissociation constants for the interaction of σF with His₈-tagged AB-AB, AB-ABR₂₀E, and ABR₂₀E-ABR₂₀E. The purified homodimers and heterodimers (the ligands) were separately immobilized on a layer of dextran that had been coupled with anti-His₈ antibodies and attached to a thin film of gold. Next, purified σF (the analyte) was applied to the surface and allowed to bind with immobilized ligand during the association phase of the analysis. After the analyte had been applied, σF was allowed to dissociate from the ligand during the dissociation phase of the analysis. Association and dissociation of σF were detected optically. Software provided with the instrument was used to calculate association rate constants from the association phase of the interactions and dissociation rate constants from the dissociation phase of the interactions. All interactions were measured in the presence of 1 mM ATP.

First we measured association and dissociation rate constants for the interaction between AB-AB (wild type homodimer) and σF, from which we derived an equilibrium dissociation constant (Kd) of 12 nM (Table I). This value was similar to that (14 nM) reported previously by Magnin et al. (19). Second, we investigated the interaction of σF with AB-ABR₂₀E. Importantly, we obtained a Kd value that was only modestly higher (28 nM) than that observed with the wild type homodimer (see also Table I). Finally, we attempted to measure the interaction of σF with ABR₂₀E-ABR₂₀E. The association rate constant observed using the mutant homodimer was so low that an equilibrium dissociation constant could not be derived, a finding that underscores the importance of the Arg-20 side chain in the interaction of σF with AB. In toto, these results indicate that complex formation requires the presence of one and only one Arg-20 side chain, a finding consistent with the idea that the σF-Arg-20 interaction occurs on only one of the two subunits of the antisigma factor.

Rate constants obtained by surface plasmon resonance can differ substantially from those obtained by other procedures and can be strongly influenced by such factors as the flow rate of the analyte over the surface of the sensor (20, 21). In this regard, we note that the apparent rate constant for dissociation (koff) for the σF-AB-AB complex would appear to indicate a relatively short half-life (~3 min). Yet other evidence from affinity chromatography indicates that the complex is long-lived (see Refs. 12 and 13; see below). With this caveat in mind, the absolute values of the rate constants and equilibrium dissociation constants presented in Table I should be treated with caution. Nonetheless, we believe that we are safe in the general conclusion that σF-AB-AB and σF-ABR₂₀E have similar dissociation rate constants and much lower constants than that for σF-AR₂₀E-ABR₂₀E.

A Complex of σF with the AB Mutant Heterodimer Is Resistant to AA-mediated Dissociation—Previous work indicated that Arg-20 on AB is responsible for contacting AA as well as σF. If, as our present results and those of Campbell et al. (18) indicate, σF contacts Arg-20 on only one of the two subunits of the antisigma factor, then Arg-20 on the other subunit might serve as a contact site for AA during the AA-mediated dissociation of the σF-AB-AB complex. If so, then AA could cause the release of σF from the σF-AB-AB complex by a simple displacement mechanism in which the anti-antisigma factor docks on the subunit with a free Arg-20 side chain. To investigate this hypothesis, we separately immobilized AB-AB and AB-ABR₂₀E on solid matrices. Next, we applied radioactive σF to the matrices, thereby creating immobilized complexes of σF-AB-AB and σF-ABR₂₀E-ABR₂₀E. Finally, we measured the release of radioactive σF from the complexes following the application of buffer, buffer containing non-radioactive σF, and buffer containing purified AA. Fig. 3a shows the results of a representative experiment, and Fig. 3b summarizes the results of four independent experiments. As observed previously, only a small proportion of the radioactive σF was released from the σF-AB-AB complex by buffer (5%; column 1 of Fig. 3b) or buffer containing unlabeled σF (13%; column 2), whereas a relatively high proportion (57%; column 4) was discharged by the anti-antisigma factor. In contrast, AA was no more effective than unlabeled σF or buffer alone in releasing radioactive σF from the σF-ABR₂₀E-ABR₂₀E complex (7%; column 3). These results are consistent with a model in which AA docks on the subunit with a free Arg-20 side chain to effect the release of σF from the other subunit.

The results of Fig. 3b also reinforce the view (12, 13) that the σF-AB-AB complex is relatively long lived and argue against an alternative model for the release of σF based on the idea the
in the kinase reaction but was unaltered in its ability to bind to $\sigma^F$ and AA. Earlier work had shown that an amino acid substitution at Glu-104 (E104K) impaired phosphorylation of AA, but kinetic analysis revealed that the defect was due to impaired binding to the AA substrate ($K_m$) rather than impaired catalysis ($k_{cat}$) (17).

The crystal structure of the $\sigma^F$-AB-AB complex reveals two candidates for amino acids that could be directly involved in the kinase reaction. One candidate is Glu-46, which is in the catalytic center of the kinase in a position from which it could promote the nucleophilicity of the attacking water molecule in the ATPase reaction. Accordingly, we built a loss of side chain substitution mutant in which Glu-46 was replaced with alanine. Unexpectedly, however, AB$^{G46A}$ proved to be impaired in its ability to bind $\sigma^F$ (data not shown). It is known that the binding of AB to $\sigma^F$ is dependent upon adenosine nucleotide, and perhaps the Glu-46 side chain is needed to retain ATP in the nucleotide binding pocket of the antisigma factor/kinase. In any event, the E46A substitution is not simply impaired in catalysis and hence could not be used to address the question of the role of phosphorylation in the AA-mediated dissociation of the $\sigma^F$-AB-AB complex.

A second candidate for a residue functioning in catalysis was Arg-105, which as inferred from the crystal structure could be involved in stabilizing the transition state of the phosphotransfer reaction as well as in contacting Ser-59 in AA, which is immediately adjacent to the site of phosphorylation (Ser-58). We built an R105A substitution mutant and found that it was unimpaired in its ability to bind to $\sigma^F$. Next, we carried out a kinetic experiment to measure the rate of incorporation of $\gamma$-32P-ATP into the substrate AA as catalyzed by AB$^{R105A}$ and as compared with wild type AB. The Michaelis-Menten curve of Fig. 4 shows that the mutant enzyme was little altered in its catalytic activity ($k_{cat}$) but was markedly impaired in its $K_m$; we obtained a value of 557 nM for the mutant enzyme as compared with 4.4 nM for the wild type kinase (17). In this regard, the R105A mutant resembles E104K, which as discussed above is also impaired in its binding to AA. Evidently, the side chains of the adjacent residues Arg-105 and Glu-104 are both needed for substrate binding but not for catalysis.

$AB^{R105A}$ is Defective in Phosphorylation of AA during Sporulation—Next, we carried out experiments monitoring phosphorylation of AA in vivo. Strains producing wild type AB or AB$^{R105A}$ were induced to sporulate, and samples were collected at 1.5 and 2 h after the onset of sporulation. Cell lysates were then prepared and subjected to IEF to separate unphosphorylated and phosphorylated (AA-P) forms of AA. AA and AA-P
were visualized by immunoblotting with polyclonal anti-AA antibodies (Fig. 5). In a strain producing wild type AB, AA was phosphorylated with normal efficiency, as observed previously (13). Lanes 1 and 2 (1.5 and 2 h of sporulation, respectively) of Fig. 5 show both AA and AA-P, with the lower band representing AA-P. In striking contrast, we detected little or no AA-P in samples from cells producing AB\(^{R105A}\) (lanes 3 and 4), suggesting that AB\(^{R105A}\) is defective in phosphorylating AA. Note that the lower band present in lanes 3 and 4 is shifted slightly higher than the bands representing AA-P in the other lanes and represents one of two background bands that are also detected in a lysate of cells of a null mutant lacking AA (lane 9).

To assess further the in vivo kinase activity of AB\(^{R105A}\), we monitored phosphorylation of AA in the absence of SpoIIE, the phosphatase responsible for dephosphorylating AA-P. As shown previously (6, 22) in cells lacking SpoIIE but producing wild type AB, AA was almost entirely in the phosphorylated form (lanes 5 and 6). Importantly, in cells lacking SpoIIE but producing AB\(^{R105A}\), a significant level of AA-P was observed, although the proportion of AA that was in the phosphorylated form was substantially lower than that observed with wild type AB (compare lanes 7 and 8 with lanes 5 and 6). We interpret these results to indicate that AB\(^{R105A}\) is impaired but not completely blocked in kinase activity. Its residual kinase activity is masked by the action of SpoIIE but in the absence of the competing phosphatase kinase activity can be detected. AB\(^{R104K}\) and AB\(^{R105A}\) Have Opposite Effects on \(\sigma^F\) Activation in Vivo and on the Dissociation of the \(\sigma^F\)AB-AB Complex in Vitro—As reported previously (17) and confirmed here, sporulating cells producing AB\(^{R104K}\) are blocked in the induction of a lacZ fusion to a gene under the control of the \(\sigma^F\). Also, and again as confirmed here, the complex of \(\sigma^F\) with AB\(^{R104K}\) (\(\sigma^F\)AB\(^{R104K}\)AB\(^{R104K}\)) is known to be immune to attack by AA (9%) release of radioactive \(\sigma^F\); column 3, Fig. 3d). We interpret these results as indicating that \(\sigma^F\) does not become activated in mutant cells simply because it is unable to escape from the \(\sigma^F\)AB\(^{R104K}\) complex.

Remarkably, however, the R105A substitution had the opposite effect on \(\sigma^F\)-directed \(\beta\)-galactosidase synthesis in vivo and on AA-mediated dissociation of the \(\sigma^F\)AB-AB complex in vitro. The results of Fig. 6 show that in cells producing AB\(^{R105A}\), \(\sigma^F\) was activated earlier and to a much greater extent than in cells producing the wild type protein. Also, the \(\sigma^F\)AB\(^{R105A}\) complex was susceptible to AA, efficiently releasing \(\sigma^F\) in response to the anti-antisigma factor (59% release of radioactive \(\sigma^F\); column 4 of Fig. 3d).

We interpret this result to indicate that AA-mediated release of \(\sigma^F\) is not dependent upon the kinase activity of AB. In further support of this interpretation, we observed that approximately half of the AA that eluted from the column with immobilized \(\sigma^F\)AB-AB was phosphorylated, as detected by isoelectric focusing, whereas almost all the AA that eluted from

Fig. 6. AB\(^{R105A}\) causes premature activation of \(\sigma^F\) in vivo. Accumulation of \(\beta\)-galactosidase (\(\beta\)-gal) was measured during sporulation in wild type cells (○), AB\(^{R105A}\) mutant cells (C), MHB10), and in spolIAB\(^{R104K}\) mutant cells (A, MHB12) harboring lacZ fused to a gene under \(\sigma^F\) control. Measurements were made at the indicated times after suspension in sporulation medium.

**DISCUSSION**

We have presented biochemical evidence in support of a model for the release of \(\sigma^F\) from \(\sigma^F\)AB-AB complex in which the anti-antisigma factor AA docks with the complex and thereby displaces the transcription factor from its inhibitor (Fig. 7). A key feature of the model is residue Arg-105, which previous genetic, biochemical, and structural analyses identified as a contact site both for \(\sigma^F\) and for AA (13, 16, 18). According to the crystal structure of the \(\sigma^F\)AB-AB complex, Arg-20 is in contact with \(\sigma^F\) on one of the two subunits of the AB dimer but is exposed to solvent on the other subunit where it could dock with AA (18). The principal findings of the present investigation were as follows. First, we have shown that the dissociation constant of the \(\sigma^F\)AB-AB\(^{R20K}\) complex is similar to that of the corresponding wild type complex. This finding sup-
ports the conclusion that the stability of the $\sigma^F$-AB-AB complex depends on the presence of Arg at position 20 on one and only one of the two AB subunits in the complex. Second, $\sigma^F$ was efficiently displaced from the wild type complex by AA but poorly from the $\sigma^F$-AB-AB$^{\text{ADP}}$ complex. This finding together with previous findings indicating that Arg-20 is a contact site for AA support the view that AA docks with the $\sigma^F$-AB-AB complex and does so by contacting Arg-20 on the subunit that is not in contact with $\sigma^F$. Thus, Arg-20 serves two distinct functions. Reflecting the asymmetry of the $\sigma^F$-AB-AB complex, Arg-20 on one subunit interacts with $\sigma^F$ and on the other subunit is exposed in a manner that allows it to contact AA.

AB is both an anti-$\sigma^F$ factor and a protein kinase that is responsible for phosphorylating and thereby inactivating AA (9–12). A point of uncertainty has been whether AA becomes phosphorylated during the displacement reaction and, if so, whether phosphorylation is required in order for AA to liberate $\sigma^F$ from the complex. Campbell et al. (18) demonstrated that a non-phosphorylatable mutant of AA (AA$^{\text{S55A}}$) in which the phosphorylated residue, Ser-58, was replaced with alanine, was able to cause the displacement of $\sigma^F$ from the complex with AB. This finding is consistent with the idea that displacement does not require concomitant phosphorylation. We have been able to obtain complementary evidence on this point through the creation of an amino acid substitution mutant of AB at residue Arg-105 (R105A). The AB$^{\text{R105A}}$ mutant was defective in its capacity to phosphorylate AA, as shown biochemically as well as in vivo. Nevertheless, the complex of the mutant protein with $\sigma^F$ ($\sigma^F$-AB$^{\text{R105A}}$-AB$^{\text{R105A}}$) was fully susceptible to undergoing dissociation in response to wild type AA. Moreover, cells producing AB$^{\text{R105A}}$ exhibited abnormally high levels of $\sigma^F$ activity during sporulation, a finding consistent with the idea that the mutant is defective in phosphorylating AA but not in releasing $\sigma^F$. The crystallographic structure of the AA-AB complex reveals that Arg-105 contacts the residue (Ser-59) that is immediately adjacent to the side chain (that of Ser-58) that undergoes phosphorylation in AA. Taken together, these findings are consistent with the idea that AB$^{\text{R105A}}$ is involved in the kinase reaction but is not needed for the interaction of AA with AB when $\sigma^F$ is displaced from the $\sigma^F$-AB-AB complex. Thus, two complementary lines of evidence, one based on a kinase-defective mutant of AB and the other on a non-phosphorylatable mutant of the substrate AA, indicate that the displacement reaction does not require concomitant phosphorylation of AA. Nevertheless, it is entirely possible that AA does indeed become phosphorylated in reacting with the complex, and previous evidence is consistent with this idea. The conclusion we draw is that AA need not be phosphorylated concomitantly in order for dissociation of the complex to take place. Rather, it seems likely that displacement and phosphorylation represent successive steps in the reaction of AA with the $\sigma^F$-AB-AB complex.

Finally, we comment on one additional feature of the interplay between AA and AB. The antisigma factor and the anti-antisigma factor are mutually antagonistic proteins. On the one hand, and as we have seen, AB that contains ATP in its nucleotide binding pocket is capable of phosphorylating and thereby inactivating AA. On the other hand, AA is capable of binding to an ADP-containing form of AB (13, 15, 23). As a result of the kinase reaction, AB is left with ADP in the nucleotide binding pocket. A fresh molecule of unphosphorylated AA can bind tightly to the ADP-containing form of AB to form a long lived complex. AB in the resulting AA-AB(ADP) complex is inert both as a kinase and as an anti-$\sigma^F$ factor. Formation of the AA-AB(ADP) complex is believed to contribute importantly to the activation of $\sigma^F$ by sequestering AB in a form in which it is unable to phosphorylate AA or to inhibit $\sigma^F$ (15, 24). Recent work by Masuda et al. reveals that AA undergoes a conformational distortion upon binding to ATP-containing AB. The authors hypothesize that this energetically unfavorable distortion does not occur when AA binds to the ADP-containing form of AB, thereby explaining the high stability of the AA-AB(ADP) complex.

These findings also shed new light on the role of residue Glu-104 in AB. As confirmed here and shown previously, the Glu-104 side chain is required for AB(ATP)-mediated phosphorylation of AA and in the AA-mediated displacement of $\sigma^F$ from the $\sigma^F$-AB-ATP$_2$ complex. It is not, however, required in order for AA to form the AA-AB(ADP) complex. Masuda et al. propose that the favorable electrostatic interaction of Glu-104 with AA compensates for the energetic cost of the conformational distortion required in order for AA to interact with the ATP-containing form of AB. Because no such distortion is expected to occur when AA interacts with the ADP-containing form of AB, Glu-104 is dispensable in AA-AB(ADP) complex formation. The findings underscore the intricacy of the interplay between the proteins that govern the activation of cell-specific transcription.

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Docking Model for Release of $\sigma^F$

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