Characterization and Regulation of *p*-Aminobenzoic Acid Synthase from *Streptomyces griseus*

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p-Aminobenzoic acid synthase (PABA synthase) of *Streptomyces griseus* catalyses the conversion of chorismic acid to *p*-aminobenzoic acid (PABA), a precursor of the aromatic *p*-aminoacetophenone moiety of candicidin, a polyene macrolide antibiotic. This enzyme uses glutamine or ammonia as amino donors for PABA formation. Enzyme extracts converted [¹⁴C]chorismic acid to labelled PABA. PABA synthase was present in *S. griseus* IMRU 3570 only during the antibiotic producing phase. No detectable levels of the enzyme were found in cell-free extracts of nonproducing mutants of *S. griseus* obtained after UV mutagenesis. PABA synthase activity was found also in *Streptomyces coelicolor* var. *aminophilus*, producer of the polyene macrolide antibiotic fungimycin, but it was not present in extracts of several other streptomycetes that do not produce aromatic polyene macrolide antibiotics. PABA synthase (amidotransferase) activity was partially purified by DEAE-Bio-gel and Sephacryl S-200 filtrations. The estimated molecular weight was 50000. PABA synthase was repressed by aromatic amino acids and PABA but not by anthranilic acid. Inorganic phosphate strongly repressed but did not inhibit PABA synthase activity.

INTRODUCTION

Candicidin is an aromatic polyene antifungal antibiotic produced by *Streptomyces griseus* IMRU 3570. It contains the amino sugar mycosamine (3-amino-3,6-dideoxy-D-mannopyranose) attached glycosidically, and a *p*-aminoacetophenone moiety that forms part of the same carbon skeleton as the macrolide ring (Zielinski *et al.*, 1979).

The *p*-aminoacetophenone aromatic moiety appears to serve as starter for the head-to-tail condensation of malonyl-CoA and methylmalonyl-CoA in the biosynthesis of the macrolide ring of candicidin (Martín & McDaniel, 1975*b*; Martín, 1977). This aromatic moiety is formed from chorismic acid via *p*-aminobenzoic acid (PABA). PABA is a precursor for the biosynthesis of candicidin as can be concluded from the following evidence: (a) the intact ring and the carboxyl group of PABA are incorporated into candicidin with a high efficiency (Liu *et al.*, 1972; Martín & Liras, 1976); (b) the inhibitory effect of tryptophan on candicidin biosynthesis is partially reversed by PABA, suggesting that the effect of tryptophan is due to starvation of the cell for PABA, thus preventing candicidin biosynthesis (Gil *et al.*, 1980).

A large amount of PABA (up to 430 µg ml⁻¹) must be synthesized in cultures of *S. griseus* which are able to produce up to 4000 µg candicidin ml⁻¹. The branch-point intermediate of the aromatic pathway giving rise to PABA in bacteria is chorismic acid. In *Escherichia coli*, *Enterobacter aerogenes* and *Bacillus subtilis*, and in the fungus *Neurospora crassa*, chorismic acid

Abbreviations: DMG buffer, dithiothreitol/magnesium chloride/glutamine/glycerol buffer; PLD medium, phosphate-limited defined medium; SPG medium, soya peptone/glucose medium; YED medium, yeast extract/glucose medium; PABA, *p*-aminobenzoic acid.

is converted to PABA, in the presence of L-glutamine as amino donor, by PABA synthase (Gibson *et al.*, 1964; Altendorf *et al.*, 1971; Kane & O'Brien, 1975). The relationship of PABA synthase to anthranilate synthase in *S. griseus* is intriguing. The *pabA* gene (PABA synthase) of *E. coli* has been sequenced and shown to be related to the *trp(G)D* gene of *E. coli* (Kaplan & Nichols, 1983). The *pab* gene of *S. griseus* has been cloned (Gil & Hopwood, 1983) and its expression is being studied in our laboratory.

It is therefore of interest to purify this enzyme and to study its regulatory pattern in *S. griseus*. The results reported here indicate that PABA synthase behaves as a typical enzyme of secondary metabolism and was detected only in strains producing aromatic polyene macrolides containing a *p*-aminoacetophenone moiety.

METHODS

Micro-organisms and growth conditions. The strains used in these studies were *Streptomyces griseus* IMRU 3570 and mutants derived from it. Non-producer mutants were obtained by UV light (260 nm) irradiation using a Desaga UV lamp located at a distance of 10 cm above the spore suspension. Under these conditions 0.1% of spores survived after 3 min of UV treatment. Non-producing mutants were selected among the surviving 0.1%. *S. coelicolor* M145, *S. coelicolor* 2280 (*pab*⁻) and *S. lividans* 66 were kindly provided by D. A. Hopwood, John Innes Institute, Norwich, UK. *S. griseus* NRRL 3851, a cephamycin-producing strain, was obtained from NRRL. *S. coelicolor* var. *aminophilus* IMRU 3865, the fungimycin producer, was supplied by L. E. McDaniel, Rutgers University, New Brunswick, NJ, USA. *S. lividans* JG10, a PABA auxotroph, was obtained from *S. lividans* TK24 by NTG mutation (Gil & Hopwood, 1983).

Cultures were grown aerobically in a medium supporting high antibiotic production which contained soya peptone (25 g l⁻¹), glucose (65 g l⁻¹) and zinc sulphate (0.5 mM) (SPG medium; Martin & McDaniel, 1975*a*). The cultures were incubated at 32 °C in 250 ml triple-baffled flasks, containing 50 ml SPG medium, in a Gallenkamp orbital shaking incubator at 225 r.p.m. The production flasks were inoculated with 5 ml of a 24 h inoculum grown in YED medium containing yeast extract and glucose (both 10 g l⁻¹). Candidicin production was determined spectrophotometrically at 380 nm after extraction with butanol (Gil *et al.*, 1980).

Preparation of phosphate-limited resting cells. Candidicin-producing cells grown in SPG medium for 24 h were collected, washed twice with sterile saline (0.8% NaCl) and resuspended at 5 mg dry cell weight ml⁻¹ in a phosphate-limited defined (PLD) medium containing (mg ml⁻¹): glucose, 50; L-asparagine, 10; ZnSO₄·7H₂O, 0.02; MgSO₄·7H₂O, 0.2; FeSO₄·7H₂O, 0.02; and CuSO₄·5H₂O, 0.02. Unless otherwise indicated, no phosphate was added to the medium. In phosphate-supplemented media, 5 mM-K₂HPO₄ was used.

A 5 ml sample of the cell suspension was incubated in 50 ml triple deep-baffled flasks (Bellco Glass Inc., Vineland, NJ, USA) at 250 r.p.m. on an orbital shaking incubator (Gallenkamp).

Preparation of cell-free extracts. Cells grown in YED and SPG media, and incubated in the PLD medium or in minimal medium (MM; Hopwood, 1969) were harvested at different times of incubation (indicated in the text), washed with saline solution at 4 °C and stored as cell paste over liquid nitrogen. The frozen cells were resuspended in 10 mM-potassium phosphate buffer, pH 6.8, containing 1 mM-dithiothreitol, 10 mM-MgCl₂, 20 mM-L-glutamine and 30% (v/v) glycerol (DMG buffer) at 20 °C. In some experiments glutamine was not added to the buffer (see text). The cells were washed twice with cold (4 °C) DMG buffer and disrupted by treatment with glass beads for 2 min in a CO₂-refrigerated MSK homogenizer (Braun, Melsungen, FRG). Cell debris was removed by centrifugation at 30000 g for 30 min at 4 °C. The clear supernatants were dialysed overnight at 4 °C against DMG buffer or filtered through Sephadex G-25 previously equilibrated with cold DMG buffer. Protein was measured by the Lowry method with bovine serum albumin as standard and appropriate blanks to correct the interference of glycerol (Zishka & Nishimura, 1970).

PABA synthase assay. The reaction mixture for PABA synthase (amidotransferase) activity contained 50 µmol Tris/HCl, pH 9.0, 10 µmol MgSO₄, 20 µmol L-glutamine and 50 µmol potassium chorismate in a final volume of 1 ml. Concentrations of protein (enzyme) varied between 1.0 and 5.0 mg ml⁻¹. Aminase activity was measured using 100 µmol NH₄Cl as amino donor instead of L-glutamine, in a buffer without glutamine. Reaction mixtures were incubated at 37 °C for 30 min and the PABA formed was measured by diazotization using a modification of the Bratton-Marshall technique (Jones & Westlake, 1974), or spectrophotofluorimetrically (Kane & O'Brien, 1975). Standard curves were prepared with pure PABA (Sigma). One unit of PABA synthase is defined as the amount of enzyme that produced 1 nmol PABA per 30 min. Specific activity is expressed as units per mg protein.

In experiments using ¹⁴C-labelled chorismic acid, the product of the reaction ([¹⁴C]PABA) was separated from anthranilic acid by TLC on silica gel G using benzene/ethanol (80:20, v/v) as solvent. PABA and anthranilic acid were detected with ninhydrin (0.1%, w/v) in acetone. In this system, PABA and anthranilic acid moved with *R_F* values of 0.43 and 0.54, respectively.

Preparation of partially purified PABA synthase. PABA synthase activity was partially purified by two sequential fractionation treatments. All the manipulations were carried out at 4 °C. The dialysed cell extract was chromatographed on a DEAE-Bio-gel (Bio-Rad) column (30 × 2.5 cm). The column was washed with 500 ml DMG buffer followed by 400 ml of a linear gradient of NaCl (0 to 1 M) in DMG buffer. The fractions of highest activity were pooled, dialysed, placed on a Sephacryl S-200 (Pharmacia) column (43 × 3.5 cm) and eluted with DMG buffer. The active fractions were used as partially purified enzyme. The molecular weight of PABA synthase (amidotransferase activity) was estimated by gel filtration on Sephacryl S-200. The enzyme was stable when intact cells were stored over liquid nitrogen, but it lost 50% of its activity in one day when purified preparations were kept at 4 °C. The stability of the enzyme was increased by glycerol addition (see Results).

Chemicals. Chorismic acid was prepared as the ammonium salt from the culture supernatant of *Enterobacter aerogenes* 62-1 as described by Gibson (1970). Ammonium chorismate was converted into the potassium salt before use. [^{14}C]Chorismic acid [4.5 μCi (166.5 kBq) nmol^{-1}] was prepared by the same method using [^{14}C]glucose [230 mCi (8.5 GBq) mmol^{-1}] as substrate. All other chemicals were obtained from commercial sources and were of the highest purity available.

RESULTS

Properties of PABA synthase in cell-free extracts

Properties of PABA synthase were examined initially using a dialysed cell-free extract prepared from *S. griseus* IMRU 3570 grown for 20 h in SPG medium. Under these conditions the enzyme had an absolute requirement for chorismic acid and either glutamine (amidotransferase activity) or ammonia (aminase activity). The amidotransferase was greater than the aminase activity by a factor of about five (an average of eight different experiments) and the optimum pH for both activities *in vitro* was 9.0 (Fig. 1). The product of the reactions was identified as PABA by TLC.

The amidotransferase activity of PABA synthase depended on the concentration of Mg^{2+} in the reaction mixture. An increase in enzyme activity was observed up to 3 mM- MgSO_4 . PABA synthase activity was partially inhibited by Na^+ , K^+ , Ca^{2+} and Fe^{2+} at 5 mM and completely inhibited by the same concentration of Mn^{2+} , Ba^{2+} , Zn^{2+} , Hg^{2+} , Cu^{2+} and Fe^{3+} . The inhibitory effect exerted by these ions was totally (e.g. Na^+ , K^+ , Ca^{2+} , Ba^{2+} , Fe^{3+}) or partially reverted (e.g. Mn^{2+} , Fe^{2+}) by 5 mM- Mg^{2+} . Addition of EDTA at concentrations above 2 mM completely inhibited the enzyme activity.

In crude cell-free extracts prepared in buffer without glutamine, glycerol or Mg^{2+} , PABA synthase activity was stable for several weeks when stored at -20 °C or on liquid nitrogen. However, it lost its activity within 24 h when stored at 4 °C. The addition of glycerol (up to 30%) to the cell-disruption buffer greatly enhanced the stability of the enzyme *in vitro*. Addition of glycerol directly to the PABA synthase reaction did not stimulate enzyme activity. Glutamine and Mg^{2+} also enhanced the stability of the enzyme.

In vitro conversion of [^{14}C]chorismic acid to PABA

To prove that PABA synthase and not anthranilate synthase was being measured, experiments were done with [^{14}C]chorismic acid (1000 c.p.m. in 100 nmol) as substrate, prepared as indicated in Methods. Reaction products were extracted with ethyl acetate (pH 4.7) and separated by TLC (see Methods). Formation of [^{14}C]PABA occurred, but no radioactive anthranilic acid was found under the conditions used.

Time-course of enzyme activity during batch cultures

PABA synthase activity was induced when *S. griseus* was grown on SPG, a medium that supported good candicidin production. The amidotransferase activity increased rapidly, reaching a maximum at 18 to 20 h, and declined thereafter to lower levels (Fig. 2). The aminase activity was about five times lower at all times. Candicidin production in SPG medium started at 15 h, coinciding with the time of appearance of PABA synthase activity. A similar pattern of enzyme formation and disappearance has been found in several enzymes of secondary metabolism (Martín & Demain, 1980). No PABA synthase activity was detected in cells grown in YED (Fig. 2) or minimal medium (not shown), two media that do not support candicidin biosynthesis.

Fig. 1. Optimal pH of amidotransferase and aminase activities of PABA synthase in crude extracts. Amidotransferase (○) and aminase (●) activities were determined as indicated in Methods, in extracts of cells grown in SPG medium for 20 h. Tris/maleate buffer (10 mM) was used in the pH range 5.5 to 6.5. Tris/HCl (10 mM) in the pH range 7.2 to 9.0 and carbonate buffer (10 mM) in the pH range 9.5 to 10.5.

Partial purification and enzyme kinetics of PABA synthase

PABA synthase was purified from batches of *S. griseus* grown in SPG medium for 20 h and disrupted as indicated in Methods. Enzyme extracts (280 mg protein in 70 ml) were dialysed against DMG buffer for 10 h at 4 °C with buffer changes every 2 h. The dialysed extracts were chromatographed on DEAE-Bio-gel followed by elution with a linear gradient of 0 to 1 M-NaCl in DMG buffer. The enzyme activity was eluted as a single symmetrical peak at a NaCl concentration of 0.6 M. The eluted enzyme was dialysed against DMG buffer for 2 h, concentrated by batchwise absorption on a small DEAE-Bio-gel column and eluted with 0.8 M-NaCl in DMG buffer. After a second dialysis the enzyme was chromatographed on a Sephacryl S-200 gel. The molecular weight of the enzyme was estimated to be 50 000 from its elution behaviour after gel filtration on Sephacryl S-200 as compared with standards of known molecular weight (Fig. 3). Purification of the enzyme was achieved only for the amidotransferase activity because the aminase activity was undetectable after column chromatography, even in the presence of MgCl_2 or glycerol (Table 1).

Double reciprocal plots were made of the reaction velocity of PABA synthase against glutamine concentration at five potassium chorismate concentrations (0.015–0.05 mM) and against potassium chorismate concentration at four glutamine concentrations (5–40 mM). Both series of plots gave sets of convergent lines, suggesting that the reaction proceeds by a sequential mechanism. The apparent K_m values for chorismic acid and for L-glutamine were 6.6×10^{-5} M and 7.1×10^{-3} M respectively.

Regulation of PABA synthase

Inhibition. *In vitro* PABA synthase (amidotransferase) activity from *S. griseus* was not subject to product inhibition by PABA. It was also insensitive to inhibition by anthranilic acid, aromatic amino acids, phosphate, ATP, glucose 6-phosphate or fructose 1,6-diphosphate.

Repression by aromatic amino acids. The effect of aromatic amino acids on PABA synthase was studied using phosphate-limited resting cells of *S. griseus*. Aromatic amino acids were added immediately following their suspension in PLD medium and samples were taken 8 h later. The results shown in Fig. 4 indicate that tyrosine, phenylalanine and tryptophan repressed the formation of PABA synthase. The extent of this repression was greater than the reduction of candidicin biosynthesis, probably because we measured total candidicin accumulated during 8 h and not the rate of antibiotic biosynthesis at a specific time.

Regulation by anthranilic acid and PABA. Candidicin biosynthesis was clearly reduced by addition of anthranilic acid and PABA which might be due to repression of PABA synthase.

Fig. 2

Fig. 3

Fig. 2. Time-course of PABA synthase (amidotransferase) activity (●) and candidicin biosynthesis (○) during batch cultures of *S. griseus*. PABA synthase activity was determined in cell-free extracts prepared from cells taken at different times from the inoculum (YED) or production (SPG) medium.

Fig. 3. Determination of the molecular weight of PABA synthase (amidotransferase) activity by gel filtration on Sephacryl S-200 previously calibrated with proteins of known molecular weight (●): A, bovine albumin (66000); O, ovalbumin (46000); L, L-lysozyme (14600); C, cytochrome *c* (12400). P, PABA synthase (○).

Table 1. *Partial purification of PABA synthase of S. griseus*

Purification step	Volume (ml)	Activity (units ml ⁻¹)	Protein (mg ml ⁻¹)	Specific activity (units mg protein ⁻¹)	Purification (fold)	Percentage recovery
Cell extract	70	38	4	9.5	—	100
DEAE-Bio-gel	20	56	2.4	23.4	2.45	49
Sephacryl S-200	16	8	0.064	125	13.15	5.6

Addition of exogenous PABA to the culture decreased the PABA synthase in the cells by 67%, but anthranilic acid had no effect. On the other hand, candidicin production was decreased by addition of anthranilic acid (52%) and PABA (27%). Similar experiments were done on resting cells to avoid interference by growth-related factors. Under these conditions, candidicin biosynthesis was inhibited (30%) by anthranilic acid, but the activity of PABA synthase was again not reduced. Since anthranilic acid inhibited candidicin production but did not repress PABA synthase, it seems that its effect was exerted at a different level.

Control of PABA synthase formation by phosphate

The well-known inhibitory effect of inorganic phosphate on candidicin biosynthesis (Martín & Demain, 1976) was correlated with the reduction of PABA synthase both in SPG and in PLD suspension medium. Increasing phosphate concentrations had a higher inhibitory effect on candidicin biosynthesis and PABA synthase activity (Fig. 5). Potassium phosphate at 5 to 10 mM almost completely repressed both PABA synthase activity and candidicin biosynthesis.

PABA synthase was derepressed by potassium phosphate starvation. Cells grown for 24 h in SPG medium supplemented with 5 mM-potassium phosphate (in which both PABA synthase and candidicin are repressed) were transferred to phosphate-limited and phosphate-supplemented (5 mM) PLD medium (Fig. 6). Transfer to phosphate-limited medium resulted in an almost immediate formation of PABA synthase and accumulation of candidicin, whereas transfer to PLD medium supplemented with 5 mM-phosphate resulted in neither derepression of enzyme nor formation of candidicin.

Fig. 4

Fig. 5

Fig. 4. Repression of PABA synthase by aromatic amino acids. Resting cell cultures of *S. griseus* in PLD medium were supplemented with either 10 mM-L-tyrosine (Tyr), L-phenylalanine (Phe) or L-tryptophan (Trp). Controls (C) were not supplemented. Candidicin biosynthesis (▨) and PABA synthase activity (□) were determined after 8 h incubation; results are expressed as $\mu\text{g ml}^{-1}$ and units mg protein^{-1} , respectively.

Fig. 5. Repression by phosphate of PABA synthase activity (□) and candidicin biosynthesis (▨). Cultures were incubated for 20 h in phosphate-supplemented or unsupplemented SPG medium. Results are expressed as $\mu\text{g (mg dry wt)}^{-1}$ for candidicin biosynthesis and units mg protein^{-1} for PABA synthase activity.

Fig. 6. Derepression of PABA synthase activity following phosphate starvation. Cells of *S. griseus* incubated in SPG medium supplemented with 5 mM-phosphate [in which both PABA synthase activity (○) and candidicin formation (△) were repressed] were collected, washed in saline solution and transferred to phosphate-free (●, ▲) and phosphate-supplemented (5 mM) (○, △) PLD medium at 0 h (arrow). △, ▲, Candidicin formed; ○, ●, PABA synthase activity.

PABA synthase activity in non-producing mutants of S. griseus and in different Streptomyces strains

A total of 32 non-producing mutants of *S. griseus* were obtained after screening 25000 colonies obtained by treatment with UV light for lack of formation of an inhibition zone against *Candida utilis*; they produced no candidicin in SPG medium as assayed by either spectrophotometry or TLC. All possible pairs of mutants were unable to produce antibiotic by cosynthesis in solid medium. None of these mutants were auxotrophs, as tested in PABA-free defined medium solidified with pure agarose (Sigma). Under these conditions, PABA auxotrophs of *S. lividans* JG10 and *S. coelicolor* 2280 failed to grow. Cell-free extracts of the non-producing mutants grown in SPG medium prepared at different times of the growth cycle had no detectable PABA synthase. Residual PABA synthase activity below the detection level (0.5 units) might, however, be sufficient to form the amount of PABA required for folic acid synthesis.

In order to test whether PABA synthase is involved exclusively in secondary metabolism or is also involved in vitamin synthesis, we assayed the enzyme activity in cell-free extracts from various prototrophic *Streptomyces* species grown in SPG medium. We could not detect PABA synthase throughout the culture cycle in *S. griseus* NRRL 3851 (producer of cephamycins A and B), *S. lividans* 66 or *S. coelicolor* M145. However, PABA synthase activity was found in *S. coelicolor* var. *aminophilus*, the fungimycin producer. Fungimycin is an aromatic polyene antibiotic similar to candicidin, containing an *n*-methyl-*p*-aminoacetophenone moiety instead of *p*-aminoacetophenone.

DISCUSSION

PABA synthase activity exhibited both aminase and amidotransferase activities in crude extracts. Enzyme activity with glutamine as amino donor was 5- to 10-fold higher than with ammonia. The aminase activity could not be assayed in purified preparations because of its high lability. Several microbial enzymes that transfer amino groups may act as amidotransferases or aminases (Buchanam, 1973). Anthranilate synthase of *S. griseus* IMRU 3570 had a similar optimal pH (8.5) and temperature (30 °C) to PABA synthase but it differs in having mainly aminase rather than amidotransferase activity (G. Naharro & J. F. Martín, unpublished results). The amidotransferase activity of the PABA synthase of *Bacillus subtilis* is 5- to 10-fold higher than the aminase activity (Kane & O'Brien, 1975), as we observed in *S. griseus*, whereas the anthranilate synthase of a chloramphenicol-producing strain of *Streptomyces venezuelae* has similar amidotransferase and aminase activities (Francis *et al.*, 1978). Sensitivity of PABA synthase to EDTA and its activation by Mg²⁺ ions have also been reported for the PABA synthase of *B. subtilis* (Kane & O'Brien, 1975). PABA synthase activity in *S. griseus* was about seven times higher at maximal levels than in *E. coli* HB101 (8.2 and 1.2 units mg protein⁻¹, respectively) (A. Rebollo, P. Liras, J. A. Gil & J. F. Martín, unpublished results) and about 1.5- to 2-fold higher than in *B. subtilis* (Kane & O'Brien, 1975).

The time-course of PABA synthase activity follows a pattern similar to that of most other enzymes of secondary metabolism (Martín & Demain, 1980). No PABA synthase activity was found in the phosphate-rich YED medium used for inoculation. PABA synthase activity in SPG medium was first detected 12 to 15 h after inoculation, coinciding with the time of depletion of inorganic phosphate in the medium and a sharp drop in the rate of RNA synthesis (Liras *et al.*, 1977).

PABA synthase, purified by gel filtration on DEAE-Bio-gel and Sephacryl S-200, had an approximate molecular weight of 50000. This compares with the PABA synthase of *B. subtilis* which is composed of two non-identical subunits A (mol. wt 31000) and X (mol. wt 19000) (Kane & O'Brien, 1975), but is slightly smaller than the PABA synthase of *E. coli* which consists of two subunits of molecular weight 48000 and 9000, respectively (Huang & Gibson, 1970), although the second (amidotransferase) subunit has recently been shown to contain 187 amino acids and have a molecular weight of 20752 (Kaplan & Nichols, 1983). In *B. subtilis* and *E. coli*, PABA synthase activity was separated into two peaks by gel filtration. However, PABA synthase of *S. griseus* could not be separated into two active fractions. This result correlates with the finding that one single 3.5 kb DNA fragment carrying the PABA synthase gene of *S. griseus* complements both *E. coli* mutants *pabA* and *pabB* that are located at 40 and 74 min of the *E. coli* map (Gil & Hopwood, 1983), although it does not exclude the possibility of two linked genes existing in this fragment (Martín & Gil, 1984).

An important result in relation to the control of antibiotic biosynthesis is that PABA synthase is strongly repressed, but not inhibited, by phosphate. Neither ATP, fructose 1,6-diphosphate or glucose 6-phosphate had any detectable inhibitory effect on PABA synthase activity *in vitro*. Derepression of PABA synthase occurred following a shiftdown in phosphate concentration (Fig. 6). Inorganic phosphate is a crucial growth-limiting nutrient for biosynthesis of polyene macrolide antibiotics (Martín, 1977). The onset of candicidin biosynthesis is triggered by phosphate depletion (Liras *et al.*, 1977). Phosphate repression of PABA synthase explains the

lack of enzyme activity in inoculum cultures grown in YED (a phosphate-rich medium) and the delay in the onset of candicidin biosynthesis in cultures supplemented with high concentrations of phosphate (Liras *et al.*, 1977). Only when phosphate is depleted does formation of PABA synthase, leading to candicidin biosynthesis, occur (Fig. 2).

PABA synthase activity was not detected in several other species of *Streptomyces*, including strains of *S. griseus* that produce cephamycin instead of candicidin. However, this enzyme was found in *S. coelicolor* var. *aminophilus* which produces fungimycin, an aromatic polyene antibiotic similar to candicidin. Hybridization of total DNA of several *Streptomyces* species with a probe carrying the PABA synthase (*pab*) gene from *S. griseus* (Gil & Hopwood, 1983) suggests that this gene is absent in *Streptomyces* species which do not produce candicidin, i.e. it appears to be a specific gene for biosynthesis of secondary metabolites.

The 32 mutants of *S. griseus* blocked in candicidin biosynthesis lacked PABA synthase. However, they were not auxotrophs when tested in defined medium solidified with pure agarose. PABA auxotrophs of *S. coelicolor* and *S. lividans* did require exogenous PABA for growth under these conditions, suggesting that the lack of PABA requirement by mutants of *S. griseus* lacking PABA synthase is due either to a residual level of this enzyme below the threshold detection level, or to the formation of enough PABA by other aromatic amidotransferases (e.g. anthranilate synthase) existing in the cell. The possible existence of two different PABA synthases for vitamin biosynthesis and for candicidin formation is currently being investigated.

Blocked mutants may also lack other enzymes involved in candicidin biosynthesis in addition to PABA synthase.

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