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Purification, Kinetic Properties, and Intracellular Concentration of SpoIIE, an Integral Membrane Protein That Regulates Sporulation in *Bacillus subtilis*

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SpoIIE is a bifunctional protein which controls σ^F activation and formation of the asymmetric septum in sporulating *Bacillus subtilis*. The *spoIIE* gene of *B. subtilis* has now been overexpressed in *Escherichia coli*, and SpoIIE has been purified by anion-exchange chromatography and affinity chromatography. Kinetic studies showed that the rate of dephosphorylation of SpoIIAA-P by purified SpoIIE in vitro was 100 times greater, on a molar basis, than the rate of phosphorylation of SpoIIAA by SpoIIAB. The intracellular concentrations of SpoIIE and SpoIIAB were measured by quantitative immunoblotting between 0 and 4 h after the beginning of sporulation. The facts that these concentrations were very similar at hour 2 and that SpoIIE could be readily detected before asymmetric septation suggest that SpoIIE activity may be strongly regulated.

The successful completion of sporulation in the gram-positive bacterium *B. subtilis* depends on the activation of the first sporulation-specific transcription factor, σ^F. The activation of σ^F in the prespore, soon after asymmetric septation, not only induces the prespore-specific program of gene expression (13) but also mediates an intracellular signal across the septum which activates the first mother cell-specific transcription factor, σ^E (27).

σ^F, together with the two regulatory proteins SpoIIAA and SpoIIAB, is synthesized before asymmetric septation (for a review, see references 14 and 27). In the predivisional cell, the anti-σ factor SpoIIAB holds σ^F in an inactive complex (2, 10, 21) and also maintains the anti-anti-σ factor SpoIIAA in an inactive form by phosphorylating it on a specific serine residue (2, 9, 18, 21, 23). After asymmetric septation, activation of σ^F in the prespore is triggered by the action of the specific phosphatase SpoIIE, which catalyzes the dephosphorylation of SpoIIAA-P (11). When dephosphorylated, SpoIIAA releases σ^F activity by binding to and sequestering the anti-σ factor SpoIIAB (4, 11, 12, 15, 19, 25).

SpoIIE is a membrane-bound protein containing, in its N-terminal region, 10 membrane-spanning segments inserted into the asymmetric septum that separates the prespore from the mother cell (3, 6) and, in its C-terminal region, a large cytoplasmic domain which includes the phosphatase activity (11). The phosphatase domain of SpoIIE has some sequence similarity to the PP2C family of eukaryotic Ser/Thr protein phosphatases (1), especially in the metal binding residues, suggesting a conserved metal binding site and a common mechanism of phosphate recognition by the metal ions (8). The fact that the serine phosphatase activity is fused to a membrane domain may help to ensure a specific orientation of the protein, which provides activation of σ^F only in the prespore (3, 11). Indeed, recent immunofluorescence studies provide evidence that SpoIIE may be localized exclusively to the prespore face of the septum (16, 29). In addition, SpoIIE is required for the proper formation of the asymmetric septum itself (7, 15). It has been suggested that SpoIIE acts to couple asymmetric septation to σ^F activity (11, 15).

Given the apparent importance of SpoIIE in the establishment of σ^F activity and thus of differential gene expression in sporulation, we have been studying the mechanism by which SpoIIE activity is itself regulated. In the present paper, we describe the purification of the full-length protein SpoIIE to apparent homogeneity and some characteristics of the reaction in which SpoIIE catalyzes the dephosphorylation of SpoIIAA-P. We have also determined the intracellular concentration of the enzyme in samples taken at various times during sporulation.

MATERIALS AND METHODS

Cloning. The wild-type *spoIIE* gene was amplified by PCR (GeneAmp XL PCR kit; Perkin-Elmer) following the protocol given by the supplier. The oligonucleotides 5′-CAGGTGGGAGATGAGACATATGGAAAAAGC-3′, generating an *Nde*I restriction site (underlined) covering the start codon (ATG) of the *spoIIE* gene, and 5′-GCGGATCCCATATATTCCCATCTTCGCCAGAAG-3′, generating a *Bam*HI restriction site (underlined) at the 3′ end of the *spoIIE* gene, were used to amplify the complete *spoIIE* gene from chromosomal DNA of *B. subtilis* SG38. The sequence of the 2,568-bp product was confirmed by means of the DNA sequencing kit, with Amplitaq DNA polymerase (Perkin-Elmer) and an ABI1373A automated sequencer (Applied Biosystems). The gene was cloned into *Nde*I/*Bam*HI-digested pET11a (Novagen). The ATG start codon of the *spoIIE* gene was located 8 nucleotides downstream of the highly efficient Shine-Dalgarno sequence of the phage T7 major capsid protein encoded by the vector plasmid. The *Escherichia coli* strain C41(DE3) was transformed with the recombinant plasmid pRB1011.

Overexpression of *spoIIE* and membrane isolation. To stimulate overproduction of SpoIIE, the *E. coli* clone containing pRB1011 was inoculated into 10 ml of 2YT medium containing ampicillin (100 μg/ml) and grown at 30°C for 1 to 2 h. This culture was used to inoculate a 3-liter volume of the same medium, which was induced with 0.7 mM IPTG (isopropyl-β-D-thiogalactopyranoside) when the A_{600} reached 0.6. The cells were harvested by centrifugation at 4°C after overnight growth for 15 h at room temperature. The cells from a 3-liter volume were resuspended in 50 ml of 100 mM Tris-HCl (pH 7.5) containing 5 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10% glycerol, and 0.1% Triton X-100 and then disrupted in a precooled French press. The homogenate was centrifuged for 10 min at 3,000 × *g* to remove the cell debris, and the membrane fraction was recovered by centrifugation for 4 h at 40,000 × *g*. To remove peripheral membrane proteins, the membrane pellet was resuspended in the same buffer supplemented with 3 M KCl and was recentrifuged for 4 h. The washed membranes were suspended in a solution containing 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mM PMSF, 10% glycerol, and 8% sucrose and stored at −70°C.

Purification. Proteins were extracted by treatment of the membrane fraction with a solution containing 5% Triton X-100, 50 mM Tris-HCl (pH 8.0), 50 mM

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NaCl, 1 mM DTT, 1 mM PMSF, and 10% glycerol for 3 h at 4°C. After centrifugation for 4 h at 40,000 × *g*, the supernatant was loaded onto a 30-ml DEAE-Sepharose column equilibrated in 50 mM Tris-HCl (pH 8.0) containing 0.5 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 10% glycerol, and 1% Triton X-100 (buffer A). Proteins were eluted with a linear gradient (0 to 0.7 M) of NaCl in the same buffer. The fractions containing SpoIIE, which eluted between 0.2 and 0.3 M NaCl, were pooled and applied to a small column (1 by 2 cm) of Affi-Gel blue (Pharmacia) equilibrated in a solution containing 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 10% glycerol, and 1% Triton X-100. The column was washed with 30 ml of the same buffer, and SpoIIE was eluted with 30 ml of a linear NaCl gradient (0.2 to 2 M). Fractions of 1.5 ml were collected. The enzymically active fractions, eluting between 0.8 and 1 M NaCl, were combined, dialyzed against a solution containing 50 mM Tris-HCl (pH 6.8), 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 10% glycerol, and 1% Triton X-100, and applied to cation-exchange SP Hi-trap (Pharmacia) which had been equilibrated in the same buffer. The column was developed at a flow rate of 1 ml/min with a 30-ml linear gradient (0 to 0.8 M NaCl). The fractions containing purified SpoIIE eluted at about 0.35 M NaCl. They were dialyzed against buffer A (pH 7.5), concentrated with the use of Centriplus concentrators (Amicon), and stored in 50% glycerol at −70°C.

Removal of Triton X-100 by Extracti-gel D. The purified enzyme was applied to a 1-ml Extracti-gel D column (Pierce) equilibrated at room temperature in a solution containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10% glycerol, 0.5 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF. Elution was performed with the same buffer. SpoIIE, which eluted in the void volume, was assayed for activity in the presence of different detergents.

Purification of the C-terminal fragment of SpoIIE. The histidine-tagged, carboxy-terminal fragment of SpoIIE was overexpressed as previously described (29) and purified from inclusion bodies by metal chelation chromatography after being solubilized with 6 M guanidine according to the Qiagen protocol. To recover the activity of the C-terminal fragment, the protein was renatured on the column (by means of a linear gradient of 6 to 0 M guanidine) over a period of 10 h, before elution with 200 mM imidazole.

Determination of protein concentration. Protein concentration was determined after electrophoresis of purified proteins and SYPRO orange staining. The gel was scanned with a FluorImager (Molecular Dynamics), and the ImageQuant software package was used to calculate the quantities of SpoIIE and C-terminal fragment in the unknown samples, with known protein molecular weight standards used for calibration.

Western blot analysis. To raise antibodies against SpoIIE, the C-terminal fragment of SpoIIE was purified from inclusion bodies by a Prep Cell (model 491; Bio-Rad) as previously described (29). The antibodies were purified by protein A Sepharose (Pharmacia), and the purified antiserum was used at a dilution of 1/2,000 for Western blot analysis.

Assay for dephosphorylation of SpoIIAA-P. SpoIIAA (50 μM final concentration) was first phosphorylated in a 1-ml reaction volume containing 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 750 μM $MgCl_2$, 100 μM ATP (including 100 μCi of [γ-^{32}P]ATP), and 5 μM SpoIIAB. After complete phosphorylation (2 h at 37°C and overnight at 4°C), SpoIIAA-P was separated from SpoIIAB on a 1-ml DEAE-Sepharose column equilibrated in a solution containing 50 mM Tris-HCl (pH 8.0) and 1 mM DTT. The fractions containing SpoIIAA-P (eluted with 200 mM NaCl) were dialyzed extensively against a solution containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 1 mM DTT to remove the [γ-^{32}P]ATP.

Dephosphorylation was carried out at 30°C in a 300-μl volume containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM DTT, 10 mM $MnCl_2$, and 5 or 15 μM [^{32}P]SpoIIAA-P. The reaction was started by the addition of either SpoIIE or the C-terminal fragment of SpoIIE (2 to 60 nM). Samples (20 μl) were taken at different times and assayed as previously described (24).

Quantification of SpoIIE and SpoIIAB during sporulation. *B. subtilis* cell extracts were prepared by incubating 1-ml cell pellets in 250 μl of 50 mM Tris-HCl (pH 7.5) containing 5 mg of lysozyme per ml, 1 mM DTT, 1 mM PMSF, and 10% glycerol for 10 min at 37°C and sonicating them briefly. These extracts were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted with purified antibodies raised either against the C-terminal fragment of SpoIIE or against SpoIIAB. Each blot included known volumes of standard solutions of purified protein. The quantities of SpoIIE and SpoIIAB in the samples were determined by the method of Lord et al. (17).

RESULTS AND DISCUSSION

Overproduction and purification of the full-length protein SpoIIE. The wild-type *spoIIE* gene from *B. subtilis* was amplified by PCR and cloned into the pET11a vector as described in Materials and Methods. *E. coli* C41(DE3) was selected for SpoIIE overproduction. This strain has been used successfully for overproduction of membrane proteins. It reproducibly yields a higher level of expression and less toxicity than BL21(DE3) (22).

To determine the conditions for maximal overexpression of *spoIIE*, a Western blot was performed on cell extracts prepared after cells were grown in various conditions and induced by IPTG. Maximal expression of *spoIIE* occurred when the cells were grown at 30°C and harvested approximately 15 h after induction (data not shown). About 75% of the total SpoIIE was membrane associated. The membrane-bound phosphatase could not be released by treatment with 3 M KCl, confirming that the enzyme is an integral membrane protein. We therefore tested various detergents {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate [CHAPS], octoglucoside, and Triton X-100} for their ability to solubilize the enzyme. Triton X-100 (5%) was found to be the most effective. To keep the protein in solution, only a 1% final concentration of Triton X-100 needed to be added to the buffers during the purification.

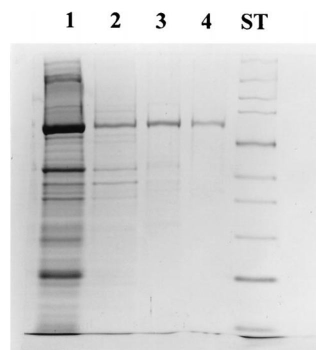

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the results of SpoIIE purification stained with Coomassie blue. The membrane extract was treated with 5% Triton X-100, and the solubilized proteins (lane 1) were applied to a DEAE-Sepharose column. The fractions containing SpoIIE were pooled (lane 2) and applied to an Affi-Gel blue column. After dialysis, the active fractions (lane 3) were loaded on a cation-exchange column. The fractions containing purified SpoIIE (lane 4) were concentrated and stored in 50% glycerol. The molecular mass standards (lane ST) are, from the top, myosin (212 kDa), MBP-β-galactosidase (158 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), glutamate dehydrogenase (55 kDa), MBP2 (42 kDa), lactate dehydrogenase M (36 kDa), triosephosphate isomerase (26 kDa), and trypsin inhibitor (20 kDa).

The solubilized membrane extract from a 3-liter culture was purified through a number of steps as described in Materials and Methods. Figure 1 shows the purity of the preparation at each stage. The final preparation was more than 95% pure as estimated from an overloaded gel stained by silver staining.

Apparent molecular weight of the purified SpoIIE. To measure the apparent molecular weight of SpoIIE, we analyzed the final enzyme preparation by gel filtration chromatography on Superose 12. The elution position of the native enzyme corresponded to an apparent M_r of 250,000 (results not shown) even when chromatography was performed in the presence of 1 M NaCl. Assuming that the micellar weight of Triton X-100 is 66,700 at 4°C (28), these results imply that the M_r of SpoIIE is about 180,000. However, the M_r of SpoIIE calculated from the gene sequence is 91,500. Since SpoIIE has a large membrane-spanning domain, the enzyme may be aggregated even in the presence of Triton X-100. Alternatively, this finding may indicate that SpoIIE is dimeric, as was recently reported for PP2C of *Plasmodium falciparum* (20).

Kinetic properties of the dephosphorylation of SpoIIAA-P. As previously described (11), SpoIIE catalyzes hydrolysis of SpoIIAA-P, a reaction dependent on either Mn^{2+} or Mg^{2+}. The reaction was completely inhibited by inorganic phosphate or by EDTA (data not shown). To study the kinetics of this reaction, we incubated SpoIIE with excess purified

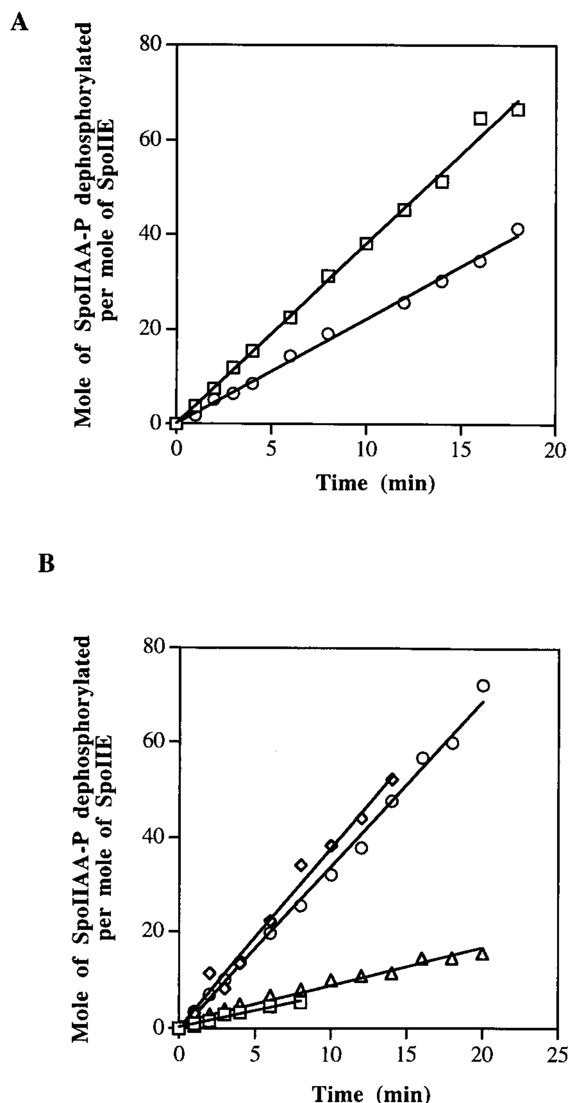

FIG. 2. (A) Time course of dephosphorylation of SpoIIAA-P by SpoIIE (squares) and by the C-terminal fragment of SpoIIE (circles). (B) Time course of dephosphorylation of SpoIIAA-P by SpoIIE in the absence of detergents (squares) and in the presence of Triton X-100 (diamonds), CHAPS (circles), and octoglucoside (triangles). The assays were as described in Materials and Methods.

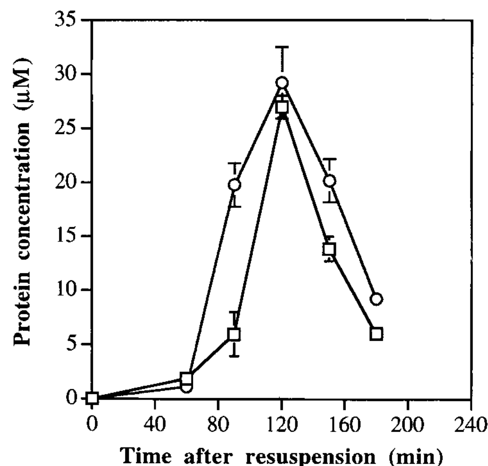

FIG. 3. Changes in intracellular concentration of SpoIIE (squares) and SpoIIAB (circles) during the first 4 h of sporulation. Samples were collected and assayed as described in Materials and Methods. Each point shown is the mean of three determinations. The error bars give the range of the three estimates at each time point; no error bars are shown where the range of three determinations is too small to fit conveniently on the figure.

[^{32}P]SpoIIAA-P and measured the amount of P_i produced. We first studied the kinetics in the presence of 1% Triton X-100. Figure 2A shows that dephosphorylation of SpoIIAA-P appeared to be linear with time. The turnover number (moles of SpoIIAA-P dephosphorylated per mole of SpoIIE) was 6×10^{-2} to 7×10^{-2} s^{-1} and was similar for all concentrations of SpoIIE from 2 to 60 nM.

We then tested the effect of various detergents on the phosphatase activity of the full-length protein, after first removing Triton X-100 with an Extracti-gel D column (see Materials and Methods) (Fig. 2B). The rate of dephosphorylation in CHAPS was similar to that found with the initial preparation in Triton X-100, but there was little activity in octoglucoside. With no detergent in the reaction mixture, the protein was still active, but at low concentrations the rate of hydrolysis of SpoIIAA-P was not proportional to the concentration of the enzyme (results not shown). The presence of either CHAPS or Triton X-100 abolished this nonlinearity of response.

The specific activity of the C-terminal fragment of SpoIIE (purified from inclusion bodies and renatured) was about 70% of that of the full-length protein (Fig. 2A). One possible interpretation of the data is that the purified full-length protein is fully active and that we recovered 70% of the activity from the C-terminal fragment by renaturation from inclusion bodies. Alternatively, it may be that all of the activity of the C-terminal fragment has been recovered but that the N terminus of the full-length protein plays a role in modulating its activity in the cell.

Intracellular concentration of the SpoIIE compared to SpoIIAB. To see whether we could find evidence for the suggestion that the phosphatase activity of SpoIIE is regulated in the cell, we determined the concentration of SpoIIE in sporulating *B. subtilis* cells and compared it with that of SpoIIAB. Samples were taken at various times during the first 4 h of sporulation and assayed by quantitative immunoblotting with purified antibodies raised against either SpoIIAB or the C-terminal fragment of SpoIIE. At 0 h of sporulation (t_0), both proteins were barely detectable. As sporulation proceeded, the intracellular concentration of both proteins increased quickly until about t_2 (Fig. 3). Thereafter, the concentration of SpoIIE declined, and the protein was hardly detectable at t_4, suggesting that it had been degraded (26). A degradation product with a molecular weight of 50,000 was detectable with anti-SpoIIE antibodies at t_4. As Fig. 3 shows, the molar ratios of SpoAB/SpoIIE were close to unity both at t_1 and at t_2. However, in the sample taken at $t_{1.5}$, the concentration of SpoIIAB exceeded that of SpoIIE by around threefold. To check whether this observation was reproducible, we took samples from another, independent sporulation experiment. In this second experiment, the molar ratios of SpoAB/SpoIIE were 1.1 at t_1, 2.8 at $t_{1.5}$, and 1.3 at t_2. We conclude that, shortly after t_1, the concentration of SpoIIAB rises much more sharply than that of SpoIIE.

SpoIIE activity may be regulated in the cell. We have previously proposed that after asymmetric septation, nonphosphorylated SpoIIAA, generated in the prespore by the activity of SpoIIE, sequesters SpoIIAB in the kinase reaction and keeps

the latter from inhibiting σ^F (19). For this mechanism to be effective, the activity of SpoIIE in hydrolyzing SpoIIAA-P to SpoIIAA would need to be no less than that of SpoIIAB in phosphorylating SpoIIAA to SpoIIAA-P, but one would not expect the activities of the two enzymes to differ enormously. In fact, however, under the conditions of the assays in vitro, the rate of dephosphorylation by SpoIIE was about 100 times higher than the rate of phosphorylation by SpoIIAB. Moreover, the time course of accumulation of nonphosphorylated SpoIIAA, previously published by Magnin et al. (19), closely follows that for the appearance of SpoIIE, which suggests that SpoIIE activity is not present in great excess. Again, the results given in Fig. 3 show that SpoIIE is detectable by immunoblotting 1 h after the initiation of sporulation, which is well before the formation of the asymmetric septum. It seems unlikely that this SpoIIE is fully active; if it were, nonphosphorylated SpoIIAA would be generated in the predivisional cell and σ^F would be liberated prematurely.

These arguments suggest that SpoIIE activity is likely to be regulated in vivo and/or that the high activity that is observed in vitro is an artifact of our having the enzyme in a soluble, rather than a membrane-bound, form. It has been suggested that in the recently discovered Spalten protein of *Dictyostelium*, the membrane-associated N-terminal domain regulates the phosphatase activity that is located in the soluble C-terminal domain (5). If SpoIIE is confined to the prespore side of the asymmetric septum of the sporulating cell (3, 6, 29), its local concentration will be substantially higher than that shown in Fig. 3. The high concentration of SpoIIE is likely to result in the rapid hydrolysis of all the SpoIIAA-P in the prespore, which will in turn ensure the rapid activation of σ^F in that compartment.

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