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Autoprocessing of the Protease That Degrades Small, Acid-Soluble Proteins of Spores of *Bacillus* Species Is Triggered by Low pH, Dehydration, and Dipicolinic Acid

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The sequence-specific protease (termed GPR) that degrades small, acid-soluble proteins (SASP) during germination of spores of *Bacillus* species is synthesized during sporulation as an inactive precursor termed P_{46}. Approximately 2 h later in sporulation, P_{46} is converted proteolytically to a smaller form, termed P_{41}, which is active in vitro, but which does not act significantly on SASP in vivo until spore germination is initiated. While it appears likely that $P_{46}\rightarrow P_{41}$ conversion is an autoprocessing event, the mechanisms regulating $P_{46}\rightarrow P_{41}$ conversion in vivo are not clear. In this work we found that $P_{46}\rightarrow P_{41}$ conversion in vitro was stimulated tremendously in an allosteric manner by pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]) plus Ca^{2+} but not by Ca^{2+} in combination with a variety of DPA analogs. The processing reaction stimulated by Ca^{2+}-DPA was seen at pH 5.1 but not at pH 6.2 or 7, and under these conditions $P_{46}\rightarrow P_{41}$ conversion exhibited a linear time course and was a first-order reaction, indicative of an intramolecular autoprocessing reaction. At pH 5.1, $P_{46}\rightarrow P_{41}$ conversion was stimulated markedly by very high ionic strength. At pHs from 5.1 to 6.6, $P_{46}\rightarrow P_{41}$ conversion also occurred when P_{46} was dehydrated to ~54% relative humidity. This processing was stimulated markedly when dehydration was carried out in the presence of DPA and NaCl but was greatly decreased when dehydration was to 10, 33, or 75% relative humidity. Since previous work has shown that $P_{46}\rightarrow P_{41}$ processing in vivo takes place (i) after a fall in forespore pH to 6.3 to 6.9 and approximately in parallel with (ii) DPA accumulation by the forespore and (iii) dehydration of the forespore, our new findings in vitro suggest that these three changes may synergistically trigger $P_{46}\rightarrow P_{41}$ autoprocessing in the developing forespore. Presumably the conditions in vivo during this autoprocessing preclude significant attack of the P_{41} generated on its SASP substrates.

Approximately 10 to 20% of the protein of dormant spores of *Bacillus* species is degraded in the first minutes of spore germination (19). The proteins degraded in this process are a group of small, acid-soluble proteins (SASP), primarily of the α/β type and γ type, which are synthesized only midway in sporulation within the developing spore. SASP degradation during spore germination is initiated by a sequence-specific protease termed GPR. This tetrameric endoprotease is synthesized as an inactive zymogen in the developing forespore, slightly before the synthesis of its SASP substrates (11, 19, 24). The subunits of this inactive zymogen, which form tetramers, are termed P_{46}. Approximately 2 h later in sporulation, P_{46} is processed proteolytically by removal of 15 (*Bacillus megaterium*) or 16 (*Bacillus subtilis*) amino-terminal residues (17), generating P_{41}, which forms tetramers which are active on SASP in vitro (19). However, the P_{41} generated in vivo normally degrades little if any SASP in the developing or dormant spore. During spore germination, P_{41} is further processed to P_{40} by removal of one additional amino-terminal residue, and P_{40} (as well as any residual P_{41}) rapidly initiates SASP degradation (17, 19). The precise function (if any) of the $P_{41}\rightarrow P_{40}$ processing is not clear, as P_{41} and P_{40} have identical catalytic activity in vitro, and under some conditions P_{41} catalyzes rapid SASP degradation in vivo (7). In contrast, it is clear that precise regulation of the timing of the conversion of $P_{46}\rightarrow P_{41}$ is crucial to achieving a normal SASP level in spores. Thus, synthesis of GPR at its normal time in sporulation as P_{41} or early conversion of P_{46} to P_{41} during sporulation results in spores with greatly reduced SASP levels (7, 17). Since the α/β-type SASP are DNA-binding proteins responsible for protecting spore DNA from a variety of types of damage, decreased α/β-type SASP levels greatly decrease spore resistance to a variety of treatments and decrease long-term spore survival (20).

There are a number of key questions concerning the $P_{46}\rightarrow P_{41}$ conversion process. One obvious question concerns the identity of the enzyme catalyzing this reaction. Previous work from our laboratory has provided a number of lines of evidence suggesting that P_{46} processes itself to P_{41} via an intramolecular cleavage reaction. This evidence includes the following. (i) The amino acid sequence around the site cleaved in $P_{46}\rightarrow P_{41}$ conversion is similar, although not identical, to the sequence recognized by P_{41} in its SASP substrates (17). Furthermore, SASP with sequences identical to the $P_{46}\rightarrow P_{41}$ cleavage site are cleaved rapidly by P_{41} (5). (ii) Overexpression of P_{46} ~500-fold during sporulation alters neither the timing nor the extent of $P_{46}\rightarrow P_{41}$ processing during sporulation (17). (iii) GPR variants in which the amino acid sequence in the $P_{46}\rightarrow P_{41}$ cleavage site has been changed to be more like the site cleaved by GPR in SASP undergo $P_{46}\rightarrow P_{41}$ processing much faster than wild-type P_{46}, both in vivo and in vitro (7). (iv) With purified P_{46} in vitro, addition of dimethyl sulfoxide promotes rapid conversion of P_{46} to P_{41} in a reaction whose rate is first order with respect to GPR concentration (8). (v) P_{41} alone is not sufficient to cause processing of P_{46} either in vivo or in vitro (7, 8, 17).

A second important question concerning $P_{46}\rightarrow P_{41}$ processing during sporulation is: how is this conversion regulated such

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that it takes place only ~2 h after P_{46} synthesis? While the answer to this question is not known, we have proposed (17, 21) that conversion of P_{46} to P_{41} is triggered by the major physiological changes which take place in the developing forespore late in sporulation, including decreased forespore pH (13), forespore accumulation of pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]), and forespore dehydration (6). We have further suggested that these same physiological changes in the forespore that trigger $P_{46}\rightarrow P_{41}$ conversion also preclude P_{41} attack on SASP until spore germination begins (17, 21). In order to begin to test these predictions in detail, we have examined the ability of pH, DPA, and dehydration to promote $P_{46}\rightarrow P_{41}$ conversion in vitro.

MATERIALS AND METHODS

Enzymes and reagents. The wild-type forms of P_{46} and P_{41} of *B. megaterium* GPR (P_{46}^{wt} and P_{41}^{wt}, respectively) were purified to ≥95% homogeneity from *Escherichia coli* strains overexpressing these proteins and stored at −80°C in 10 mM (P_{41}^{wt}) or 25 mM (P_{46}^{wt}) Tris-HCl (pH 7.4)–5 mM $CaCl_2$–20% glycerol as described previously (7, 17). The variant form of *B. megaterium* P_{46} (termed P_{46}^{S}) in which a serine residue is substituted for the normal valine at position 18 was also isolated and purified as described previously (7, 17). P_{46}^{S} was used for most of the experiments in this communication because previous work showed that P_{46}^{S} is processed to P_{41} more rapidly than is P_{46}^{wt}, either during sporulation or upon addition of dimethyl sulfoxide (7, 8). Consequently, we reasoned that P_{46}^{S} might autoprocess more readily to P_{41} in vitro upon stimulation by low pH, DPA, or dehydration than would P_{46}^{wt}. Chemicals used in this work were from either the Aldrich Chemical Co. or the Sigma Chemical Co. Pyridinedicarboxylic acid stock solutions were adjusted to ~pH 7 with NaOH.

Analytical procedures. The assay for GPR catalytic activity on a SASP mixture was carried out as described previously (10). Values for the catalytic activity of samples of P_{46} incubated under various conditions were compared to those for the catalytic activity of purified P_{41}^{wt}, which was set at 100%. Direct analysis of the conversion of P_{46} to P_{41} was by electrophoresis on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels, the gels being stained with Coomassie brilliant blue R (7, 9, 17). In some experiments, protein resolved by electrophoresis was transferred to polyvinylidene difluoride paper (Immobilon; Millipore Corp.), the paper was stained lightly with Coomassie blue and then destained, and appropriate bands were subjected to automated protein sequenator analysis (7, 17, 23). Protein concentrations were determined by the method of Lowry et al. (12).

Analysis of P_{46}-to-P_{41} conversion. Routine incubations for monitoring P_{46}-to-P_{41} conversion were carried out at 26°C in 50 μl of 0.1 M buffer at various pHs, containing 5 mM $CaCl_2$, 2% glycerol, 2.4 mg of P_{46}^{S} per ml, and other additions as noted for individual experiments. Control experiments showed that the presence of glycerol did not affect any of the $P_{46}\rightarrow P_{41}$ conversions studied. The buffers used for this work were the following: pH 5.1, succinate; pH 6.2, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) or 2-(*N*-morpholino) ethanesulfonic acid (MES); pH 6.6, MES; pH 7.0, Tris; and pH 7.8, Tris. The succinate, HEPES, and MES buffers were adjusted to the appropriate pH with NaOH; the Tris buffers were adjusted with HCl. The pHs of P_{46} incubation mixtures with these buffers were within 0.15 unit of the stock buffer pH. At various times during incubation, aliquots (usually 2 to 4 μl) were taken for GPR assay; dilutions (if needed) were made on ice in GPR dilution buffer, 50 mM Tris-HCl (pH 7.4)–5 mM $CaCl_2$–20% glycerol. Since P_{46} has <0.001% of the catalytic activity of P_{41} on SASP (7, 8), these assays could easily detect ~0.01% conversion of P_{46} to P_{41}. Aliquots (5 μl) for analysis of $P_{46}\rightarrow P_{41}$ conversion by SDS-polyacrylamide gel electrophoresis (PAGE) were generally diluted directly in gel-loading buffer, boiled, and cooled, and aliquots (1 to 3 μg of GPR) were analyzed by SDS-PAGE as described above. In experiments with dilute GPR or with KCl, GPR was first precipitated with trichloroacetic acid and the precipitate was rinsed three times with 80% ethanol–20% 0.1 M Tris-HCl (pH 8), dried, and then dissolved in gel-loading buffer prior to electrophoretic analysis.

Control of rH during $P_{46}\rightarrow P_{41}$ conversion. For control of relative humidity (rH) to drive $P_{46}\rightarrow P_{41}$ conversion, we constructed small, constant-rH chambers as follows. The bottoms of small (3.5-cm-diameter) plastic petri dishes were cemented to the bottom of a large (9-cm-diameter) petri dish, and the area outside the small petri dish was filled with various saturated salt solutions (plus excess salt) chosen to give different rHs at room temperature (16). Small pieces of parafilm were placed in the small petri dish, and aliquots (50 or 100 μl) of GPR incubation mixtures were placed on the Parafilm. The whole apparatus was then sealed with the top of the large petri dish and vacuum grease and incubated at room temperature. Parafilm containing the dehydrated samples was removed at various times, and the GPR was dissolved with two or three aliquots of 50 μl of protease dilution buffer prior to GPR assay or SDS-PAGE. Visual examination of the GPR droplets in these petri dishes indicated that equilibration with the vapor phase was almost complete in 24 h. Weighing of samples incubated in these chambers also showed that samples reached constant weight after about 1 day. The saturated salt solutions used in these experiments and the rH over them were as follows: NaCl, 75%; $Mg(NO_3)_2$, 54%; $MgCl_2$, 33%; and $ZnCl_2$, 10% (16).

Preparation of figures. All figures showing photographs of stained gels were generated with Adobe Photoshop Version 2.5.1 and a Macintosh computer.

RESULTS

Stimulation of $P_{46}\rightarrow P_{41}$ conversion by DPA and low pH. As noted in part previously (8, 17), prolonged incubation of P_{46}^{S} at pHs between 5.1 and 7.8 resulted in no significant (≤0.1%) generation of P_{41}, as determined either by PAGE or by assay of GPR catalytic activity (Fig. 1A and data not shown). However, addition of DPA plus Ca^{2+} resulted in significant generation of P_{41} during incubation at pH 5.1 but not at pH 6.2, 7, or 7.8 (Fig. 1B and data not shown). Ca^{2+} alone, even at 60 mM, did not cause $P_{46}\rightarrow P_{41}$ conversion at pH 5.1, and DPA without Ca^{2+} was also ineffective (data not shown). Reducing the pH of the incubations with DPA and Ca^{2+} to 4.7 also did not increase $P_{46}\rightarrow P_{41}$ conversion over that seen at pH 5.1 (data not shown). That the GPR band generated upon incubation at pH 5.1 with Ca^{2+} and DPA was truly P_{41} was shown not only by its migration at the position of authentic P_{41} but also by the fact that this new band had both the catalytic activity (Fig. 1B, lane 3) and the amino-terminal sequence (data not shown) of P_{41}. Strikingly, the stimulation of $P_{46}\rightarrow P_{41}$ conversion at pH 5.1 by DPA was specific for this isomer of pyridinedicarboxylic acid, as a number of other isomers were ineffective (Fig. 2). Similar results were obtained in this latter experiment when P_{41} generation was measured by GPR catalytic activity (data not shown). Glutamic acid (50 mM), which is also accumulated to high levels by developing forespores (14, 22), did not

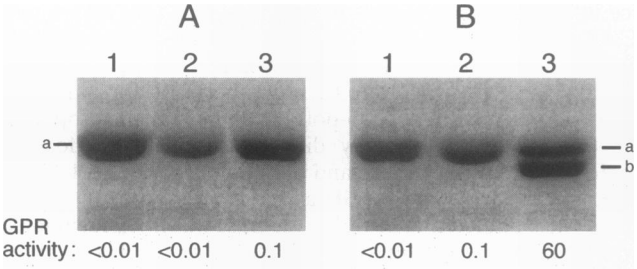

FIG. 1. Analysis of $P_{46} \rightarrow P_{41}$ conversion at various pHs with and without DPA. $P_{46}{}^{S}$ was incubated under standard conditions at pH 7.8 (lane 1), pH 6.2 (lane 2), or pH 5.1 (lane 3). (A) Incubation with 5 mM $CaCl_2$ only; (B) incubation with 50 mM DPA and 60 mM $CaCl_2$. After 18 h, aliquots were subjected to SDS-PAGE and the gel was stained with Coomassie blue. The bands labeled a and b denote P_{46} and P_{41}, respectively. The values below the lanes are the percent conversion of P_{46} to P_{41} as measured by assay of GPR catalytic activity.

promote $P_{46} \rightarrow P_{41}$ processing at pH 5.1 with $CaCl_2$ (60 mM) (data not shown).

Measurement of the kinetics of $P_{46} \rightarrow P_{41}$ conversion at pH 5.1 plus Ca^{2+}-DPA showed that this reaction was relatively linear at 40, 50, and 70 mM DPA (Fig. 3A and B and data not shown). Identical kinetics were obtained if DPA was present from time zero in this reaction or if P_{46} was preincubated for 1 h at pH 5.1 prior to addition of DPA and $CaCl_2$ (data not shown). This finding suggests that P_{46} is relatively stable at pH 5.1. Strikingly, the plot of the dependence of the rate of the conversion reaction on the DPA concentration gave a curve suggesting that DPA acted cooperatively in promoting $P_{46} \rightarrow P_{41}$ conversion (Fig. 4). This observation further suggests that DPA activates $P_{46} \rightarrow P_{41}$ conversion in an allosteric manner. Unfortunately, we could not readily test Ca^{2+}-DPA concentrations higher than 70 mM because this was the approximate limit of Ca^{2+}-DPA solubility under these conditions. Surprisingly, the rate of $P_{46} \rightarrow P_{41}$ conversion at pH 5.1 with 50 mM DPA and 60 mM $CaCl_2$ was approximately twofold slower at 37°C than it was at 26°C (data not shown).

Previous work has suggested that the GPR variant ($P_{46}{}^{S}$) with the valine-to-serine substitution at residue 18 more readily processes to P_{41} than does $P_{46}{}^{wt}$, both in vivo and in vitro (7, 8). This was also observed in the current study, as the rate of generation of P_{41} from $P_{46}{}^{wt}$ was ~10-fold slower than that from $P_{46}{}^{S}$ at pH 5.1 with Ca^{2+}-DPA (compare Fig. 3B and C). However, continued incubation of $P_{46}{}^{wt}$ under these conditions eventually resulted in >90% conversion to P_{41} (data not shown).

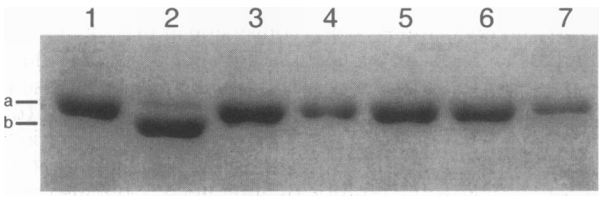

FIG. 2. Test of DPA analogs for stimulation of $P_{46} \rightarrow P_{41}$ conversion. $P_{46}{}^{S}$ was incubated under standard conditions at pH 5.1 with the following additions: lane 1, 100 mM NaCl; lane 2, 70 mM DPA and 80 mM $CaCl_2$; lane 3, 70 mM pyridine-2,3-dicarboxylic acid and 80 mM $CaCl_2$; lane 4, 70 mM pyridine-2,4-dicarboxylic acid and 80 mM $CaCl_2$; lane 5, 70 mM pyridine-2,5-dicarboxylic acid and 80 mM $CaCl_2$; lane 6, 70 mM pyridine-3,4-dicarboxylic acid and 80 mM $CaCl_2$; and lane 7, 70 mM pyridine-3,5-dicarboxylic acid and 80 mM $CaCl_2$. After 18 h, aliquots were removed and subjected to SDS-PAGE. The bands labeled a and b denote the migration positions of P_{46} and P_{41}, respectively.

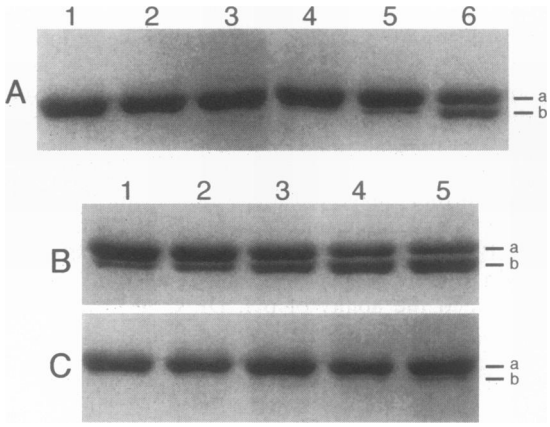

FIG. 3. Time course of conversion of $P_{46}{}^{S}$ and $P_{46}{}^{wt}$ at different DPA concentrations. (A and B) $P_{46}{}^{S}$ at 0.6 mg/ml or (C) $P_{46}{}^{wt}$ at 0.6 mg/ml was incubated at pH 5.1 with (A) 40 mM DPA and 50 mM $CaCl_2$ or (B and C) 70 mM DPA and 80 mM $CaCl_2$. At various times during incubation, samples were analyzed by SDS-PAGE. The incubation times for the samples in the various lanes were as follows: 1, 10 min; 2, 20 min; 3, 40 min; 4, 80 min; 5, 3 h; and 6, 16 h. The bands labeled a and b denote the migration positions of P_{46} and P_{41}, respectively.

The relatively linear rate of conversion of P_{46} to P_{41} at pH 5.1 plus Ca^{2+}-DPA was consistent with this reaction being intramolecular autoprocessing. This was confirmed by measuring the rate of $P_{46} \rightarrow P_{41}$ conversion at GPR concentrations from 1.5×10^{-5} to 1.5×10^{-7} M (Fig. 5). The similar rate of $P_{46} \rightarrow P_{41}$ conversion over this GPR concentration range suggests that $P_{46} \rightarrow P_{41}$ conversion is a first-order reaction, which is also consistent with it being intramolecular autoprocessing. These data cannot definitively rule out a model in which the rate-limiting step in $P_{46} \rightarrow P_{41}$ conversion is a slow conformational change in P_{46} tetramers, followed by their rapid bimolecular processing by a P_{41} tetramer. However, this model (i) requires that the K_m for interaction of a P_{41} tetramer with conformationally changed P_{46} tetramer be extremely low ($<10^{-8}$ M) and (ii) does not readily explain the generation of the first molecule of P_{41} from P_{46}. Consequently, we greatly favor a model in which conversion of P_{46} to P_{41} is an intramolecular (i.e., intratetramer) catalytic event.

Stimulation of $P_{46} \rightarrow P_{41}$ conversion by high ionic strength and dehydration. The stimulation of $P_{46} \rightarrow P_{41}$ processing by low pH and DPA was not completely surprising, as these conditions mimic those in the developing forespore at the time of $P_{46} \rightarrow P_{41}$ processing during sporulation (6, 21). An additional change in the conditions in the forespore at this time is dehydration (6, 21), and one effect that might be expected from forespore dehydration is a large increase in ionic strength. Consequently, it was not surprising that $P_{46} \rightarrow P_{41}$ processing was also stimulated by a very high concentration of NaCl (Fig. 6). Again, this stimulation was seen only at pH 5.1, not at pH 6.2 or 7. KCl at 1.5 M stimulated $P_{46} \rightarrow P_{41}$ processing, as did 1.5 M NaCl (data not shown).

In order to directly test the effect of dehydration on $P_{46} \rightarrow P_{41}$ processing, we equilibrated samples of P_{46} in closed chambers at various rHs. These samples generally reached constant weight after ~24 h. Starting solutions of $P_{46}{}^{S}$ (2.4 mg/ml) in 100 mM buffer with 50 mM DPA and 60 mM $CaCl_2$ had 14 g of water per g dry weight. This value was reduced to: (i) 0.05 g

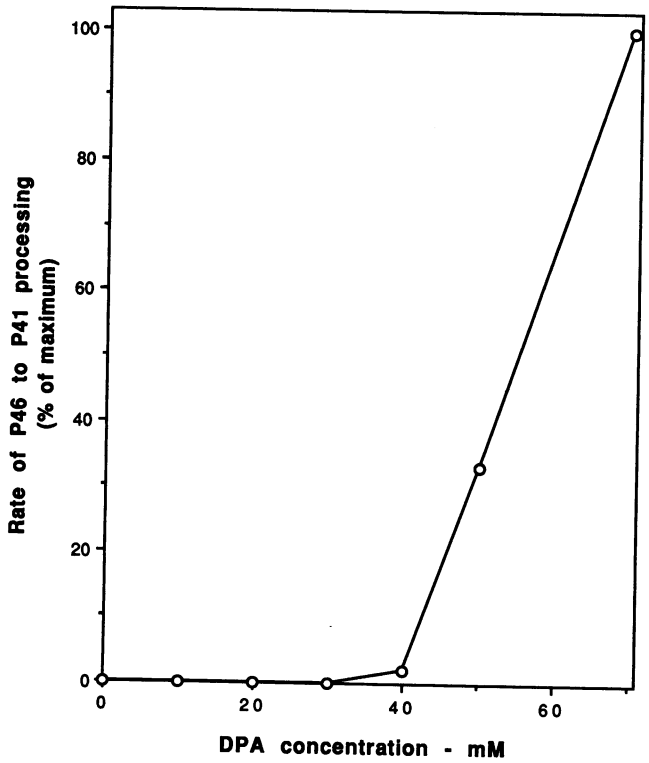

FIG. 4. Variation of the rate of $P_{46} \rightarrow P_{41}$ conversion as a function of DPA concentration. $P_{46}{}^{S}$ was incubated at pH 5.1 under standard conditions with increasing concentrations of DPA and with $CaCl_2$ concentrations to give 10 mM in excess over DPA. The rate of $P_{46} \rightarrow P_{41}$ conversion was measured as shown in Fig. 3; the rate of conversion at 70 mM DPA was set at 100%, and all other values are expressed relative to this number.

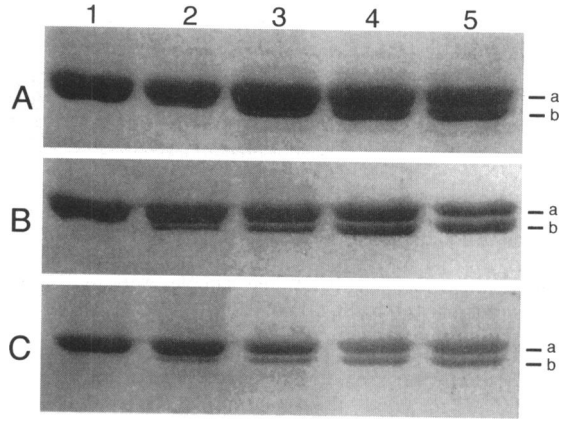

FIG. 5. Rate of $P_{46} \rightarrow P_{41}$ conversion at different GPR concentrations. $P_{46}{}^{S}$ at 2.4 mg/ml (1.5×10^{-5} M) (A), 0.24 mg/ml (1.5×10^{-6} M) (B), or 0.024 mg/ml (1.5×10^{-7} M) (C) was incubated at pH 5.1 with 50 mM DPA and 60 mM $CaCl_2$. At various times, samples were taken and analyzed by SDS-PAGE. The incubation times of the samples in the various lanes were as follows: 1, 0 min; 2, 10 min; 3, 20 min; 4, 40 min; 5, 80 min. The bands labeled a and b denote the migration positions of P_{46} and P_{41}, respectively.

of water per g dry weight upon equilibration over saturated $ZnCl_2$ (rH, 10%); (ii) 0.13 g of water per g dry weight upon equilibration over saturated $MgCl_2$ (rH, 33%); (iii) 0.32 g of water per g dry weight upon equilibration over saturated $Mg(NO_3)_2$ (rH, 54%); and (iv) 0.65 g of water per g dry weight upon equilibration over saturated NaCl (rH, 75%). Strikingly, dehydration of P_{46} at pH 6.2 plus DPA and NaCl (see below) over $Mg(NO_3)_2$ yielded a significant amount of $P_{46} \rightarrow P_{41}$ conversion in 1 to 2 days as measured by either SDS-PAGE or GPR activity (Fig. 7, lanes 4 and 5). In contrast, there was only minimal generation of P_{41} upon more extensive dehydration (i.e., over $ZnCl_2$ or $MgCl_2$ [Fig. 7, lanes 1 to 3]), and only a small amount of P_{41} was generated upon less dehydration (i.e., over NaCl [Fig. 7, lanes 6 and 7]). Analysis of the kinetics of $P_{46} \rightarrow P_{41}$ conversion under these conditions showed that over $Mg(NO_3)_2$ generation of P_{41} continued for up to 5 days, even though constant weight had been achieved in 1 day (Fig. 8). In contrast, during incubation for up to 5 days over either $ZnCl_2$ or $MgCl_2$ there was essentially no change in the level of P_{41} after the first day (Fig. 7, lanes 1 to 3 and data not shown).

Just as $P_{46} \rightarrow P_{41}$ conversion in solution was stimulated by low pH, DPA, and NaCl, these factors also greatly stimulated $P_{46} \rightarrow P_{41}$ conversion upon dehydration over $Mg(NO_3)_2$ (Fig. 9A and B). Indeed, these various components, in particular DPA and NaCl, appeared to act synergistically. Addition of DPA or NaCl to initial concentrations higher than 50 or 300 mM, respectively, gave no greater effect, and initial DPA and NaCl concentrations of $\leq$20 mM and 100 mM, respectively, resulted in significantly less processing (data not shown). We also observed the generation of a small amount of a band migrating just below P_{41} upon dehydration at pH 5.1 (Fig. 9A, lanes 2, 4, 6, and 8). Previous work (7) has shown that extended incubation of P_{41} results in intramolecular removal of an additional eight amino-terminal residues from P_{41}, generating an active form termed P_{39}. Presumably this is the band migrating below P_{41}, although this has not yet been shown directly.

DISCUSSION

Previous work has shown that $P_{46} \rightarrow P_{41}$ processing takes place during sporulation significantly after the fall in pH of the developing forespore and approximately in parallel with accumulation of DPA and forespore dehydration (13, 17). Thus it is not surprising that we found that decreased pH, increased DPA, and dehydration, as well as high ionic strength, stimulate $P_{46} \rightarrow P_{41}$ conversion in vitro. The precise contribution of these latter variables in promoting $P_{46} \rightarrow P_{41}$ conversion in vivo is, however, not completely clear. Thus, in vitro Ca^{2+}-DPA promoted $P_{46} \rightarrow P_{41}$ conversion in solution only at pH 5.1. While there is a drop of ~1.2 in forespore pH prior to P_{46} processing during sporulation, measurements of final forespore pH have given pHs of 6.3 to 6.9 (13, 18). Thus, low pH

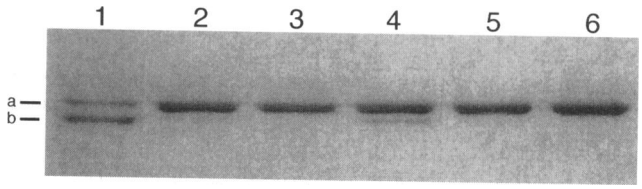

FIG. 6. Effect of NaCl on $P_{46} \rightarrow P_{41}$ conversion. $P_{46}{}^{S}$ was incubated under standard conditions at pH 5.1 (lanes 1 and 4), pH 6.2 (lanes 2 and 5), or pH 7 (lanes 3 and 6) with either 4 M NaCl (lanes 1 to 3) or 1.5 M NaCl (lanes 4 to 6). After 18 h of incubation, samples were analyzed by electrophoresis on an SDS-polyacrylamide gel. The bands labeled a and b denote the migration positions of P_{46} and P_{41}, respectively.

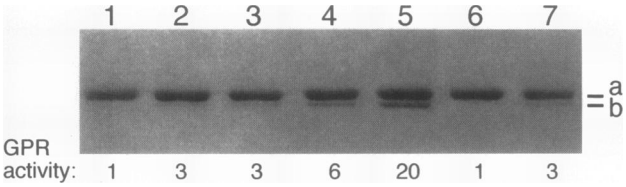

FIG. 7. Effect of dehydration to various rH values on $P_{46}\rightarrow P_{41}$ conversion. Aliquots (100 μl) of P_{46}^{S} at pH 6.2 with 100 mM NaCl, 50 mM DPA, and 60 mM $CaCl_2$ were dehydrated in environments of varying rH for various lengths of time, samples were redissolved, and aliquots were analyzed by SDS-PAGE or by GPR assay. The samples analyzed in the various lanes were as follows: 1, dried over $ZnCl_2$ for 3 days; 2 and 3, dried over $MgCl_2$ for 1 and 2 days, respectively; 4 and 5, dried over $Mg(NO_3)_2$ for 1 and 2 days, respectively; and 6 and 7, dried over NaCl for 1 and 2 days, respectively. The bands labeled a and b denote the migration positions of P_{46} and P_{41}, respectively. The values below the lanes are the percent conversion of P_{46} to P_{41} as determined by assay of GPR catalytic activity.

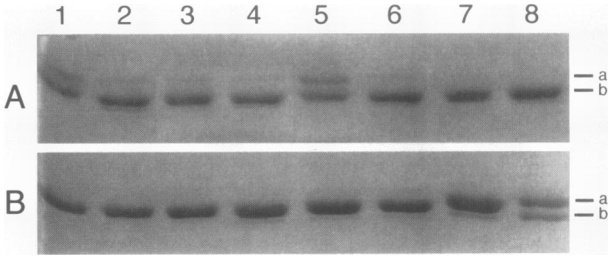

FIG. 9. Effect of pH, DPA, and NaCl on $P_{46}\rightarrow P_{41}$ conversion upon dehydration. Aliquots (50 μl) of P_{46}^{S} at either pH 5.1 (A) or pH 6.6 (B) were dehydrated over $Mg(NO_3)_2$. The samples in lanes 1 and 2 had only 5 mM $CaCl_2$, samples in lanes 3 and 4 had 50 mM DPA and 60 mM $CaCl_2$, samples in lanes 5 and 6 had 300 mM NaCl and 5 mM $CaCl_2$, and samples in lanes 7 and 8 had 300 mM NaCl, 50 mM DPA, and 60 mM $CaCl_2$. After dehydration for either 1 day (lanes 1, 3, 5, and 7) or 2 days (lanes 2, 4, 6, and 8), samples were redissolved and aliquots were analyzed by SDS-PAGE. The bands labeled a and b denote the migration positions of P_{46} and P_{41}, respectively.

and DPA alone cannot explain P_{46} processing in vivo, although certainly the low forespore pH seems likely to be necessary for this processing.

Although DPA in combination with Ca^{2+} does not promote significant $P_{46}\rightarrow P_{41}$ conversion in vitro at pH 6.2 to 8, studies in vivo have shown that *B. subtilis* mutants that cannot accumulate high DPA levels in the forespore do not process P_{46} to P_{41} (17). Consequently, the stimulation of rapid $P_{46}\rightarrow P_{41}$ processing in vitro by DPA, even though it occurred only at pH 5.1, seems most significant. Despite its identification as a predominant spore component almost 40 years ago (15), precise roles for DPA in spores have remained obscure (6). Our observation of the stimulation of $P_{46}\rightarrow P_{41}$ conversion by DPA, which is specific to this isomer of pyridinedicarboxylic acid, provides the first specific role for DPA in sporulation. The fact that the rate of $P_{46}\rightarrow P_{41}$ conversion shows such a cooperative increase as a function of DPA concentration suggests that DPA stimulates this reaction in an allosteric manner, presumably by inducing a conformational change in P_{46}. An alternative explanation, that DPA promotes tetramer formation which in turn accelerates $P_{46}\rightarrow P_{41}$ conversion, seems quite unlikely, since $P_{46}\rightarrow P_{41}$ conversion is a first-order reaction. Unfortunately, no data are currently available on the association constant for tetramer formation in GPR, and it is possible that at the lowest GPR concentration tested in this experiment the enzyme is fully in the tetramer form. However, that GPR is an allosteric enzyme is certainly consistent with this enzyme being a tetramer (10). Presumably the cooperative response of $P_{46}\rightarrow P_{41}$ conversion to increasing DPA concentrations ensures that rapid P_{41} generation takes place only when massive DPA uptake, along with forespore dehydration, is taking place (6, 21). We also note that DPA, which appears to exist in spores as a chelate with a divalent cation, predominantly Ca^{2+}, is present in amounts which would give ≥1 M DPA if it were soluble (6). The precise state of DPA in spores is, however, unclear at present.

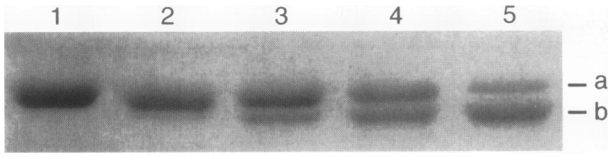

FIG. 8. Time course of $P_{46}\rightarrow P_{41}$ conversion at 54% rH. Aliquots (100 μl) of P_{46}^{S} solutions were incubated at pH 6.2 with 50 mM DPA and 60 mM $CaCl_2$ over a saturated $Mg(NO_3)_2$ solution giving an environment of 54% rH. At various times, samples were redissolved and aliquots were analyzed by SDS-PAGE. The time of incubation of the samples in the various lanes was as follows: 1, time zero; 2, 1 day; 3, 2 days; 4, 3 days; and 5, 5 days. The bands labeled a and b denote the migration positions of P_{46} and P_{41}, respectively.

The third variable that promoted $P_{46}\rightarrow P_{41}$ processing in vitro was dehydration, which would also be expected to increase the ionic strength (which also stimulated processing). Strikingly, extreme dehydration (over $ZnCl_2$ or $MgCl_2$) caused very little $P_{46}\rightarrow P_{41}$ conversion, and this small amount of conversion did not increase with time. We interpret these results to indicate that processing takes place only while dehydration at these low rHs takes place but stops once a very low water content is reached. Presumably under these latter conditions there is not sufficient free water to allow continued enzyme action or protein movement. In contrast, at an intermediate rH (54%), $P_{46}\rightarrow P_{41}$ conversion proceeds continuously over 5 days, while at higher rHs (75% rH or in solution) there is not sufficient dehydration to trigger processing. Measurements of the amount of water in the core of dormant spores have given values of 0.25 to 0.5 g/g dry weight, depending on the species (6). These values are in the range that we found gave reasonable $P_{46}\rightarrow P_{41}$ conversion in vitro. We note, however, that both in vivo and in our in vitro system we have no good estimate of the amount of water associated with GPR nor of the amount of free water.

An obvious question concerning $P_{46}\rightarrow P_{41}$ processing is what changes in the protein are involved in this reaction. Previous work (8) has shown that there is a significant conformational change associated with the $P_{46}\rightarrow P_{41}$ conversion, and this processing is stimulated by dimethyl sulfoxide, which is known to cause conformational changes in proteins (1). This is further supported in the current work, as dehydration, which can also cause conformational changes in proteins (2–4), also promoted $P_{46}\rightarrow P_{41}$ conversion. The precise nature of the conformational change required for $P_{46}\rightarrow P_{41}$ conversion is by no means clear, but we speculate that it requires disruption of hydrophobic interactions. This speculation is supported by at least three pieces of evidence. (i) P_{46}^{S}, in which a hydrophilic serine has been substituted for a hydrophobic valine, processes to P_{41} much more readily than does P_{46}^{wt}. (ii) Dimethyl sulfoxide, which can disrupt hydrophobic interactions (1), readily stimu-

lates $P_{46} \rightarrow P_{41}$ conversion. (iii) $P_{46} \rightarrow P_{41}$ conversion is faster at 26°C than at 37°C, as might be expected for a reaction which requires disruption of hydrophobic interactions.

A second question about $P_{46} \rightarrow P_{41}$ processing concerns the precise contribution in vivo of the three (or four) variables which can influence $P_{46} \rightarrow P_{41}$ processing in vitro. While it is clear that P_{46} can process itself to P_{41} rapidly enough in vitro to account for $P_{46} \rightarrow P_{41}$ conversion in vivo, to date this has been observed only at rather low pH or by addition of dimethyl sulfoxide (8). Under more physiological conditions (i.e., during dehydration), the reaction with $P_{46}{}^{S}$ is still rather slow, and it is even slower with $P_{46}{}^{wt}$. Presumably the hydrophobic valine at residue 18 in $P_{46}{}^{wt}$ makes the processing reaction more unfavorable than with $P_{46}{}^{S}$. Consequently, rapid processing of $P_{46}{}^{wt}$ may require higher concentrations of DPA or a lower pH than does $P_{46}{}^{S}$. Clearly, our in vitro conditions, in particular our mode of dehydration, do not duplicate those in the forespore during the period of GPR processing. However, it is also possible that there are additional effectors we have not yet identified.

A final point concerns the reasons that P_{41} once generated during sporulation does not attack SASP in the developing or dormant spore. The results with $P_{46} \rightarrow P_{41}$ processing may prove most informative in this regard as they suggest that when P_{46} is dehydrated to a very low water content there is minimal $P_{46} \rightarrow P_{41}$ conversion, presumably only as the sample passes through a more optimum water content. However, once the sample has reached a very low water content, there is no further processing. This suggests that in vivo P_{41} may be generated rapidly as the forespore is dehydrating and can act only slowly on SASP under these conditions. However, as rapid forespore dehydration continues, the even more dehydrated P_{41} is then completely inactive on SASP, which are thus stable indefinitely in the dormant spore. This idea is currently being tested in vitro. We also note that any P_{46} not converted to P_{41} during rapid forespore dehydration will not subsequently be converted to P_{41} once full forespore dehydration has been achieved. This would explain the fact that normally approximately 20 to 40% of P_{46} is not converted to P_{41} during sporulation (11, 17).

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