A Phosphotransferase Activity of the *Bacillus subtilis* Sporulation Protein Spo0F That Employs Phosphoramidate Substrates†

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ABSTRACT: Transient phosphorylation at an aspartate residue on the Spo0F protein is a central step in the phosphorelay signal transduction pathway controlling sporulation in *Bacilli*. The response regulator Spo0F∼P is stable to hydrolysis ($t_{1/2}$ > 24 h at 23 °C in the absence of Mg^{2+}), allowing the use of nondenaturing PAGE to separate the phosphorylated and non-phosphorylated forms of Spo0F. Using this novel assay, phosphoramidate containing compounds were found to specifically phosphorylate Spo0F, a reaction that requires the presence of a divalent metal, but mixed phosphate−carboxylate compounds did not act as phospho donors. Rapid hydrolysis of Spo0F∼P generated with phosphoramidate by proteins downstream in the phosphorelay (Spo0B and Spo0A) is consistent with phosphorylation at the active site of Spo0F. The initial rate of Spo0F∼P formation from phosphoramidate displays Michaelis−Menten kinetics, providing evidence for the proposal that response regulators, such as Spo0F, function as phosphoryl transferase enzymes (McCleary et al., 1993). The results establish that SpoOF functions as a phosphoryl transferase that uses exclusively a phosphoramidate rather than an acyl phosphate as substrate during autophosphorylation.

Transient phosphorylation at aspartate residues is part of a ubiquitous system used in the control of some prokaryotic and eukaryotic cellular signaling processes. Phosphorylation transfers employing acyl phosphate intermediates are characteristic of "two-component" systems. Two-component systems typically consist of an autophosphorylating histidine protein kinase and a response regulator protein, which is activated by phosphorylation at an aspartate residue. Although numerous examples of such systems have been identified in bacteria (Parkinson & Kofoid, 1992), so far only a few have been found in yeast (Maeda et al., 1994) and plant (Chang et al., 1993) species. Sporulation in *Bacillus subtilis* is controlled by an expanded version of the two-component signal transduction theme termed the phosphorelay (Hoch, 1993). In the first step of this series of phosphorylation reactions, one or more histidine protein kinases (KinA and KinB) bind ATP and autophosphorylate at a histidine residue. The phosphate on these kinases then is transferred to Spo0F, a response regulator, and Spo0F∼P serves as a substrate for the third protein, Spo0B, which ultimately transfers the phosphate to Spo0A, a response regulator acting as both a repressor and an activator of transcription. This multiprotein phosphorelay controls the levels of Spo0A∼P, the activated form of Spo0A. Sporulation may be regulated in both a positive manner by sporulation initiation signals that stimulate autophosphorylation of histidine kinases and in a negative manner by protein phosphatases that specifically dephosphorylate Spo0F (Perego et al., 1994) and Spo0A (Ohlsen et al., 1994). This phosphorelay is unique among "two-component" signal transduction systems in that phospho transfer occurs between two response regulators, Spo0F and Spo0A, through a unique phosphoryl transferase, Spo0B.

Several response regulatory proteins such as CheY (Lukat et al., 1992), NRI (Feng et al., 1992), BvgA (Boucher et al., 1994), OmpR (McCleary & Stock, 1994), and ComA (Roggiani & Dubnau, 1993) have been shown to be phosphorylated and activated by acetyl phosphate. Response regulators show a varying range of specificity for phospho donors. For example, NRI is phosphorylated by acetyl phosphate, carbamoyl phosphate, and phosphoramidate (PA)[1] (Feng et al., 1992), whereas CheB is only phosphorylated by PA (Lukat et al., 1992). Since NRI was not activated by phosphoenol pyruvate, a higher energy compound than PA, response regulator phosphorylation does not correlate exclusively with the energy level of the phospho donor. This apparent substrate specificity and activation of response regulators by phospho donors has been advanced as evidence that response regulators autophosphorylate using phospho donors as substrates. Whether these phospho donors act as phosphorylating reagents that react with accessible residues or act as substrates in an enzymatic reaction has not been fully resolved. It has been suggested that phosphorylation of response regulators by small molecules could have functional significance since this reaction could link two-component systems to the metabolic state of the cell, and indeed in the cases of NRI (Feng et al., 1992), OmpR (McCleary & Stock, 1994), and PhoB (Wanner & Wilmes-Riesenberg, 1992), acetyl phosphate has been shown to offer

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[1] Abbreviations: IPTG, isopropyl β-D-thiogalactopyranoside; PMSF, phenylmethanesulfonyl fluoride; βME, β-mercaptoethanol, EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid); PA, phosphoramidate.

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regulatory control. To examine if this observation could relate to the regulation of Spo0F, a novel method to assay for protein acyl phosphates employing nondenaturing PAGE has been developed based on the observation that Spo0F∼P has moderate stability ($t_{1/2}$ > 24 h at ambient temperature).

Using this assay, it has been shown that Spo0F can be phosphorylated by molecules containing phosphonitrogen bonds but not by molecules containing acyl phosphate bonds. Spo0F∼P produced with PA was specifically hydrolyzed when incubated with both Spo0B and Spo0A, implying that the biologically active aspartate pocket of Spo0F was specifically phosphorylated. Specific phosphorylation of Spo0F with both PA and KinA∼P required a divalent metal ion, suggesting that both phospho donors phosphorylate Spo0F by the same chemical mechanism. This report describes these properties in detail and also shows that the initial rate of Spo0F∼P formation displays saturation kinetics, a feature characteristic of an enzyme catalyzed reaction, providing evidence that Spo0F autophosphorylates using phosphoramidate substrates.

EXPERIMENTAL PROCEDURES

DEAE-Trisacryl LS was obtained from Biosepra (Marlborough, MA) and hydroxylapatite from Bio-Rad. Phosphocreatine, acetyl phosphate, and carbamoyl phosphate were purchased from Sigma. Dilithium acetyl [^{32}P]phosphate was prepared by the method of Stadtman (1957); monopotassium phosphoramidate, by the method of Sheridan *et al.* (1972), and phosphopyridine and phosphopicoline, by the method of Skoog and Jencks (1984). Phosphohistidine was prepared as a mixture of the 1- and 3-isomers by the method of Hultquist (1966). A molar absorptivity of $\epsilon_{275nm} = 7000\ M^{-1}\ cm^{-1}$ was used to determine the Spo0F concentration. The concentrations of all other proteins and unpurified Spo0F were determined using the Bradford dye binding assay with bovine serum albumin as a standard (Bradford, 1976). KinA (Perego et al., 1989), Spo0B (Burbulys et al., 1991), and Spo0A (Grimsley et al., 1994) were isolated by standard procedures. CheY was a gift from Dr. F. Dahlquist, University of Oregon. CheY∼P was prepared from acetyl [^{32}P]phosphate as described in the literature (Lukat et al., 1992).

Isolation of Spo0F. The *spo0F* containing *Ssp*1 fragment from the plasmid pJH4183 (Trach et al., 1988) was cloned into the *Sma*1 site of pKK223-3 (Pharmacia). The vector containing insert, correctly oriented relative to the *tac* promoter, was designated pKK0F. Mutant *spo0F187* (Spo0FD54N) was PCR amplified from genomic DNA that was isolated from the spo$^-$ *B. subtilis* strain MUV7-187 (unpublished results) using oligos 0F5 (5′-GGT CGG TAC CTC ATA TGA TGA ATG AAA AAA TTT TAA TCG-3′) and 0F3P (5′-GGA CGG ATC CGT CAG TTA GAC TTC AGG GGC AG-3′). Using artificial *Nde*I and *Bam*HI restriction sites in oligos 0F5 and 0F3P, respectively, the resulting PCR product was cloned into these same restriction sites on pET20b (Novagen, Madison, WI), giving the plasmid pET0F187. The appropriate *Escherichia coli* strain (DH5α for pKK0F or BL21DE3 for pET0F187) containing the plasmid pKK0F was grown at 37 °C in LB medium supplemented with 100 μg/mL ampicillin. Upon reaching an OD_{600} of 0.7−0.8, the cultures were induced by the addition of IPTG to a final concentration of 2 mM. After 5−6 h of additional growth, cells were harvested by centrifugation and stored at −20 °C. Spo0F was purified by a three-step procedure entailing cell lysis and DEAE-Trisacryl and hydroxylapatite column chromatography. Cells (20 g) were thawed on ice and resuspended in 60 mL of 25 mM Tris (pH 7.8), 10 mM KCl, 1 mM $MgCl_2$ (DEAE buffer) supplemented with 5 mM βME, 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 1 μM leupeptin, and 1 μM pepstatin. Cells were disrupted by sonication on ice using 5 × 30 s bursts from a Heat Systems (New York) Model W-220F sonicator equipped with a 0.5 in. tip. The sample temperature was maintained below 6 °C by incubation on ice. After sonication, the solution was made to 1 mM in PMSF and centrifuged at 15 000 rpm in a Sorvall SS-34 rotor for 60 min at 4 °C. The supernatant was applied at 1.0 mL/min to a 5.0 × 8.0 cm column of DEAE-Trisacryl LS previously equilibrated in DEAE buffer. The column was washed with 2.5 L of DEAE buffer at 4.0 mL/min, and then Spo0F was eluted with a linear gradient from 10 to 210 mM KCl in DEAE buffer at 2.0 mL/min. With the aid of SDS−PAGE analysis and molecular weight markers, Spo0F was found to elute 40% into the gradient. Spo0F containing fractions were applied directly to a 5.0 × 2.0 cm column of hydroxylapatite previously equilibrated in 25 mM Bis-Tris (pH 7.3) and 50 mM KCl (HA buffer) supplemented with 2 mM $MgCl_2$. After loading at 0.2 mL/min, the column was washed with 500 mL of HA buffer, followed by the same buffer adjusted to 800 mM in $MgCl_2$ at a flow rate of 0.5 mL/min until the absorbance of the effluent at 280 nm was greater than 0.05, at which point the column flow was stopped for at least 2 h. Elution was resumed in the same buffer at a flow rate of 0.5 mL/min, and Spo0F eluted in a single peak at ∼1 mg/mL. The pooled protein was dialyzed against HA buffer containing 0.02% NaN_3. Spo0F purity was confirmed by SDS Tris/Tricine PAGE (Figure 1) (Schagger & von Jagow, 1987) and nondenaturing PAGE. Using electrospray mass spectroscopy, the protein was found to be a single species of 14 230 Da, which is the mass calculated for intact Spo0F.

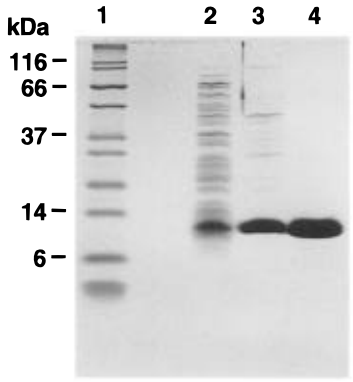

FIGURE 1: Purification of Spo0F. A sample from each purification step (∼5 μg) was analyzed by SDS Tris/Tricine PAGE. Lane: 1, standards; 2, cell free extract; 3, DEAE-Trisacryl column pool; 4, hydroxylapatite column pool.

Nondenaturing PAGE Analysis of Spo0F and Spo0∼P. Nondenaturing PAGE was performed as described by Hedrick and Smith (1968) except that ammonium persulfate was used to initiate polymerization in the 4% stacking gel. Reactions were quenched by the addition of 250 mM EDTA in an equal volume of 250 mM Tris Cl^- (pH 6.8), containing 50% glycerol, 0.005% bromophenol blue, and 0.005% phenol

Table 1: Half-Life of Spo0F∼P in Differing Electrophoresis Buffers at Various Temperatures

	$t_{1/2}$ (h)[b]			
	SDS buffers[a]	nondenaturing buffers		
buffer	37 °C	37 °C	25 °C	14 °C[c]
125 mM Tris, pH 6.8[d]	15	3	30	>100
375 mM Tris, pH 8.9[e]	8	5	25	>100
25 mM Tris, pH 8.3,[f] 192 mM Glycine	nd[g]	nd	40	>100

[a] Contain 0.1% SDS (w/v). [b] Values are the mean ± SEM of 2−3 determinations. [c] Less than 15% of the phosphoproteins were hydrolyzed after 100 h. [d] Stacking, [e] separation, and [f] electrode buffers. [g] Not determined.

red. The samples were then frozen in a dry ice−ethanol bath to prevent hydrolysis. For analysis, samples were electrophoresed through a 4% stacking and 13% resolving gel. It was found necessary to maintain the gel buffer below 14 °C in order to obtain well-resolved protein bands. Coomassie blue stained gels were digitized using a Speedlight gel documentation system (B&J Scientific, Encinitas, CA). The digitized data were analyzed using ImageQuant software (Molecular Dynamics, San Jose, CA). The percentage of Spo0F∼P formed was calculated from the proportion of Spo0F that migrated with an R_f of 0.49. Free Spo0F migrated with an R_f of 0.55. Radioactivity was visualized in both stained and unstained gels by autoradiography using gels that were vacuum dried on a Bio-Rad Model 585 gel dryer to improve detection of radioactivity. The percentage of Spo0F∼P formed with KinA was also determined from the specific activity of the [γ-^{32}P]ATP used and the amount of radioactivity counted in radioactive bands excised from SDS−PAGE gels.

Phosphorylation of Spo0F with KinA. KinA (1 μM) was first autophosphorylated in 50 mM EPPS (pH 8.5), 50 mM KCl, 20 mM $MgCl_2$, 5% glycerol (v/v) (kinase buffer), and 1 mM ATP containing 60 μCi of [γ-^{32}P]ATP. After 5 min at 23 °C, Spo0F was added to the reaction to a concentration of 25 μM using a 25- to 100-fold concentrated stock of protein. The mixture was incubated for 2 h at 23 °C, prior to removing a 20 μL sample for nondenaturing PAGE analysis.

Phosphorylation of Spo0F with Small Molecules. Reactions were carried out in a solution containing 50 mM EPPS, pH 6.5, 50 mM KCl, and 125 mM $MgCl_2$ (phosphorylation buffer) at 23 °C using increasing concentrations of phospho donors up to 250 mM. After initiating the reaction with 25 μM Spo0F, aliquots were removed at 15, 30, 60, and 90 min and were analyzed by nondenaturing PAGE.

Hydrolysis of Spo0F∼P in Electrophoresis Buffers. Spo0F was phosphorylated with KinA as described above. The reaction mixture (1 mL) was then applied to a HiLoad 26/50 Sephacryl S-200 column equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 50 mM KCl (gel filtration buffer) at 4 °C, and the column was washed at 1 mL/min. KinA and ATP were resolved from Spo0F/Spo0F∼P by this method. Fractions containing Spo0F∼P were identified by SDS−PAGE analysis as described by Burbulys *et al.* (1991). Spo0F∼P, thus obtained, was concentrated in a Centricon 3 microconcentrater. Spo0F∼P at 0.3 mg/mL was incubated in the electrophoresis buffers and at the temperatures detailed in Table 1. At intervals from 1 to 100 h, 15 μL aliquots were removed and analyzed by SDS−PAGE. The amount of radioactivity associated with excised Spo0F bands was quantitated as described above. Using data in which between 10% and 90% of the phosphoprotein was hydrolyzed, the first order rate constant for the hydrolysis of Spo0F∼P was obtained from a 1n(% Spo0F∼P) vs time plot.

Hydrolysis of Spo0F∼P by Phosphorelay Proteins. To generate Spo0F∼P, Spo0F (0.71 mg/mL) was incubated with 250 mM PA in phosphorylation buffer for 2 h at 23 °C. To remove unreacted PA, the reaction was quenched by the addition of EDTA to 250 mM, and the sample was applied to a 2.6 × 25 cm column of Trisacryl GF05M, previously equilibrated in HA buffer supplemented with 0.1 mM EDTA. Elution was carried out at 1.0 mL/min, and 3 mL fractions were collected. Those containing Spo0F∼P were identified by nondenaturing PAGE analysis. Samples of Spo0F∼P (10 μM) were then incubated with KinA or Spo0B (1 μM), or with Spo0A (10 μM) alone, or with Spo0B (1 μM) and Spo0A (10 μM) in kinase buffer at 37 °C. At 2, 5, 10, and 20 min, 20 μL aliquots were removed and analyzed by nondenaturing PAGE.

Metal Dependence. The effect of Mg^{2+} on the KinA mediated phosphorylation of Spo0F was carried out as described above but in the presence of 0−100 mM $MgCl_2$, and the reaction was buffered at pH 6.5. At intervals from 1 to 120 min, aliquots were removed and analyzed by nondenaturing PAGE. Spo0F was also phosphorylated with 250 mM PA in phosphorylation buffer, but 2−500 mM $MgCl_2$ or 1 mM EDTA was used. After initiating the reactions by adding Spo0F to a final concentration of 25 μM, aliquots were removed at 1 and 3 h and analyzed by nondenaturing PAGE. The data from the 60 min time points are shown for both KinA∼P and PA mediated reactions in Figure 6. To determine the effect of Mg^{2+} on the rate of Spo0F∼P hydrolysis, duplicate samples of Spo0F∼P (0.3 mg/mL) were incubated in phosphorylation buffer at 23 °C with concentrations of $MgCl_2$ ranging from 0 to 250 mM. At intervals from 0.25 to 70 h, aliquots were removed and analyzed by nondenaturing PAGE. The first order rate constant for the hydrolysis of Spo0F∼P was determined as described above.

Initial Rate Studies. Duplicate samples of Spo0F (0.3 mg/mL) were preincubated in phosphorylation buffer at 23 °C for 5 min. Phosphorylation was initiated by adding PA to the reaction at concentrations from 6 to 500 mM. Aliquots were taken from 2 to 40 min for analysis by nondenaturing PAGE. From the linear portion of the percent Spo0F∼P formed versus time plot, initial rates were estimated. Initial rate measurements were analyzed by Michaelis−Menten and Lineweaver−Burk methods (Segel, 1994). Results were accommodated to the appropriate equations using the program Slidewrite 5.0 with a weighting factor calculated from the inverse of the range seen between duplicate data points.

RESULTS

Nondenaturing PAGE. Because many of the small phosphorylating molecules are difficult to obtain as radiolabeled derivatives, a nonradioactive assay for Spo0F∼P formation was developed. Nondenaturing PAGE was used to separate Spo0F from Spo0F∼P. Spo0F purified by the method described in the previous section migrates as a single band

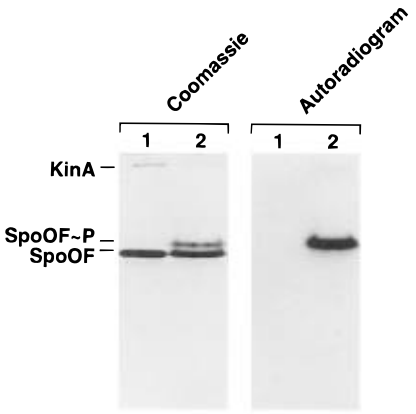

FIGURE 2: Spo0F resolved from Spo0F∼P by nondenaturing PAGE. Coomassie blue stained nondenaturing PAGE gel and autoradiogram of the same gel. Lane: 1, Spo0F kinase reaction without ATP; 2, Spo0F kinase reaction with [γ-^{32}P]ATP.

with an R_f relative to bromophenol blue of 0.55, whereas Spo0F∼P prepared with KinA and [γ-^{32}P]ATP runs as a mixture of two bands with R_f values of 0.55 and 0.49 (Figure 2). The slower migrating band was attributed to Spo0F∼P since only this band contained radioactivity. Spo0F∼P is hydrolyzed by both acid and base treatment (Burbulys et al., 1991), and Spo0F∼P is hydrolyzed completely within 1 h in the presence of 0.2 M pyridine (data not shown). These properties verify that Spo0F is phosphorylated at a carboxylate group (Fujitaki & Smith, 1984). Because acyl phosphates are known to be unstable to hydrolysis (Koshland, 1951), the stability of Spo0F-P in various electrophoresis buffers was determined (Table 1). Using our nondenaturing PAGE conditions, the separation was completed within 2 h of running at 4 °C; thus from the half-life values in Table 1, at 14 °C, more than 98% of the phosphate would remain attached to Spo0F during the separation. From the ratio of the two resolved bands, Spo0F was calculated to be 45% phosphorylated by KinA in this instance. When radioactivity bound to Spo0F was quantitated using the SDS−PAGE method, Spo0F was also determined to be 45% phosphorylated, in agreement with results from nondenaturing PAGE. The percent of Spo0F phosphorylated with KinA varied between 15% and 70%, depending on the particular preparation of KinA used. Factors affecting KinA phosphorylation activity are not clearly defined. Using pyruvate kinase and phosphoenolpyruvate to regenerate ATP from ADP (Lukat et al., 1991), Spo0F∼P levels as high as 85% can be achieved. ADP is a competitive inhibitor with respect to ATP of phosphotransfer between KinA and Spo0F;[2] thus, blocking the accumulation of ADP may relieve this product inhibition. The high levels of Spo0F∼P produced by the combined action of KinA and pyruvate kinase suggests that the purified Spo0F protein could be completely phosphorylated, if optimal conditions could identified. Interestingly, Spo0F∼P was more stable in SDS−PAGE buffers than in nondenaturing PAGE buffers.

In comparative experiments, it was observed that complete hydrolysis occurred with the chemotaxis protein, CheY∼P, during nondenaturing PAGE electrophoresis (data not shown), a result most probably arising from the inherent instability of CheY∼P ($t_{1/2}$ ∼0.5 min) (Ganguli et al., 1995).

[2] Grimshaw *et al.*, unpublished results.

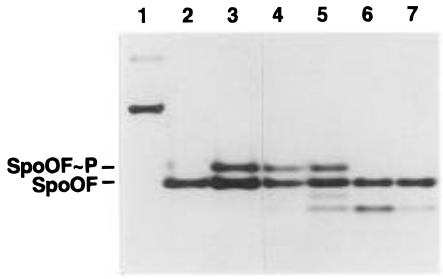

FIGURE 3: Nondenaturing PAGE showing that Spo0F is phosphorylated by either KinA or PA in the presence of 20 or 125 mM magnesium, respectively. The phosphorylation was carried out as described under Experimental Procedures. Lane: 1, BSA standard; 2, Spo0F; 3, Spo0F∼P prepared with KinA; 4, Spo0F∼P formed after 1 h with 150 mM PA; 5, Spo0F∼P formed after 3 h with 150 mM PA; 6, Spo0F after 12 h incubation with 150 mM PA, 2 mM EDTA, and no divalent metal; 7, Spo0F after 1 h incubation with 150 mM PA, 2 mM EDTA, and no divalent metal.

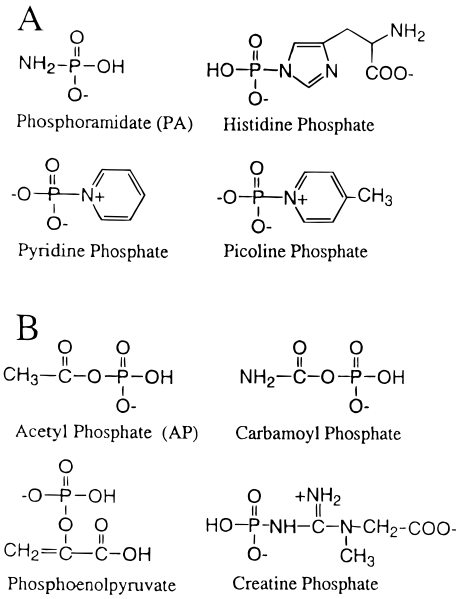

FIGURE 4: Structures of phospho donor compounds that (A) phosphorylate and (B) do not phosphorylate Spo0F.

Is Spo0F Phosphorylated by Small Molecular Weight Phosphorylating Agents? This question was addressed by incubating Spo0F with several phosphate containing compounds and assaying for Spo0F∼P formation using nondenaturing PAGE (PA is shown in Figure 3; all are summarized in Figure 4). Compounds known to phosphorylate other two-component response regulators (PA, acetyl phosphate, and carbamoyl phosphate) were assayed (Feng et al., 1992), as well as compounds known to phosphorylate carboxylate compounds (pyridine phosphate, picoline phosphate, and histidine phosphate) (Herschlag & Jencks, 1990). Spo0F was phosphorylated by all phosphoramidate compounds assayed (Figure 4), except phosphocreatine, but Spo0F was not phosphorylated by any of the phosphate ester compounds shown nor by the nucleotides ATP, ADP, GTP, and GDP.

Site of Phosphorylation. To determine if Spo0F was phosphorylated by KinA∼P and small molecular weight phospho donors in an equivalent manner, Spo0F∼P samples generated by KinA or phosphoramidate were compared by nondenaturing PAGE. Whether prepared with PA or KinA∼P, Spo0F∼P exhibited a similar nondenaturing electrophoretic profile, showing two predominant protein bands with R_f

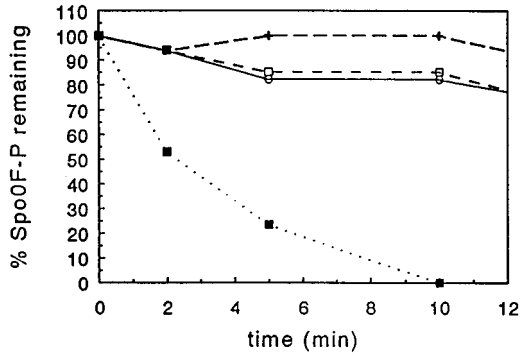

FIGURE 5: Spo0B and Spo0A specifically hydrolyze Spo0F∼P generated from PA. Reaction mixtures containing Spo0F∼P alone (○), Spo0F∼P and Spo0B (+), Spo0F∼P and Spo0A (□), or Spo0F∼P, Spo0B, and Spo0A (■) were incubated in kinase buffer at 37 °C. The percentage of SpoOF remaining phosphorylated was determined as described under Experimental Procedures.

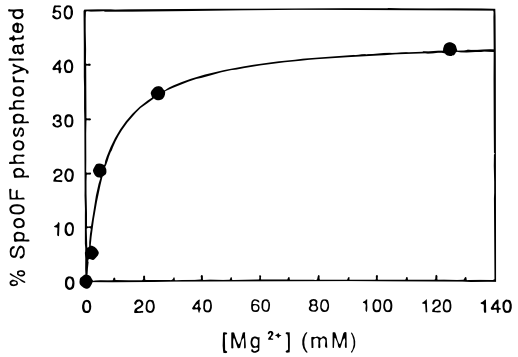

FIGURE 6: Magnesium is required to form Spo0F∼P. Phosphorylation reactions were carried out at 23 °C as described under Experimental Procedures. Spo0F∼P prepared with 250 mM PA (●) in the presence of increasing concentrations of $MgCl_2$ after 1 h as determined by nondenaturing PAGE analysis.

values of 0.55 and 0.49 relative to bromophenol blue (Figure 3). In contrast, a slower migrating Spo0F∼P band was not observed with the Spo0F mutant, D54N (data not shown), whether treated with PA or KinA∼P.

To determine whether the active site of Spo0F is phosphorylated with PA, Spo0F∼P prepared with PA was incubated with other phosphorelay proteins. Incubation of Spo0F∼P with Spo0B alone at 1/10 the concentration of Spo0F∼P did not appear to stimulate the loss of phosphate (Figure 5), as measured by the loss of the slow migrating band on nondenaturing PAGE. Although Spo0B alone is known to accept phosphate from Spo0F∼P (Burbulys et al., 1991), in the absence of ^{32}P label the sensitivity of native PAGE assay is limited; thus, small changes in Spo0F∼P levels resulting from transfer to Spo0B would not be detected by this technique. Incubation of Spo0F∼P with Spo0A at equimolar concentration with Spo0F∼P did not stimulate the loss of phosphate faster than the control of Spo0F∼P alone. In contrast, addition of Spo0B at one-tenth molar and Spo0A equimolar to the concentration of Spo0F∼P resulted in the loss of phosphate from Spo0F∼P at a rate much greater than the control value (Figure 5). This suggests that Spo0F∼P derived from the PA reaction behaved identically to that generated by KinA plus ATP in the natural reaction since phospho transfer only occurs when the phosphorelay chain is completed (Burbulys et al., 1991).

Spo0F treated with small molecules containing a phosphonitrogen bond exhibited two additional minor protein bands on nondenaturing PAGE that migrated faster than unphosphorylated Spo0F. These minor bands displayed R_f values of 0.58 and 0.63 relative to bromophenol blue, and they appeared at a much slower rate than the predominant phosphorylated form of Spo0F. The minor phosphorylated forms constituted ≤1% and 5% of the total stain intensity after 1 and 3 h, respectively. In contrast, the major phosphorylated form constituted 35% and 45% of the total stain intensity after 1 and 3 h, respectively (Figure 3). These phosphorylation yields have been reproduced with many different preparations of Spo0F. When Spo0F∼P is separated from PA by gel filtration and incubated at room temperature, the banding pattern of Spo0F on nondenaturing PAGE returns to a single band with the same relative mobility as unphosphorylated Spo0F over several days. The pattern of the minor bands mirrored the pattern of the major bands; however, the greater mobility of these minor bands relative to the Spo0F∼P band argued that the fast migrating bands arose from phosphorylation at additional sites other than the active site.

Metal Requirement. Phosphorylation of Spo0F with KinA∼P or PA required a divalent metal ion. For example, when Spo0F was treated with PA in the absence of a divalent metal, the slower migrating protein band attributed to Spo0F∼P was absent (Figure 3, lane 6). Similarly, Spo0F could not be phosphorylated by KinA and ATP in the absence of divalent ion. In addition to Mg^{2+}, the divalent cations Mn^{2+}, Ca^{2+}, Zn^{2+}, and Fe^{2+} stimulated the formation of Spo0F∼P from PA. However, it was impossible to compare the potency of these various metals at stimulating the phosphorylation reaction since all of the metals except Mg^{2+} formed insoluble precipitates with PA. Thus, it was concluded that the specific phosphorylation of Spo0F required divalent metal ions. Interestingly, only one of the two minor protein bands, the fastest migrating band at R_f 0.63, developed when Spo0F was treated with PA in the absence of divalent metal ions, suggesting at least one of the side reactions was also ion catalyzed.

In order to discover the Mg^{2+} concentration required to form the maximum amount of Spo0F∼P, the percentage of Spo0F∼P formed from PA in the presence of increasing concentrations of Mg^{2+} was determined. The percentage of Spo0F∼P formed increased as the Mg^{2+} concentration increased, but reached a maximum of 45% at 125 mM Mg^{2+}. Identical results were found after 1 or 3 h of incubation, indicating that a steady state had been reached. The results gave a saturation profile typical of titration data (Figure 6). It was calculated that Mg^{2+} stimulated Spo0F phosphorylation to 50% at 8 ± 2 mM. The results confirmed that phosphorylation of Spo0F by PA required divalent metal ions and that maximal phosphorylation of Spo0F by PA required high concentrations of metal ion.

Increasing the Mg^{2+} concentration also increased the rate of Spo0F∼P hydrolysis 4-fold from 0.035 h^{-1} at 0 mM Mg^{2+} to 0.14 h^{-1} at 2 mM Mg^{2+}, but no further increases in the rate of Spo0F∼P hydrolysis occurred when the Mg^{2+} concentration was increased above 2 mM. This saturation of Spo0F∼P hydrolysis rate suggests that a maximum amount of Mg^{2+} is bound to Spo0F∼P at 2 mM concentrations of Mg^{2+}.

Kinetic Profile of the Phosphorylation of Spo0F by PA. To determine whether the phosphorylation of Spo0F by PA exhibited the kinetic profile expected for enzyme catalysis

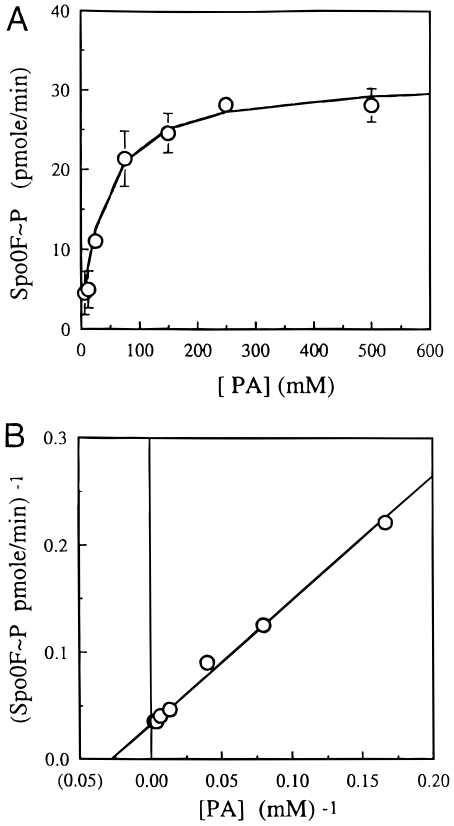

FIGURE 7: Phosphorylation of Spo0F with PA exhibits Michaelis−Menten kinetics. (A) Initial velocity for the phosphorylation of Spo0F at increasing concentrations of PA in phosphorylation buffer at 23 °C. (B) The data from A linearized in a Lineweaver−Burk plot.

or chemical reaction, the initial rate of this reaction was measured at increasing concentrations of PA in saturating concentrations of Mg^{2+} ion. The results conformed to a saturation profile indicative of enzyme catalysis (Figure 7A). Transformation of the data by the Lineweaver−Burk method yielded the linear plot shown in Figure 7B. From a nonlinear least squares fit of the untransformed data to the Michaelis−Menten equation, a K_m value for PA of 39 mM and a k_{cat} value of 0.01 min^{-1} were calculated. Similar values were calculated using the Lineweaver−Burk method. For comparison, the rate of phosphotransfer from KinA to Spo0F under our experimental conditions was found to be 18-fold faster at 0.18 min^{-1}, assuming Spo0F is the catalytic agent.[3] The dependence of this reaction on the concentration of KinA was not determined, but the KinA mediated reaction proceeded at micromolar concentrations of KinA.

DISCUSSION

Spo0F∼P can be separated from Spo0F by nondenaturing PAGE (Figure 2), a technique that separates proteins on the basis of both conformation and charge (Hedrick & Smith, 1968). The mixture of bands exhibited by the Spo0F sample implies that Spo0F is never completely phosphorylated. This may result either from incomplete phosphorylation of Spo0F with KinA or from the hydrolysis of Spo0F∼P during electrophoresis. Results support the former conclusion because Spo0F∼P has been shown to be stable to hydrolysis in electrophoresis buffers at temperatures below 14 °C. In contrast, Spo0F∼P hydrolyzes during electrophoresis at 37 °C, giving a smear of unresolved bands. The quantitative nature of the nondenaturing PAGE analysis was further confirmed by the identical values repeatedly recorded for the percentage of Spo0F∼P when measured by nondenaturing PAGE. In radioactive measurements using SDS−PAGE, similar results were observed. Thus, the greater stability of Spo0F∼P to hydrolysis ($t_{1/2}$ > 100 h at 14 °C) allows this technique to be used routinely for estimating the extent of protein phosphorylation in the phosphorelay. Hydrolysis of CheY∼P during electrophoresis indicates that not every response regulator can be analyzed by this technique. However, many phosphorylated response regulators such as OmpR∼P (Igo et al., 1989) and VanR∼P (Wright et al., 1993) have similar stabilities to hydrolysis as Spo0F∼P; thus it should be possible to use nondenaturing PAGE to analyzed these "stable" response regulators. Interestingly, Spo0F∼P was more stable in SDS than in nondenaturing PAGE buffer at 37 °C, implying that the native protein stimulated dephosphorylation of itself. This result suggests that Spo0F could possess an autophosphatase activity like many other response regulatory proteins (Lukat et al., 1990).

Phosphorylation of Spo0F by several potential phospho donors was assayed by the nondenaturing PAGE procedure. In general, the results (Figures 3 and 4) indicated that Spo0F was phosphorylated by compounds containing phospho-nitrogen bonds but not by compounds containing phospho-anhydride bonds. When the structures of compounds that are reactive with Spo0F are compared, compounds that chemically and structurally mimic the histidine phosphate of KinA∼P, the natural substrate for Spo0F, are found to be phospho donors. This apparent substrate specificity may result from the greater chemical reactivity of phosphoramidate compounds over acyl phosphate compounds (Herschlag & Jencks, 1990). An exception to the general trend is demonstrated by the observation that Spo0F does not react with creatine phosphate. Although possessing a phosphoramidate linkage, creatine phosphate has a guanidino group that is distinct from the ringed structure exhibited by histidine, pyridine, and picoline phosphates. CheB shows a phospho donor specificity similar to Spo0F, since CheB reacts with PA but not acetyl phosphate (Lukat et al., 1992). Many response regulators such as NRI, CheY, and BvgA (Lukat et al., 1992; Feng et al., 1992; Boucher et al., 1994) show a broader specificity for phospho donor structure by reacting with both phosphoramidate and acyl phosphate compounds. This suggests that proteins reactive with acetyl phosphate, such as CheY, have a reaction site that is more tolerant of this mixed anhydride phosphorylating agent or have evolved structural elements that promote this more difficult reaction. Thus the Spo0F structure places restrictions on the molecular dimensions of the species that can be phospho donors in addition to appearing to require the reactivity of the phosphoramidate linkage.

Carbamoyl phosphate, acetyl phosphate, and PA are all reactive compounds with phospho donor potential greater than ATP (Stryer, 1981); thus, these compounds may nonspecifically react with accessible amino acid residues on proteins. However, at least in the case of NRI, it has been shown that phosphorylation occurs at the active site (Feng et al., 1992). As a result, it was important to determine if small molecules specifically phosphorylated Spo0F at the active site tricarboxylate pocket. In experiments designed

[3] Grimshaw *et al.*, unpublished results.

to examine this issue, it was observed that Spo0F∼P generated with KinA∼P or PA exhibited a similar electrophoretic profile, indicating that Spo0F was phosphorylated in an equivalent manner by both reagents. Furthermore, the specific hydrolysis of Spo0F∼P by incubation with both Spo0B and Spo0A implies that the phosphate on Spo0F∼P generated from PA was transferred to Spo0A through Spo0B as occurs in the natural reaction (Burbulys et al., 1991). In addition, the Spo0F D54N mutant is not phosphorylated by either KinA∼P or PA. This result confirms the participation of aspartate 54 in the phosphorylation process and supports the observation that the active site of Spo0F is phosphorylated by PA.

Spo0F∼P prepared from PA also contained two minor electrophoretic bands that may have resulted from phosphorylation at secondary sites (Figure 3). The slow appearance of minor phosphorylated forms of Spo0F implies that the reaction between Spo0F and PA is not entirely specific for the active site; however, the faster rate at which Spo0F is converted to the predominant phosphorylated form argues that phosphorylation at the active site is favored.

A divalent metal ion was found to be essential for the specific phosphorylation of Spo0F by both PA and KinA. The extent of Spo0F phosphorylation increased as the Mg^{2+} concentration increased, exhibiting a half-maximal effect at 8 ± 2 mM. Because phosphate containing compounds, such as acetyl phosphate and PA, have dissociation constants for magnesium greater than 100 mM (Kluger et al., 1975; Herschlag & Jencks, 1990), it was concluded that the half-maximal constant of ∼8 mM did not arise from the binding of Mg^{2+} to PA, but from binding of Mg^{2+} to Spo0F. Consistent with this idea, the dissociation constant for Mg^{2+} binding to Spo0F determined by multidimensional NMR is of the same order of magnitude (19 mM) (Feher et al., 1995). In contrast, the dissociation constant for the binding of Mg^{2+} to CheY is 0.5 mM, 16-fold lower than the constant for Spo0F (Lukat et al., 1990). Magnesium stimulated the rate of Spo0F∼P hydrolysis by up to 4-fold, showing that divalent cations play a mechanistic role in the hydrolysis reaction. Even at saturating concentrations of Mg^{2+}, Spo0F∼P hydrolyzed slowly, exhibiting a half-life of 5 h at 23 °C and neutral pH, which places Spo0F∼P among the most stable of the known phosphorylated response regulators (Parkinson & Kofoid, 1992). The difference in Mg^{2+} binding affinity and phosphoprotein stability between Spo0F and CheY illustrate that there are functional differences between these two proteins despite the sequence and secondary structure similarities (Feher et al., 1995). An essential catalytic role of Mg^{2+} in phosphorylating reactions has been proposed to be the neutralizing of negative charges on the carboxylate and phosphate groups, thus eliminating repulsive forces that would keep these groups apart (Kluger et al., 1975). In fact, studies on phosphoryl transfer between pyridine phosphate and acetate establish that Mg^{2+} aids catalysis by chelating both the carbonyl and the phosphoryl oxygens in the transition state (Herschlag & Jencks, 1990). Consequently, only Spo0F containing bound magnesium would be expected to phosphorylate at the active site carboxylates with PA or KinA∼P, as is observed.

The phosphorylation of Spo0F by small molecules shows some structural specificity, a feature consistent with the proposal that response regulators are enzymes that transfer phosphate to themselves (Sanders et al., 1992). Although PA phosphorylates the active site of Spo0F, this reactive compound (Chanley & Feageson, 1963) appears to nonspecifically phosphorylate Spo0F at secondary sites, implying that this phospho donor molecule reacts with other accessible amino acid residues. Thus, phosphorylation at the active site is not sufficient evidence alone to prove that Spo0F catalyzes the transfer of phosphate to itself. The question of whether phosphorylation of Spo0F results from nonspecific chemical reactions or enzyme catalysis at the active site was addressed by studying the effect of phospho donor concentration on the initial rate of the reaction. The results demonstrated that the rate of phosphorylation of Spo0F saturated as the PA concentration was increased, exhibiting a Michaelis−Menten profile and a linear Lineweaver−Burk plot. The kinetic evidence combined with the substrate, metal, and active site specificity of this reaction argue that Spo0F phosphorylates itself at the active site pocket using phosphoramidates as substrates. On the basis of sequence homology, we conclude that other response regulators should also have this autophosphorylation activity. From this small molecule autophosphorylation activity, it can be inferred that the catalytic amino acids necessary for phosphotransfer reside on the response regulator protein Spo0F. Consequently, response regulators such as Spo0F could not only be considered phosphatases of their cognate kinase (Hess et al., 1988; Sanders et al., 1992), but also be considered phosphotransferases (Lukat et al., 1992).

The autophosphorylation of Spo0F with PA as a substrate was catalytically inefficient compared to the reaction with KinA∼P as a substrate; nevertheless, Spo0F significantly stimulates this reaction since the analogous chemical reaction between PA and acetate does not occur (Jencks & Gilchrist, 1965). The large Michaelis constant for PA of 40 mM suggests that Spo0F has a weak affinity for PA. The Michaelis constant for the reaction with KinA∼P as a substrate was not determined, but this reaction occurred at micromolar concentrations of KinA, 3 orders of magnitude lower than when PA was used as a substrate. Even though imidazole phosphates, such as that formed on KinA∼P, are less reactive than PA (Chanley & Feageson, 1963), the reaction between KinA∼P and Spo0F is 18-fold faster than the reaction between PA and Spo0F. This observation suggests that KinA contributes to the catalysis. KinA∼P may contribute to catalysis by inducing a conformational change in Spo0F or by providing binding contacts that stabilize the transition state. One such contact may be between KinA and Mg^{2+} ion.

Spo0F was not phosphorylated to completion by PA or KinA and ATP. The level of Spo0F phosphorylation may be a steady-state value reached once the rate of Spo0F phosphorylation and Spo0F∼P hydrolysis have reached a balance. Since radiolabeled PA is unavailable, it was not possible to measure the rate of Spo0F∼P decay using a simple pulse−chase experiment; thus the actual rate of dephosphorylation is difficult to measure since the acyl phosphate of Spo0F∼P in the PA reaction may be hydrolyzed by several mechanisms. For example, in addition to an autohydrolytic activity of Spo0F, the amine moiety of PA or ammonia (even when reduced by buffer action) generated by the hydrolysis of PA would be expected to stimulate phospho-aspartate decay (Koshland, 1951). Indeed, preliminary studies on the role of amines in Spo0F∼P hydrolysis show that PA accelerates the rate of loss of radiolabel from

[^{32}P]Spo0F∼P. Therefore, the exclusive use of the measured phosphorylation and hydrolysis rate constants to calculate an accurate steady-state level of Spo0F∼P is not possible. The extent of Spo0F∼P formed with KinA and ATP would also depend on the relative rates of phosphorylation and dephosphorylation. Although Spo0F is phosphorylated by the histidine kinase, KinA, at a faster rate than PA, this faster phosphorylation rate may be compensated by a faster dephosphorylation rate. Consistent with this idea, histidine kinases are known to act as phosphatases of their cognate response regulators (Ninfa & Magasanik, 1986). Complete phosphorylation of Spo0F may be unnecessary since this protein serves as a secondary messenger in the phosphorelay.

The autophosphorylation of response regulators with small molecular weight phospho donors may function to link the two-component systems to the metabolic state of the cell. Indeed, AlgR, which controls mucoidy in *Pseudomonas aeruginosa*, is activated by nutrient deprivation (Deretic et al., 1992). Similarly, sporulation in *Bacillus subtilis* is stimulated by nutrient deprivation, and it is possible that the phosphorelay proteins may be activated by metabolic signaling molecules (Hoch, 1993). Unlike CheY (McCleary & Stock, 1994), NRI (Feng et al., 1992), and PhoB (Wanner & Wilmes-Riesenberg, 1992), Spo0F is not phosphorylated by acetyl phosphate and thus is not subject to regulation by the Pta-AckA pathway; however, Spo0F may be subject to regulation by another metabolic pathway. Based on the substrate specificity that we observed for phosphorylation of Spo0F, a putative sporulation signaling molecule produced by such a pathway should have a phosphoramidate group.

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