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Contribution of Partner Switching and SpoIIAA Cycling to Regulation of σ^F Activity in Sporulating *Bacillus subtilis*

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σ^F, the first compartment-specific transcription factor in sporulating *Bacillus subtilis*, is negatively regulated by an anti-σ factor, SpoIIAB. SpoIIAB has an alternative binding partner, SpoIIAA. To see whether (as has been proposed) SpoIIAB's binding preference for SpoIIAA or σ^F depends on the nature of the adenine nucleotide present, we used surface plasmon resonance to measure the dissociation constants of the three complexes SpoIIAA-SpoIIAB-ADP, σ^F-SpoIIAB-ADP, and σ^F-SpoIIAB-ATP. The results suggested that SpoIIAB's choice of binding partner is unlikely to depend on the ATP/ADP ratio in the cell. The intracellular concentrations of σ^F, SpoIIAB, SpoIIAA, and SpoIIAA-phosphate (SpoIIAA-P) were measured by quantitative immunoblotting between 0 and 3 h after the beginning of sporulation (t_0 to t_3). σ^F and SpoIIAB were barely detectable at t_0, but their concentrations increased in parallel to reach maxima at about $t_{1.5}$. SpoIIAA-P increased steadily to a maximum at t_3, but nonphosphorylated SpoIIAA was detectable only from $t_{1.5}$, reached a maximum at $t_{2.5}$, and then declined. Kinetic studies of the phosphorylation of SpoIIAA catalyzed by SpoIIAB suggested that the reaction was limited by a very slow release of one of the products (SpoIIAA-P or ADP) from SpoIIAB, with a turnover of about once per 20 min. This remarkable kinetic property provides an unexpected mechanism for the regulation of σ^F. We propose that when SpoIIE (which dephosphorylates SpoIIAA-P) is active at the same time as SpoIIAB, SpoIIAA cycles repeatedly between the phosphorylated and nonphosphorylated forms. This cycling sequesters SpoIIAB in a long-lived complex and prevents it from inhibiting σ^F.

Early in the sporulation of *Bacillus subtilis*, the cell divides asymmetrically to form two compartments that remain attached to each other, a small prespore and a larger mother cell. Although the two compartments have identical genomes, they show different patterns of gene expression. The prespore gradually becomes transformed into the mature spore, which is eventually released by lysis of the mother cell. Gene expression in the two compartments depends on sigma factors that show both spatial and temporal specificity: in the prespore, σ^F is followed by σ^G, and in the mother cell, σ^E is followed by σ^K (9, 16). The first sporulation-specific sigma factor, σ^F, is crucial, since it is responsible for both the transcription of the gene for σ^G (24) and the activation of σ^E (12, 14). Although σ^F is present in the predivisional cell and in the mother cell (11, 13, 19, 21), it is inactive in both; shortly after asymmetric septation, however, it becomes activated in the prespore. Thus, both the maintenance of σ^F in an inactive form and its subsequent compartment-specific activation are essential for the success of sporulation.

The results of genetic experiments (23) suggest that σ^F is controlled by *spoIIAA* and *spoIIAB*, the first two genes in an operon whose third gene encodes σ^F itself. In the past few years, several interactions of SpoIIAB with σ^F and with SpoIIAA have been described in detail. SpoIIAB binds to and inhibits σ^F (6, 19), and this binding is reported to be dependent on ATP (1). Alternatively, SpoIIAB can interact in either of two ways with SpoIIAA. In the presence of ADP, the two proteins form a complex (1) that contains ADP (17); but in the presence of ATP, SpoIIAB catalyzes the transfer of phosphate to serine-58 of the SpoIIAA molecule (19, 20). The resulting SpoIIAA-P differs in conformation from SpoIIAA and cannot bind to SpoIIAB, even when ADP is present (17).

Further genetic experiments proved that σ^F is also regulated by *spoIIE* (18), a gene recently shown to encode a protein phosphatase that hydrolyzes SpoIIAA-P (7). Although the SpoIIE molecule has an additional function (4, 10), genetic dissection has shown that it is the protein phosphatase activity that is specifically required to activate σ^F (10).

Two (compatible) mechanisms have been suggested to account for the regulation of σ^F by means of these various interactions. In the first, proposed by Alper et al. (1) and endorsed by Diederich et al. (5), it was suggested that regulation depended on the concentrations of ATP and ADP within the cell (or compartment). With a high ATP concentration or a low ADP concentration (or both), SpoIIAB bound to and inhibited σ^F; however, with a low ATP concentration or a high ADP concentration (or both), SpoIIAB switched partners, binding instead to SpoIIAA and so liberating σ^F. This mechanism, called the adenosine-nucleotide switch by Alper et al. (1), thus emphasizes the noncovalent interactions among the three SpoIIA proteins.

The second proposed mechanism emphasizes the covalent change in SpoIIAA that is brought about when SpoIIAB catalyzes its phosphorylation by ATP. That this phosphorylation is essential for regulation of σ^F activity is known from the experiments of Diederich et al. (5), who showed that regulation was profoundly altered by replacing the serine residue at the phosphorylation site of SpoIIAA by alanine on the one hand or aspartic acid on the other. Equally, it is known that σ^F activity is dependent on the hydrolysis of SpoIIAA-P by the SpoIIE phosphatase (7) and is correlated with the accumulation of the resulting SpoIIAA (3, 10).

In the experiments reported in the present paper, we have sought to estimate the quantitative contribution of these different mechanisms to the regulation of σ^F activity. To study the

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equilibrium position of the SpoIIAA-SpoIIAB complex and the σ^F-SpoIIAB complex, we decided to determine (i) the dissociation constants for each of these and (ii) the intracellular concentrations of the three proteins in samples taken at various times during sporulation. We have also examined the reaction in which SpoIIAB catalyzes the phosphorylation of SpoIIAA. This reaction turns out to have unusual kinetic features, which allow us to suggest a novel mechanism for the regulation of σ^F.

MATERIALS AND METHODS

Induction of sporulation. *B. subtilis* SG38 was induced to sporulate as described previously (10). Times (hours) after resuspension in starvation medium are called t_0 and t_1, etc.

Overproduction and purification of proteins. SpoIIAA, SpoIIAA-P, and SpoIIAB were overproduced and purified as described by Magnin et al. (17). For overproduction of σ^F, the *Escherichia coli* clone (19) grown at 30°C was induced with 1 mM isopropyl-β-D-thiogalactopyranoside when the A_{600} reached 0.5 and was harvested after 3 h. The cells from 1 liter were resuspended in 50 ml of 100 mM Tris-HCl (pH 8.0), containing 10 mM EDTA, 5 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride, and disrupted in a precooled French press. The homogenate was centrifuged for 1 h at 15,000 × *g*, and the supernatant was loaded onto a 30-ml DEAE-Sepharose column equilibrated in 20 mM Tris-HCl (pH 8.3) containing 1 mM DTT. Proteins were eluted with a linear gradient (0 to 1 M) of NaCl in the same buffer. The peak containing σ^F (which was eluted at 0.25 M NaCl) was mixed with an equal volume of 2× loading buffer (50 mM Tris-HCl [pH 7.3], 2 mM DTT, 5 mM ATP, 5 mM $MgSO_4$) and loaded onto a column containing immobilized SpoIIAB, which had been prepared by binding 8 mg of purified SpoIIAB to 800 mg of cyanogen bromide-activated Sepharose 4B as described in the manufacturer's instructions. The flowthrough was passed through the column again, and the column was then washed with 30 ml of 25 mM Tris-HCl (pH 7.3) containing 1 mM DTT, 1 mM ATP, and 1 mM $MgSO_4$. σ^F was eluted with 50 mM Tris-HCl (pH 7.3) containing 1 mM DTT and 0.6 M NaCl and was shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to be at least 95% pure. It was dialyzed against 50 mM Tris-HCl (pH 7.5) containing 1 mM DTT and 50 mM NaCl, an equal volume of glycerol was added, and the preparation was kept at −20°C.

Kinetic and equilibrium constants of the protein-protein interactions. Kinetic and equilibrium constants of the protein-protein interactions were measured by surface plasmon resonance (SPR) with a BIAcore 2000 (Pharmacia) instrument. To prepare the sensor chip, a 100-μl portion of a stock solution of purified SpoIIAB (800 μg ml^{-1}) was dialyzed into phosphate-buffered saline containing 1 mM DTT at 4°C. SpoIIAB was immobilized to one flow cell of a Sensorchip CM5 (Pharmacia) by the amine coupling method described by the manufacturers, with HBS buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.4], 150 mM NaCl, 0.0005% Surfactant P 20; Pharmacia) as the running buffer. A sample of the SpoIIAB solution was diluted fivefold in 10 mM Na acetate (pH 4.0), and 70 μl of this mixture was applied to the flow cell (yielding about 1,500 resonance units of SpoIIAB). Binding experiments were always carried out within 12 h of the immobilization of SpoIIAB.

SpoIIAA, SpoIIAAS58A, and σ^F preparations were dialyzed into binding buffer (100 mM Tris-HCl [pH 7.4], 5 mM $MgCl_2$, 0.5 mM DTT) at 4°C. SpoIIAA and SpoIIAAS58A interactions were measured with 20-μl injections at a flow rate of 20 μl min^{-1}. Samples of the analyte proteins were diluted into binding buffer supplemented with 1 mM ADP (or ATP) to give protein concentrations ranging from 0.25 to 8 μM; the running buffer had the same constitution as the dilution buffer. σ^F interactions were performed with 40-μl injections at a flow rate of 20 μl min^{-1}, with the dilution and running buffer mentioned above and with the protein analyte concentration ranging from 1.0 to 6.6 μM. After injections, undissociated analyte was removed by regenerating the Sensorship surface with a 50-μl injection of binding buffer containing 0.5 M NaCl at a flow rate of 10 μl min^{-1}. For interactions with σ^F, a second regeneration step was sometimes needed. In control experiments, similar injected analytes were passed over a neighboring flow cell lacking immobilized SpoIIAB; any changes in SPR were subtracted from the sensorgrams obtained with the experimental analyte proteins. Moreover, no specific interactions were observed when analyte proteins were injected over a flow cell with immobilized SpoIIAB in the absence of ADP or ATP. Apparent kinetic constants (k_{on} and k_{off}) were obtained by use of the BIAevaluation software (Pharmacia).

SDS-PAGE and nondenaturing PAGE. SDS-PAGE and nondenaturing PAGE were performed as described by Diederich et al. (5). In our hands, σ^F runs as a doublet on nondenaturing gels.

Intracellular protein concentrations. Assays of σ^F, SpoIIAB, and total SpoIIAA were made by immunoblotting with purified antibodies as described previously (10), after the proteins from cell extracts had been separated by SDS-PAGE. Each blot included known volumes of standard solutions of purified proteins, and the quantities of SpoIIA proteins in the unknown samples were determined by densitometric analysis of the unknowns and of a set of known samples that covered the required range. To assay SpoIIAA and SpoIIAA-P separately, the cells were sonicated in isoelectric-focusing sonication buffer (22); extracts were then applied to prerun 5-ml isoelectric-focusing slab gels as described by Pollard (22) (but containing 5% acrylamide), which were run at 150 V at room temperature for 18 h. The gels were equilibrated at room temperature for 10 min in 20% methanol–20 mM Tris–192 mM glycine (pH 8.3), and the proteins were transferred electrophoretically to nitrocellulose filters and subjected to immunoblotting with anti-SpoIIAA antibody.

Assay for phosphorylation of SpoIIAA. Phosphorylation was carried out at 30°C in a 500-μl reaction volume containing 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 750 μM $MgCl_2$, 75 μM SpoIIAA, and 250 μM ATP, including 100 μCi of [γ-^{32}P]ATP (4,500 Ci $mmol^{-1}$). The reaction was started by the addition of SpoIIAB (0.32 or 0.64 μM final concentration). Samples (10 μl) were taken every 30 s and precipitated with 20% trichloroacetic acid containing 1 mg of bovine serum albumin ml^{-1} and 1 mM ATP. The precipitates were collected on cellulose nitrate filters which had been presoaked in wash solution (10% trichloroacetic acid, 0.25 mM ATP), washed with 40 ml of wash solution, and transferred into counting vials, and 2 ml of scintillation liquid (OptiScint; LKB) was added to each vial. Radioactivity was determined with a Beckman LS 5000 TD scintillation counter; a time-zero blank was subtracted from each value.

RESULTS

Kinetic and equilibrium constants of the protein-protein interactions. We examined the kinetic characteristics of the interactions between SpoIIAB and its different partners by SPR. This technique requires the immobilization of one of the reacting proteins (the ligand) on the surface of a dextran layer attached to a thin film of gold. The flow of an interacting protein (the analyte) over the ligand leads to a detectable optical change at the surface of the gold film, from which the kinetics of binding of the two proteins can be determined. When the analyte ceases to flow, the dissociation of the protein-protein complex leads to a reversal of the optical change, and the kinetics of dissociation can likewise be determined. By dividing (in the usual way) the kinetic constant for the dissociation by the kinetic constant for the association, one can determine the dissociation constant, K_d, for the protein-protein complex. It is this dissociation constant that is of particular significance in a study of partner switching.

We decided first to compare the results from SPR, a relatively new technique, with those we had previously obtained by more conventional means. It is known that SpoIIAB can interact to form a complex with a mutant SpoIIAA protein (SpoIIAAS58A) in which the serine residue at the site of phosphorylation has been replaced by alanine (5). We recently showed with the help of nondenaturing PAGE that this interaction is quite weak in the presence of ADP but stronger in the presence of ATP (15). To compare these results with those from SPR experiments, we used SpoIIAB as the ligand and analyzed its interaction with SpoIIAAS58A in the presence of ADP or ATP. SPR confirmed the results obtained with nondenaturing PAGE, i.e., that the protein-protein complex was more stable in the presence of ATP than in the presence of ADP, and further showed that this difference was largely due to a higher rate of dissociation of the SpoIIAAS58A-SpoIIAB complex in the presence of ADP (Table 1).

We then determined the reproducibility of the SPR analyses with respect to different batches of purified proteins. A comparison of the data of rows 3 and 4 of Table 1 shows that the use of different preparations of SpoIIAB with a single preparation of SpoIIAA yielded K_d values that differed by a factor of about 1.5. A roughly similar factor separated K_d values obtained when SpoIIAA was the analyte and SpoIIAB was the ligand from those obtained from the reciprocal experiment (results not shown). We concluded that it would be unwise to put much weight on an apparent difference in K_d values of twofold or less.

We had expected the σ^F-SpoIIAB complex to be much more stable (that is, to have a much lower K_d value) in the presence of ATP than in the presence of ADP, as suggested previously

TABLE 1. Analysis of binding of SpoIIA proteins to purified SpoIIAB[a]

Analyte	Nucleotide	k_{on} (10^5 M^{-1} s^{-1})		k_{off} (10^{-3} s^{-1})		K_d (nM)
		Range	Mean	Range	Mean	
SpoIIAAS58A	ADP	1.1–3.9 (5)[b]	1.7	250–490 (5)	380	2,230
	ATP	2.0–2.6 (5)	2.3	83–107 (5)	97	430
SpoIIAA	ADP	2.5–4.1 (5)	3.3	12–12 (5)	12	36
	ADP	2.1–4.2 (5)	3.3	14–19 (5)	17	51
σ^F	ADP	1.6–3.7 (3)	2.4	5.6–10 (3)	7.3	31
	ATP	2.3–6.0 (4)	4.0	4.9–6.1 (4)	5.5	14

[a] A single SpoIIAB preparation was used as the ligand for the data presented in rows 1 to 3, and a second SpoIIAB preparation was used for those presented in rows 4 to 6.

[b] The values in parentheses indicate the numbers of observations made.

(1, 5). In fact, the two nucleotides gave a difference in K_d values of little more than twofold (Table 1, rows 5 and 6), with the rate constants for the dissociation of the σ^F-SpoIIAB-ATP complex and the σ^F-SpoIIAB-ADP complex being almost indistinguishable. Moreover, when ADP was used as the nucleotide to promote the formation of the protein-protein complexes, the K_d for SpoIIAA-SpoIIAB was, if anything, higher than that for σ^F-SpoIIAB (compare the data of rows 4 and 5 of Table 1). When we repeated these experiments with ADP that had been treated with ATPase to remove any traces of contaminating ATP, the results (not shown) were not significantly different from those in Table 1.

Duncan et al. (8) have suggested that SpoIIAA can interact with and help to dissociate the σ^F-SpoIIAB-ATP complex. We found that the injection of SpoIIAA (10 μM) during the phase of dissociation of the complex increased the rate of dissociation some two- to threefold, whereas the injection of SpoIIAA-P was without effect (results not shown).

Visualizing protein-protein complexes by nondenaturing PAGE. To confirm that σ^F and SpoIIAB form a stable complex in the presence of ADP, we incubated the two proteins with 50 μM ADP and subjected the mixture to nondenaturing PAGE, without added nucleotide in the running buffer. For comparison, we used mixtures of σ^F and SpoIIAB with ATP and of SpoIIAA and SpoIIAB with ADP and ran these mixtures on the same gel. Figure 1 shows that the SpoIIAA-SpoIIAB-ADP complex dissociated to a substantial extent during the electrophoresis, leaving a smear between the running positions of the two proteins. Dissociation was less evident with the σ^F-SpoIIAB-ADP complex and still less with the σ^F-SpoIIAB-ATP complex. Thus, these results confirm in qualitative terms those shown in Table 1, rows 3 to 6.

Intracellular concentrations of the *spoIIA* proteins. The results reported to date do not speak in favor of a mechanism by which SpoIIAB's switch of partners between SpoIIAA and σ^F depends on changes in the concentrations of ATP or ADP. However, irrespective of the nucleotide concentrations, the equilibrium positions in the cell of the interactions between SpoIIAB and its partners will depend critically on the relative intracellular concentrations of the interacting proteins. Accordingly, we took samples of sporulating *B. subtilis* at various times between the beginning of sporulation (t_0) and 3 h later (t_3) and subjected them to quantitative immunoblotting with antibodies purified from antisera raised against SpoIIAA, SpoIIAB, and σ^F. In addition, we used isoelectric focusing to separate SpoIIAA from SpoIIAA-P in the cell extracts and measured the concentrations of the nonphosphorylated and phosphorylated forms individually.

The results of these experiments showed that SpoIIAB and σ^F were barely detectable at t_0. As sporulation proceeded, the intracellular concentration of both proteins rose rapidly, to reach 7 to 8 μM after 1.5 to 2.0 h; thereafter, they both began to decline slowly (Fig. 2a). The concentration of SpoIIAB monomer was always (within experimental error) equal to that of σ^F. (In fact, SpoIIAB is dimeric, but its complex with σ^F has the stoichiometry $\sigma^F{}_2 \cdot SpoIIAB_2$ [1, 6].) Total SpoIIAA (that is, the phosphorylated and nonphosphorylated forms together) followed a different pattern, showing a more gradual increase in concentration between t_1 and t_3. By separating SpoIIAA-P from SpoIIAA, we showed that the accumulation of the latter lagged behind that of the former: at t_1, only SpoIIAA-P was detectable, but by $t_{1.5}$, the nonphosphorylated form of SpoIIAA was readily seen, and thereafter, the intracellular concentrations of the two forms increased in parallel until $t_{2.5}$ (Fig. 2b).

Kinetic properties of the phosphorylation of SpoIIAA. In the presence of ATP, SpoIIAB catalyzes the transfer of phosphate to the serine-58 residue of the SpoIIAA molecule (5, 19, 20). To study the kinetics of this reaction, we incubated SpoIIAB with excess SpoIIAA in the presence of [γ-^{32}P]ATP and measured the formation of SpoIIAA-P. Figure 3 shows that phosphorylation of SpoIIAA appeared to follow a biphasic curve. Initially, the turnover number (moles of SpoIIAA phosphorylated per mole of SpoIIAB per second) was 2.3×10^{-3} to 2.5×10^{-3} s^{-1}, but after a short time, it fell abruptly to a steady-state value of 0.6×10^{-3} to 0.8×10^{-3} s^{-1}, corresponding to a turnover of once in about 20 min. By repeating the experiment with different concentrations of SpoIIAB, we showed that the intercept of the steady-state rate on the *y* axis corresponded to 0.8 to 1.0 mol of SpoIIAA phosphorylated per mol of $SpoIIAB_2$. The significance of this finding is discussed below.

DISCUSSION

Using a different technique (high-performance liquid chromatography gel permeation), we recently found the K_d value for the SpoIIAA-SpoIIAB complex formed in the presence of ADP to be about 13 μM (17). This figure, it is now clear, was much too high; the erroneous value seems to have been due to insensitivity of the detector that measures protein concentra-

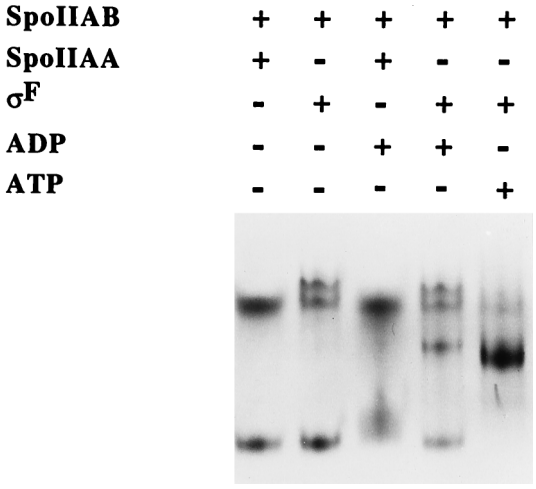

FIG. 1. Effect of adenine nucleotides on the stability of the complexes between SpoIIAB and its binding partners. The nondenaturing gel shown was subjected to electrophoresis in a running buffer lacking nucleotide. The reaction mixtures (30 μl) all contained SpoIIAB (2.5 μM) with (as indicated) various combinations of SpoIIAA (2.5 μM), σ^F (2.5 μM), ADP (50 μM), and ATP (50 μM).

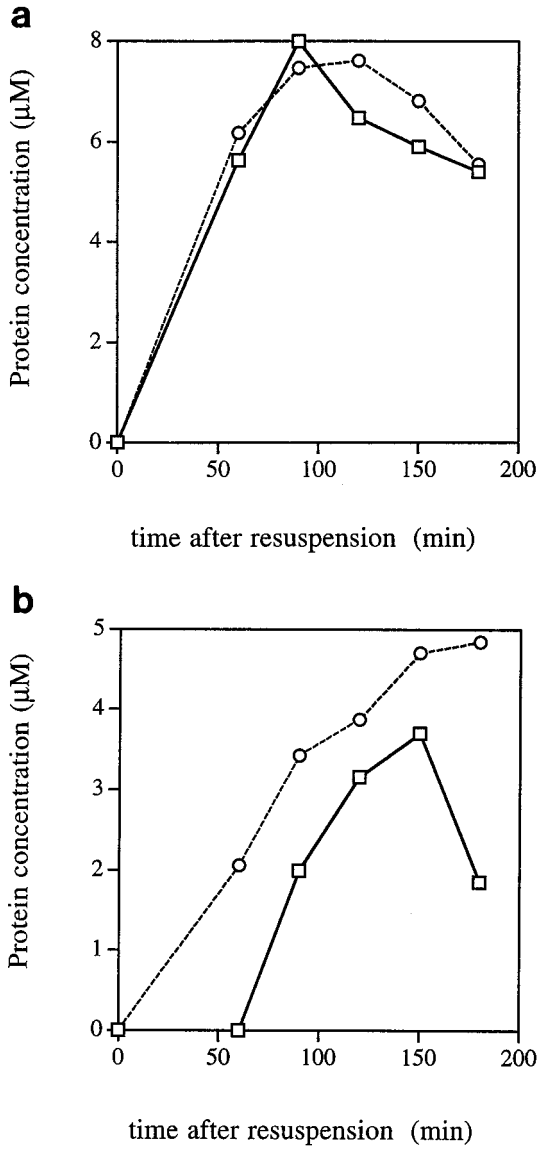

FIG. 2. Changes in intracellular concentration of SpoIIA proteins during the first 3 h of sporulation. Samples were collected and assayed as described in Materials and Methods. The total intracellular volume of the sporulating culture was estimated by making viable cell counts at the beginning of sporulation and assuming the volume of each cell to be 1.8×10^{-15} liter; any inaccuracy in this estimation will, of course, affect all four protein concentrations equally. (a) SpoIIAB (squares) and σ^F (circles); (b) nonphosphorylated SpoIIAA (squares) and phosphorylated SpoIIAA (circles).

tion in the eluate from the high-performance liquid chromatography column. The value for the K_d of the SpoIIAA-SpoIIAB complex (about 40 to 50 nM) that we now obtain by SPR (Table 1) is in good agreement with that estimated by Duncan et al. (8) (less than 120 nM). Similarly, our value of 14 nM for the K_d of the σ^F-SpoIIAB interaction in the presence of ATP agrees well with that (less than 70 nM) reported by Duncan et al. (8).

The results reported in Table 1 are in conflict with the idea that SpoIIAB's choice of protein partner for a noncovalent complex depends simply on the adenine nucleotide present, with ADP promoting the formation of a SpoIIAA-SpoIIAB complex and ATP promoting the formation of a σ^F-SpoIIAB complex. The fact that the K_d for the σ^F-SpoIIAB interaction in the presence of ATP is significantly lower than that for the SpoIIAA-SpoIIAB interaction in the presence of ADP suggests that the default partner for SpoIIAB is σ^F, and this comparison implies that (if the choice of partner were regulated by the ATP/ADP ratio) a considerable excess of ADP over ATP would be needed to cause SpoIIAB to switch its preference to SpoIIAA. However, in the presence of ADP, the σ^F-SpoIIAB complex is at least as stable as the SpoIIAA-SpoIIAB complex, so that even if an extremely low ATP/ADP ratio were achieved (which on metabolic grounds is hardly conceivable), the σ^F-SpoIIAB complex would not be disfavored. (We have previously shown that SpoIIAB has approximately equal affinities for ATP and ADP [15].) Another argument against the view that the SpoIIAA-SpoIIAB interaction is favored specifically by ADP is the fact (mentioned above) that the interaction between SpoIIAB and the nonphosphorylatable mutant protein SpoIIAAS58A is promoted much more strongly by ATP than by ADP (15) (Table 1). Taken together, the results suggest that there is no clear-cut distinction between ADP and ATP in terms of their effects on SpoIIAB's partner preference, except that ATP cannot be used to promote a reversible interaction between SpoIIAB and the wild-type SpoIIAA because SpoIIAA would then be phosphorylated to SpoIIAA-P (see reference 15).

Thus, the results of the experiments with SPR do not favor the view that noncovalent interactions dependent on the ATP/ADP ratio determine the choice of binding partner for SpoIIAB. Instead, they support the view that it is the ability of SpoIIAA to be phosphorylated (5) and dephosphorylated (3, 7, 10) that is important for normal control of σ^F. How does this control through phosphorylation and dephosphorylation work?

We believe that the key to this question is to be found in the unusual kinetic properties of the enzymatic reaction in which SpoIIAA is phosphorylated (Fig. 3). When SpoIIAA, SpoIIAB, and ATP were incubated together, phosphorylation was at first relatively rapid; however, after one SpoIIAA molecule had been phosphorylated per SpoIIAB dimer, the rate of reaction suddenly decreased. This result suggests that the rate-limiting step in the reaction is not the binding of the two proteins or the transfer of the phosphate group to SpoIIAA, but some later event. One possibility is the slow dissociation of a SpoIIAB–SpoIIAA-P complex. Another possibility (perhaps

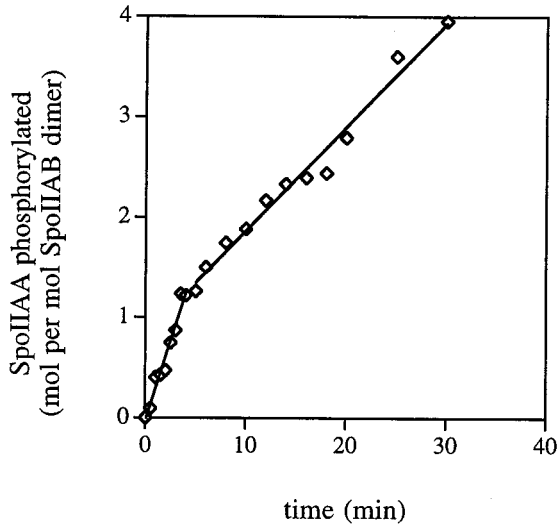

FIG. 3. Time course of phosphorylation of SpoIIAA. The assay was as described in Materials and Methods.

more likely) is that a SpoIIAB-ADP complex, liberated after the phosphorylation of SpoIIAA, exchanges only slowly with ATP to form the catalytically active SpoIIAB-ATP. (Experiments now in progress in this laboratory are designed to distinguish between these two cases.) Although we shall discuss these two possibilities separately, it turns out in either event that the existence of such a rate-limiting step has important consequences for SpoIIAB's ability to choose between SpoIIAA and σ^F as its binding partner.

Assuming first that the rate-limiting step is the dissociation of a SpoIIAB–SpoIIAA-P complex, imagine an equimolar mixture of SpoIIAA, SpoIIAB, and σ^F in the presence of excess ATP, with changes in protein-protein interaction being measured by assaying the activity of σ^F. (For the present purpose and the remainder of the Discussion, we consider the concentration of each protein [including SpoIIAB] in terms of its monomer, since a SpoIIAB dimer can bind either two monomers of SpoIIAA [17] or two monomers of σ^F [1, 6].) Initially, each molecule of SpoIIAB will be free to interact with either SpoIIAA or σ^F. But the fates of these two complexes are different. Every molecule of SpoIIAB that binds to σ^F will dissociate spontaneously in about 3 min (Table 1) or, if the σ^F-SpoIIAB complex interacts with SpoIIAA, in about 1 min (see above and reference 8). But every molecule of SpoIIAB that binds to and phosphorylates SpoIIAA will remain in a complex with SpoIIAA-P for some 20 min (Fig. 3). After the three proteins have interacted for a few minutes, most of the SpoIIAB will be trapped in this complex, and consequently, most of the σ^F in the hypothetical mixture will be active. As the SpoIIAB–SpoIIAA-P complex gradually dissociates, the SpoIIAB that is released will be able to bind to and inhibit σ^F, with the result that the activity of σ^F will slowly fall. Since the affinity of SpoIIAB for SpoIIAA-P is very low (17) and that for σ^F is very high (Table 1), by the time the phosphorylation of SpoIIAA is complete and all of the SpoIIAB–SpoIIAA-P complex has dissociated, the activity of σ^F will fall essentially to zero.

Next, assume the same hypothetical mixture but with the phosphorylation of SpoIIAA limited by a slow exchange of nucleotides between SpoIIAB-ADP and ATP. Again, every molecule of SpoIIAB that binds to σ^F will dissociate in 3 min or less. But every molecule of SpoIIAB that phosphorylates SpoIIAA will be liberated from the enzymatic reaction as a long-lived SpoIIAB-ADP complex. As the affinity of SpoIIAB-ADP for SpoIIAA is only slightly less than that for σ^F (see Table 1), a substantial fraction (e.g., one-third) of these complexes will bind to SpoIIAA, and a corresponding number of σ^F molecules will be active. As soon as the ADP in each SpoIIAB-ADP complex is exchanged for ATP, SpoIIAA will be phosphorylated and once again a long-lived SpoIIAB-ADP complex will be formed. Thus, in this case, too, after the three proteins have interacted for a few minutes, a good part of the SpoIIAB in the mixture will be in complexes with SpoIIAA and therefore unavailable to inhibit σ^F. Gradually, however, the SpoIIAA in the mixture will become phosphorylated, and in the end, all of the SpoIIAB will bind to σ^F.

If at this point SpoIIE phosphatase is introduced into the hypothetical mixture, some of the accumulated SpoIIAA-P will be hydrolyzed back to SpoIIAA. However, any molecule of SpoIIAA that is generated in this way can immediately be attacked by a molecule of SpoIIAB liberated from the σ^F-SpoIIAB complex, phosphorylated, and released as SpoIIAA-P. As long as SpoIIE and SpoIIAB are both active, SpoIIAA will cycle between the phosphorylated and nonphosphorylated forms. The concentration of SpoIIAA that is cycling in this way will be determined by the relative activities of SpoIIE and SpoIIAB, but the higher that concentration is, the more SpoIIAB will be sequestered in a long-lived complex and the less will be available to inhibit σ^F.

With this idea in mind, we can discuss our measurements of the intracellular concentrations of the SpoIIA proteins. Figure 2a shows that at all times between t_0 and t_3, the concentrations of SpoIIAB and σ^F are roughly equal. In other words, there is just enough SpoIIAB to inhibit σ^F provided that none of the SpoIIAB is involved in a long-lived complex with SpoIIAA-P or with ADP; conversely, every molecule of SpoIIAB that takes part in forming such a complex will do so at the expense of its ability to inhibit σ^F. Phosphorylated SpoIIAA cannot bind to SpoIIAB (17), and therefore its concentration is not directly relevant to the activity of σ^F. However, any nonphosphorylated SpoIIAA that is present is in direct competition with σ^F for binding to SpoIIAB; hence (if our model is correct), the activity of σ^F should be related to the steady-state concentration of nonphosphorylated SpoIIAA. This model agrees with the suggestion made by Duncan et al. (8), on the basis of quite different experiments, that the activity of σ^F is regulated primarily by the concentration of nonphosphorylated SpoIIAA (see also references 3 and 10). In practice, our experimental measurements of the concentration of nonphosphorylated SpoIIAA at different times during sporulation accord well with the known time course of σ^F activity (21). Figure 2b shows that nonphosphorylated SpoIIAA is barely detectable in the predivisional cell, but it becomes readily measurable by 90 min after the induction of sporulation and increases in concentration for an hour thereafter. Broadly similar conclusions have been derived by Lewis et al. (13) from the results of immunofluorescence experiments.

We therefore propose that the activation of σ^F requires both SpoIIAB and SpoIIE to be active simultaneously. In the predivisional cell, there are only small quantities of the SpoIIA proteins (11, 19), but what SpoIIAA is present is phosphorylated (see reference 10) (Fig. 2b), suggesting that SpoIIAB is active but SpoIIE is not. At this point, SpoIIAB has no binding partner other than σ^F, and consequently, all the σ^F in the cell will be inhibited. SpoIIE, which is located in the sporulation septum (2), starts to hydrolyze SpoIIAA-P shortly after asymmetric septation (3, 10), and there is no reason to suppose that SpoIIAB is inactivated at this time. We therefore assume that soon after the formation of the asymmetric septum, both enzymes will be active. This simultaneous activity leads to cycling of SpoIIAA, as discussed above, hence to the sequestration of SpoIIAB in a long-lived complex, and hence to the liberation of σ^F.

Our model does not bear directly on the question of the asymmetric activation of σ^F (i.e., why its activity is liberated in the prespore and not the mother cell). Duncan et al. (7) have suggested a means by which the activity of SpoIIE can be confined to the prespore; assuming that this (or some other model for limiting the activity of SpoIIE in space) is correct, the cycling of SpoIIAA and the consequent activation of σ^F will take place only in the prespore. One of the consequences would be that the concentration of SpoIIAA in the prespore (which occupies only one-fifth or less of the total volume of the sporulating cell) will be severalfold higher than that shown in Fig. 2b. Such a concentration would be sufficient, 2 h after the beginning of sporulation, to sequester all of the SpoIIAB and liberate all of the σ^F in the prespore.

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ADDENDUM

Recent experiments in this laboratory (19a) have now demonstrated that the long-lived species referred to in the Discussion is a SpoIIAB-ADP complex and that it is the slow dissociation of this complex that is the rate-limiting step in the phosphorylation of SpoIIAA catalyzed by SpoIIAB.

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