Δ-1-Piperideine-6-carboxylate dehydrogenase, a new enzyme that forms α-aminoadipate in *Streptomyces clavuligerus* and other cephamycin C-producing actinomycetes

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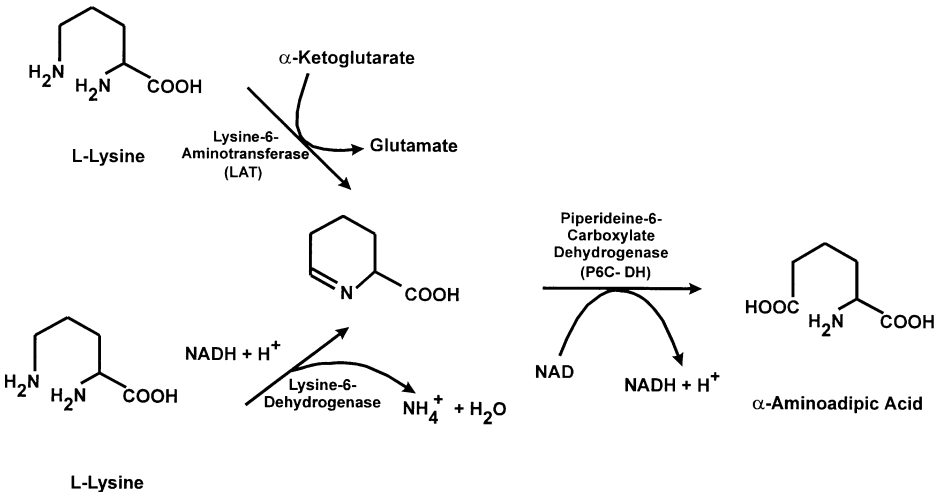
Δ-1-Piperideine-6-carboxylate (P6C) dehydrogenase activity, which catalyses the conversion of P6C into α-aminoadipic acid, has been studied in the cephamycin C producer *Streptomyces clavuligerus* by both spectrophotometric and radiometric assays. The enzyme has been purified 124-fold to electrophoretic homogeneity with a 26 % yield. The native protein is a monomer of 56.2 kDa that efficiently uses P6C (apparent K_m 14 μM) and NAD^+ (apparent K_m 115 μM), but not $NADP^+$ or other electron acceptors, as substrates. The enzyme activity was inhibited (by 66 %) by its end product NADH at 0.1 mM concentration. It did not show activity towards pyrroline-5-carboxylate and was separated by Blue-Sepharose chromatography from pyrroline-5-carboxylate dehydrogenase, an enzyme involved in the catabolism of proline. P6C dehydrogenase reached maximal activity later than other early enzymes of the cephamycin pathway. The P6C dehydrogenase activity was decreased in ammonium (40 mM)-supplemented cultures, as was that of lysine 6-aminotransferase. P6C dehydrogenase activity was also found in other cephamycin C producers (*Streptomyces cattleya* and *Nocardia lactamdurans*) but not in actinomycetes that do not produce β-lactams, suggesting that it is an enzyme specific for cephamycin biosynthesis, involved in the second stage of the two-step conversion of lysine to α-aminoadipic acid.

INTRODUCTION

Products formed from the catabolism of L-lysine are precursors of many microbial and plant secondary metabolites such as β-lactams, nourseothricins, etamycin, pipecolic acid and piperideine alkaloids [1,2]. The mechanisms of bacterial catabolism of lysine are very diverse. In some microorganisms lysine degradation starts with decarboxylation to cadaverine mediated by a lysine decarboxylase [3]. In other bacteria lysine catabolism is initiated by a mono-oxygenase, resulting in the formation of 5-aminovalerimide [4], by the deamination of lysine by a lysine 6-aminotransferase (LAT) (e.g. in *Flavobacterium* sp.) [5] or by an L-lysine 6-dehydrogenase as in *Agrobacterium tumefaciens* [6]. These two last reactions lead to the formation of Δ-1-piperideine 6-carboxylate (P6C) (Scheme 1).

In species of *Streptomyces*, lysine catabolism generally occurs through cadaverine [6]. In addition, β-lactam-producing actinomycetes possess a LAT activity specific for the synthesis of α-



Scheme 1 Conversion of lysine into α-aminoadipic acid by LAT and P6C dehydrogenase

The reaction catalysed by the lysine-6-dehydrogenase of *A. tumefaciens*, used to produce P6C, is also shown.

Abbreviations used: ACV, α-aminoadipyl-cysteinyl-valine; LAT, lysine 6-aminotransferase; P6C, Δ-1-piperideine-6-carboxylate; P5C, pyrroline 5-carboxylate; TSB, trypticase soy broth.

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aminoadipic acid, a precursor of the β-lactam antibiotics (reviewed in [7]). The gene encoding this enzyme (*lat*) is located in the cephamycin C gene cluster both in *Nocardia lactamdurans* [8] and *Streptomyces clavuligerus* [9], whereas it is absent from the genome of most other actinomycetes, confirming that this enzyme is specific for secondary metabolism.

P6C (the cyclic form of α-aminoadipic semialdehyde) was identified as the product of the deamination catalysed by LAT [10]. Recently we have established that α-aminoadipic acid or its lactam form (6-oxopiperidine-2-carboxylate), but neither P6C nor its analogue pipecolic acid, is the inmediate precursor involved in the formation of α-aminoadipyl-cysteinyl-valine (ACV) by ACV synthetase [11]. This finding indicates that a new enzymic step is required in the cephamycin pathway to oxidize P6C acid to α-aminoadipic acid.

We therefore proposed that a P6C dehydrogenase should be present in *N. lactamdurans*, *S. clavuligerus* and other β-lactam producers (Scheme 1). The lack of available P6C precluded the purification of the P6C dehydrogenase. However, we synthesized the compound recently [12] and therefore it was of great interest to characterize the P6C dehydrogenase in β-lactam-producing micro-organisms.

An enzyme involved in the catabolism of proline, pyrroline-5-carboxylate dehydrogenase, which converts pyrroline-5-carboxylate (P5C) (structurally related to P6C) to glutamic acid, has been recently described in *Streptomyces coelicolor* [13]. Here we describe the presence of a P6C dehydrogenase in the cephamycin C producers *S. clavuligerus*, *Streptomyces cattleya* and *N. lactamdurans*, and the purification to homogeneity and characterization of this enzyme, which is different from P5C dehydrogenase.

MATERIALS AND METHODS

Strains and culture conditions

S. clavuligerus ATCC 27064 was used as the source for purification of P6C dehydrogenase. *N. lactamdurans* MA4213 (provided by V. Vinci, Merck and Co, Elkton, VA, U.S.A.), *Streptomyces lividans* 1326, *S. coelicolor* A3(2), *S. cattleya* NRRL 8057 and *Streptomyces fradiae* DSM 40063 were used to test the presence of P6C dehydrogenase. *S. lividans* 1326 was used as source of proline oxidase to prepare labelled P5C.

S. clavuligerus spores were inoculated into 500 ml triple-baffled flasks containing 100 ml of trypticase soy broth (TSB) medium (30 g/l, pH 7.2). After incubation for 48 h at 28 °C in an orbital incubator at 250 rev./min, 5 % (v/v) of this seed culture was used to inoculate a batch culture in TSB medium under the same conditions. Cephamycin C production by the cultures was routinely determined by bioassay with *Escherichia coli* Ess22-31 (a β-lactam-supersensitive mutant) as test organism, as described by Romero et al. [14] and confirmed by HPLC on a C_{18} reverse-phase column [15].

To prepare cell extracts, *S. clavuligerus* cells were collected by centrifugation, washed with 0.9 % NaCl and suspended in 1/25 of the initial volume of buffer A [20 mM Tris/HCl (pH 8.0)/0.1 mM dithiothreitol/0.4 mM EDTA/3 mM $MgCl_2$/5 % (v/v) glycerol]. Cell extracts were obtained by sonication at 4 °C (six cycles of 10 s with 1 min intervals for refrigeration in ice) with a Branson B-12 sonifier. After centrifugation at 10000 ***g*** for 30 min the extract was desalted by filtration through PD-10 columns (Pharmacia) equilibrated with buffer A.

Enzyme assays

P6C dehydrogenase

Two different assays were used to quantify P6C dehydrogenase: (1) a spectrophotometric assay based on the determination of NADH formed (increase in absorbance at 340 nm) and (2) a radioactive assay based on the formation of α-aminoadipic acid from labelled P6C (obtained from [^{14}C]lysine by the action of lysine 6-dehydrogenase). The reaction mixture for the spectrophotometric assay contained 1.2 mM NAD^+, 120 μM P6C and enzyme (0.7–100 μg of protein) in 100 mM Mops/HCl buffer, pH 8.7 (final volume 500 μl). The reaction mixture was incubated for 10 min at 37 °C. One unit of enzyme was defined as the enzyme activity able to produce 1 nmol of NADH per min. The reaction mixture for the radiometric assay contained 10 mM L-lysine, 0.2 μCi of L-[U-^{14}C]lysine (296 mCi/mmol) (ICN Pharmaceuticals), 7 mM NAD^+, 0.3 units of L-lysine 6-dehydrogenase from *Agrobacterium tumefaciens* [EC 1.4.1.15] and *S. clavuligerus* P6C dehydrogenase (7–200 μg protein) in 100 mM Mops/HCl buffer, pH 8.0 (final volume 250 μl). The reaction was incubated at 30 °C for 60 min and stopped by the addition of an equal volume of methanol. After precipitation of the proteins, 10 μl of the supernatant was applied to silica gel TLC plates (DC-Alufolien Kieselgel 60, Merck, Darmstadt, Germany), which were developed in ethanol/ammonium acetate/acetic acid (6:1:1, by vol). The labelled products were identified and quantified with an Instant Imager autoradiography unit (Packard). Under these conditions L-lysine migrated with an R_F of 0.12, P6C an R_F of 0.28 and α-aminoadipic acid an R_F of 0.40. The P6C formed was also detected by spraying the plates with a solution of *o*-aminobenzaldehyde in ethanol (0.02 %) and incubation for 10 min at 30 °C. Lysine and α-aminoadipic acid were also detected by spraying with ninhydrin (0.02 % in acetone) and heating at 150 °C for 10 s.

LAT

LAT was assayed as described by Kern et al. [10]. One unit of enzyme was defined as the activity that is able to form 1 nmol of P6C per min. Specific activity is given as units per mg of protein.

Lysine 6-dehydrogenase

Lysine 6-dehydrogenase was quantified spectrophotometrically, as described by Misono et al. [6]. One unit of this enzyme was defined as the activity that is able to form 1 nmol of P6C per min.

Proline oxidase

Proline oxidase activity was determined in a 48 h culture of *S. lividans* as reported by Dendinger and Brill [16]. *S. lividans* has a specific activity of 1 unit/mg of protein in 48 h cultures.

P5C dehydrogenase

Two different assays were used. (1) The spectrophotometric assay was based on the formation of NADPH described in [16]. The reaction mixture contained 2.4 mM NADP, 7.2 mM P5C (Sigma, St. Louis, MO, U.S.A.), 40 mM Tris/HCl buffer, pH 8.0, and enzyme (100 μg of protein) in a final volume of 500 μl. The reaction was performed at 37 °C for 15 min. One unit is defined as the activity that is able to produce 1 nmol of NADPH per min. (2) Alternatively a two-step radiometric reaction was established based on the formation of glutamic acid from labelled proline, with *S. lividans* cell-free extracts as the source of proline oxidase. The first reaction contained 10 mM L-proline, 0.2 μCi of [U-^{14}C]proline (279 mCi/mmol; ICN), 50 mM Mops/HCl, pH 6.5, and 100 μl of *S. lividans* cell-free extract in a final volume of 180 μl. The reaction mixture was incubated for 30 min at 30 °C and then filtered at 4 °C through Ultra-freeMC (Millipore) at 3220 ***g*** to separate the labelled reaction product (P5C) from the

protein. Formation of P5C was shown by adding *o*-aminobenzaldehyde to an aliquot. In the second step the pH was adjusted to 8.0, after which NAD^+ (2.4 mM) and enzyme (approx. 100 μg of protein) were added to give in a final volume of 250 μl. The mixture was incubated at 30 °C for 30–120 min; the reaction was then stopped with methanol (250 μl). To establish the formation of labelled glutamic acid, the mixture was centrifuged at 20000 ***g*** for 5 min and 25 μl of supernatant was spotted on Kieselgel 60 and developed in phenol/water (3:1 v/v). The radioactive spots were detected with an Instant Imager (Packard) autoradiography unit. In addition, proline (R_F 0.50) and glutamic acid (R_F 0.07) were detected with ninhydrin (0.02 % in acetone), and P5C (R_F 0.45) was detected by spraying with *o*-aminobenzaldehyde (0.02 % in acetone).

Purification of P6C dehydrogenase

Cell extracts with a protein concentration of 5 mg/ml were applied to a pseudoaffinity Blue-Sepharose CL6B column (7 cm × 1.6 cm) (Pharmacia) previously equilibrated with buffer A [20 mM Tris/HCl (pH 8.0)/0.1 mM dithiothreitol/0.4 mM EDTA/3 mM $MgCl_2$/5 % (v/v) glycerol]. The column was washed with buffer A (flow rate 0.34 ml/min) until the absorbance at 280 nm in the effluent was zero; a linear 0–6 mM NAD^+ gradient (total volume 120 ml) was then applied.

The active fractions were applied to a 3-ml bed DEAE-Sepharose fast-flow column (2.5 cm × 1.6 cm) (Pharmacia) equilibrated in buffer A. A 0–400 mM NaCl gradient was then applied; the enzyme was eluted at 190 mM NaCl. Active fractions from DEAE-Sepharose were applied to a Sephadex-G75 column (68 cm × 2.6 cm) (Pharmacia) equilibrated with modified buffer A (50 mM Tris/HCl supplemented with 0.4 mM NAD^+).

Chemical synthesis of P6C

The synthesis of P6C was performed as described previously [12]. The final product in the synthesis, allysine ethylene acetal (18.9 mg), was deblocked immediately before use by mixing it with 40 mg of Amberlyst-15 (Sigma), in 1 ml of water for 10 min at room temperature, yielding P6C, which was characterized by NMR and quantified with *o*-aminobenzaldehyde by measuring the absorbance at 465 nm. Fresh P6C was prepared before each assay.

Protein analysis

SDS/PAGE of proteins was performed as described by Laemmli [17] by using a Mini-Protean II electrophoresis cell (Bio-Rad). Total protein was quantified by the Bradford colorimetric method.

RESULTS

Purified LAT does not convert lysine into α-aminoadipic acid

Initial experiments were based on the hypothesis that LAT might be able to convert lysine directly into α-aminoadipic acid. By using purified LAT from *S. clavuligerus* (or *N. lactamdurans*) we tested whether α-aminoadipic acid was formed during the assay. However, no trace of α-aminoadipic was found when the reaction products were analysed by HPLC after derivatization with *o*-phthaldialdehyde [18]. Formation of P6C was always found in the LAT assays. Therefore the lack of α-aminoadipic acid formation in the reaction indicates that P6C dehydrogenase activity must occur in the cells, which is absent from the purified LAT preparations.

P6C dehydrogenase activity in *S. clavuligerus*

To test the presence of a P6C dehydrogenase in β-lactam-producing micro-organisms, we initially developed a coupled assay with a preparation of pure, thermostable lysine 6-dehydrogenase from *Agrobacterium tumefaciens* (kindly provided by H. Misono) as a source of P6C. Under these conditions a lysine-dependent formation of NADH was found in *S. clavuligerus* cell extracts. On the basis of these results a coupled radiometric assay for P6C dehydrogenase with labelled lysine was established (see below).

To confirm that P6C was the substrate for this new enzymic activity, it was synthesized chemically [12]. Results with synthetic P6C unequivocally proved that this compound is converted into α-aminoadipic acid by *S. clavuligerus* cell extracts. The availability of the pure substrate allowed us to purify the enzyme and to study its kinetics with the spectrophotometric method. The reaction was linear up to the formation of 80 nmol of NADH.

P6C dehydrogenase is a monomer of 56.2 kDa

The enzyme was eluted from the Blue-Sepharose chromatography column at 0.7 mM NAD^+. This step gave a 76.9-fold purification with a yield of 47.4 % (Table 1). The active fractions were applied to DEAE-Sepharose and the enzyme was eluted at 190 mM NaCl with a 111.6-fold purification. The final step of Sephadex-G75 gel filtration resulted in a 124-fold purification, giving a specific activity of 3307 units/mg of protein with a yield of 26.5 %. P6C dehydrogenase activity in the active fractions was determined by both the radiometric and spectrophotometric assays. The purified enzyme was nearly homogeneous electrophoretically.

SDS/PAGE of proteins in the active fractions at different purification stages (Figure 1) showed that the best purification step was obtained by filtration through Blue-Sepharose CL6B, which eliminated most of the proteins in the extracts to yield a preparation with two major proteins of 56.5 and 33 kDa.

Gel filtration through Sephadex G-75, with BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa) and chymotrypsinogen A (25 kDa) as markers, gave a K_{av} of 0.066 for the P6C dehydrogenase, corresponding to a molecular mass of 56.2 kDa. This molecular mass agrees well with that deduced for

Table 1 Purification of the P6C dehydrogenase of *S. clavuligerus*

Stage	Protein (mg)	Activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Cell-free extract	411.4	13090.8	26.6	1	100
Blue-Sepharose CL6B	3.03	6207.6	2049.4	76.9	47.4
DEAE-Sepharose fast flow	1.56	4641.2	2975.1	111.6	35.4
Sephadex G-75	1.05	3472.5	3307.1	124.1	26.5

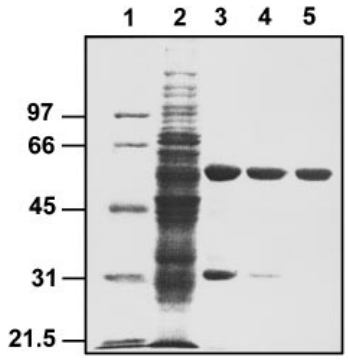

Figure 1 SDS-PAGE (10%) of P6C dehydrogenase preparations at different stages of purification

Lane 1, molecular mass standards [phosphorylase B (97.4 kDa), BSA (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa); positions (in kDa) indicated at the left]; lane 2, cell-free extracts; lane 3, eluate from Blue-Sepharose; lane 4, DEAE-Sepharose ion exchange; lane 5, Sephadex G-75 gel filtration.

the large protein present in SDS/PAGE (Figure 1, lanes 3–5) and indicates that native protein is a monomer.

Stability and substrate kinetics of P6C dehydrogenase

With the standard spectrophotometric assay at pH 8.7, pure P6C dehydrogenase showed Michaelis–Menten-type kinetics with respect to P6C with an apparent K_m of 14.02 ± 1.19 μM (mean $\pm$ S. E. M.) with an NAD^+ concentration of 1.2 mM and P6C concentrations ranging from 5 μM to 1 mM. NAD^+, but no $NADP^+$ or FAD, was used as electron-acceptor substrate in the dehydrogenation of P6C to α-aminoadipic acid. The apparent K_m for NAD^+ was 115.8 ± 8.9 μM (mean $\pm$ S. E. M.) when a fixed P6C concentration of 120 μM was used and NAD^+ concentrations ranged from 1 μM to 1 mM. The K_m values were calculated with the Enzfitter Program (Elsevier Biosoft).

The optimal pH of the enzyme was studied in 100 mM Mops/HCl buffer in the pH range 6.0–9.5. The optimal pH was pH 8.7.

The P6C dehydrogenase activity was lost during purification steps that required high pressure, such as ultrafiltration and FPLC chromatography. Pure P6C dehydrogenase showed a half-life of 15 days at -20 °C in 50 mM Tris/HCl buffer, pH 8.0, containing 0.1 mM dithiothreitol, 0.4 mM EDTA, 3 mM $MgCl_2$, 0.4 mM NAD^+ and 5% (v/v) glycerol. The half-life at 4 °C was 3 days.

Enzyme activity effectors

Compounds related to P6C, such as lysine, ornithine, pipecolic acid or the reaction product (α-aminoadipic acid) at 1 mM concentration did not affect the enzyme activity *in vitro*. Several inhibitors of NAD^+-dependent dehydrogenases were tested as effectors of the enzyme, but none of ATP, ADP, AMP, 3-acetylpyridine adenine dinucleotide, nicotinamide hypoxanthine dinucleotide phosphate, inorganic phosphate or citric acid at 1 mM had any inhibitory effect. However, NADH, the final product of the reaction, inhibited by 66% the activity at 0.1 mM concentration.

Time course of P6C dehydrogenase in *S. clavuligerus* cultures: regulation by ammonium ions and pipecolic acid

To correlate the presence of P6C dehydrogenase activity with the production of cephamycin C, three enzymes of the pathway, P6C dehydrogenase, LAT and isopenicillin N synthase, were measured in *S. clavuligerus* cultures in TSB medium. In parallel the effects of ammonium ions (40 mM) and pipecolic acid (10 mM) (a structural analogue of P6C) were studied. As shown in Figure 2, LAT is formed as a very early enzyme during the batch cultures, with a maximal specific activity of 1500 units/mg of protein at

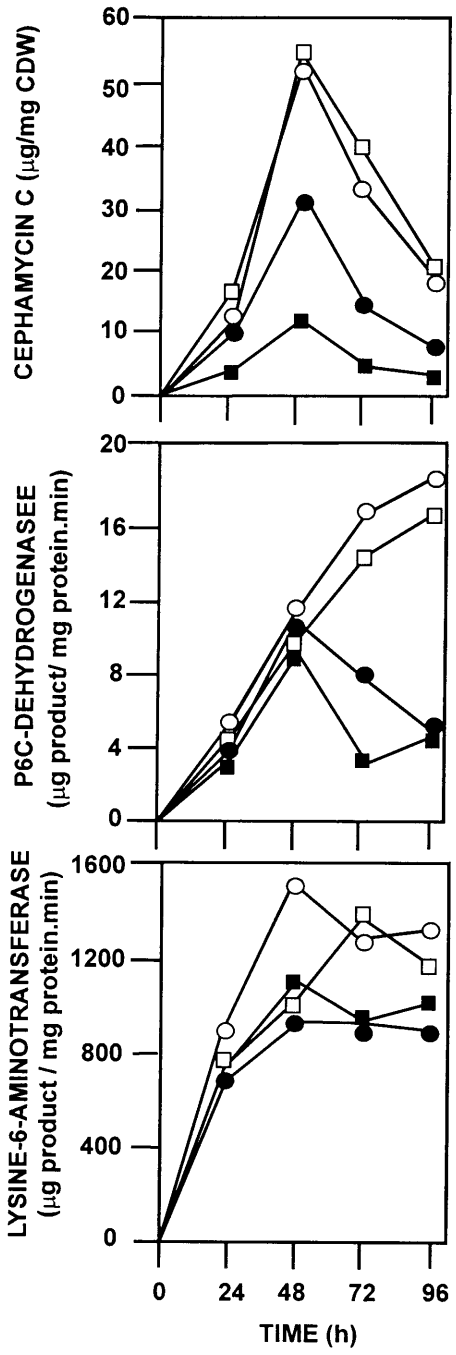

Figure 2 Time course of the enzymes involved in the formation of α-aminoadipic acid in cultures of *S. clavuligerus*

Top panel, cephamycin C production; middle panel, P6C dehydrogenase activity; bottom panel, LAT activity. Symbols: ○, control *S. clavuligerus* cultures; □, cultures supplemented with lysine 10 mM; ●, cultures supplemented with pipecolic acid (10 mM); ■, cultures supplemented with ammonium ions (40 mM).

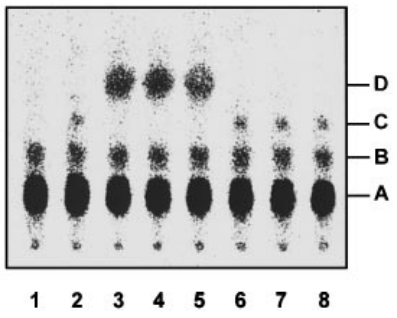

Figure 3 Autoradiography of the products (separated by TLC) of coupled reactions with different sources of P6C dehydrogenase

Lysine 6-dehydrogenase of *A. tumefaciens* was used as the source of P6C. Lane 1, control without any enzyme; lane 2, control with lysine 6-dehydrogenase. Lanes 3–8, reactions with lysine 6-dehydrogenase and extracts from *S. clavuligerus* (lane 3), *N. lactamdurans* (lane 4), *S. cattleya* (lane 5), *S. lividans* (lane 6), *S. fradiae* (lane 7) and *S. coelicolor* (lane 8). The spots correspond to lysine (A), an unknown degradation product of labelled lysine present in the commercial preparation of [^{14}C]lysine (B), P6C (C) and α-aminoadipic acid (D). Note the formation of labelled α-aminoadipic acid in reactions with extracts of *S. clavuligerus*, *N. lactamdurans* and *S. cattleya*.

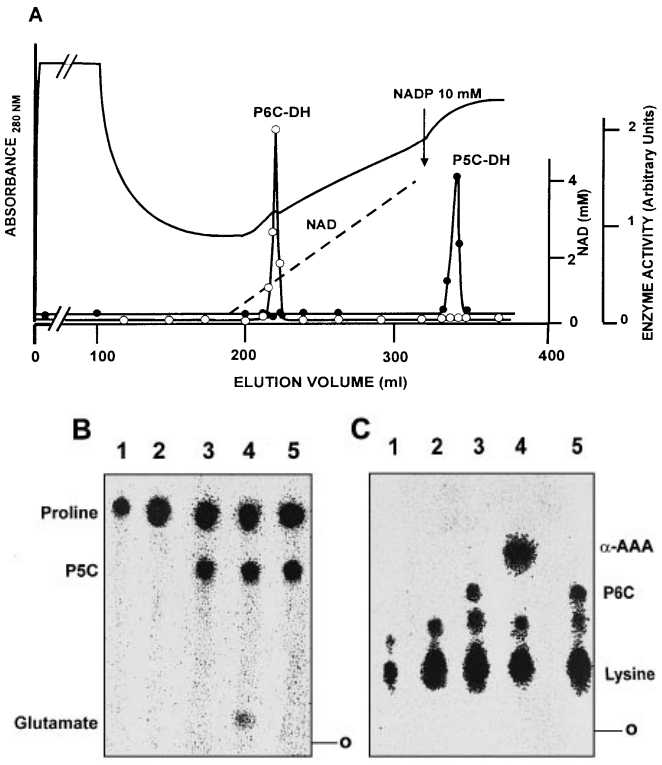

Figure 4 Substrate specificity of P6C dehydrogenase and P5C dehydrogenase

(**A**) Separation of P6C dehydrogenase (○) and P5C dehydrogenase (●) by Blue-Sepharose chromatography. Solid line, absorbance at 280 nm; broken line, NAD^+ gradient. (**B**) Products formed in a P5C DH radiometric assay separated by TLC with phenol/water (3:1, v/v) as solvent system. Lane 1, proline (0.005 μCi); lane 2, control reaction without proline oxidase; lane 3, control reaction without P5C dehydrogenase; lane 4, complete reaction; lane 5, complete reaction containing P6C dehydrogenase instead of P5C dehydrogenase. (**C**) Products formed in a P6C DH radiometric assay separated by TLC with ethanol/ammonium acetate/acetic acid (6:1:1, by vol.) as solvent system. Lane 1, lysine (0.005 μCi); lane 2, control reaction without L-lysine dehydrogenase; lane 3, control reaction without P6C dehydrogenase; lane 4, Complete reaction; lane 5, complete reaction with P5C dehydrogenase instead of P6C dehydrogenase.

48 h of incubation, decreasing steadily thereafter. P6C dehydrogenase activity reached a peak later than LAT, following a time course similar to that of isopenicillin N synthase and penicillin N expandase [19].

The addition of ammonium (40 mM) to the cultures resulted in a clear decrease in the formation of cephamycin C (77% decrease in antibiotic at 48 h). Both P6C dehydrogenase (which was affected particularly after 48 h) and LAT activities were depressed in ammonium-supplemented cultures (Figure 2, middle and bottom panels). Ammonium also exerted a strong regulatory effect on isopenicillin N synthase (results not shown).

Interestingly, the addition of pipecolic acid (10 mM) to the cultures negatively regulates cephamycin biosynthesis. This analogue of P6C, which is not an inhibitor of P6C dehydrogenase activity *in vitro* (see above), clearly decreased the production of cephamycin C (60% of the antibiotic in control cultures at 48 h) and repressed P6C dehydrogenase (48% of control activity at 72 h) and LAT (70–73% of control activity at 48 and 72 h) (Figure 2, middle and bottom panels). No effect of pipecolic acid was found on isopenicillin N synthase (results not shown).

P6C dehydrogenase occurs in β-lactam-producing but not in other *Streptomyces* species

It was of great interest to study the P6C dehydrogenase activity in different *Streptomyces* strains to establish whether this is a specific enzyme for β-lactam antibiotic biosynthesis. P6C dehydrogenase activity was measured in TSB cultures (48 h) of three producers of cephamycin C (*N. lactamdurans*, *S. clavuligerus* and *S. cattleya*) and three non-producers (*S. lividans*, *S. coelicolor* and *S. fradiae*) with the lysine 6-dehydrogenase radioactive assay. This assay, based on the formation of labelled P6C by the lysine 6-dehydrogenase added in the first reaction, works independently of the presence or absence of LAT activity in the strains. The results (Figure 3) showed that the three actinomycetes that are producers of β-lactam antibiotics do not accumulate the spot of P6C (R_F 0.28) (formed by lysine 6-dehydrogenase) but convert this compound into a spot of labelled α-aminoadipic acid (R_F 0.40) by the action of P6C dehydrogenase. P6C is retained unconverted in reactions with extracts of cephamycin non-producer strains *S. coelicolor*, *S. lividans* and *S. fradiae* (Figure 3, lanes 6, 7 and 8), indicating the lack of P6C dehydrogenase in these strains.

P6C dehydrogenase does not use pyrroline-5-carboxylic acid as substrate

To test whether P6C dehydrogenase and P5C dehydrogenase, an enzyme of proline catabolism, used the same substrates, both enzyme activities were separated by Blue-Sepharose chromatography in which both of them were retained. An NAD^+ gradient (0–4 mM) allowed the release of P6C dehydrogenase; the P5C activity was eluted when buffer A was supplemented with 10 mM $NADP^+$ (Figure 4A). P6C dehydrogenase (eluted from Blue-Sepharose) did not use P5C as substrate at pH 8.0, as shown by the spectrophotometric assay. In contrast, P5C dehydrogenase did not use P6C. To confirm the substrate specificity the activities of pure preparations of P6C dehydrogenase and partly purified P5C dehydrogenase were tested on P6C and P5C with the radiometric assay. No formation of glutamate was found when P5C was used as substrate by P6C dehydrogenase (Figure 4B, lane 5); neither was a spot of α-aminoadipic acid formed from P6C by P5C dehydrogenase (Figure 4C, lane 5), indicating that

each of these dehydrogenases is specific for its respective substrate.

DISCUSSION

It was thought initially that LAT might convert L-lysine into α-aminoadipic acid, which is the direct precursor of the ACV tripeptide that is later converted into β-lactams. However, as described in this article, L-lysine is converted by LAT into P6C but not into α-aminoadipic acid. In addition, studies *in vitro* with purified ACV synthetase clearly proved that α-aminoadipic acid, but not P6C or pipecolic acid, was required for the synthesis of ACV [11].

In this article we provide evidence that in the β-lactam producers *S. clavuligerus*, *N. lactamdurans* and *S. cattleya*, but not in other *Streptomyces* species that are unable to synthesize β-lactam antibiotics, there is a specific dehydrogenase that converts P6C into α-aminoadipic acid. This enzyme uses NAD^+ (apparent K_m 115.8 μM), but not $NADP^+$ or FAD, as electron acceptor during the oxidation of P6C to α-aminoadipic acid. P6C dehydrogenase showed a high affinity for P6C (apparent K_m 14.02 μM).

Interestingly the P6C dehydrogenase was found to be different from P5C dehydrogenase, a proline catabolism enzyme that converts P5C into glutamic acid. Both enzymes were separated by Blue-Sepharose affinity chromatography; pure P6C dehydrogenase was unable to convert P5C into glutamic acid under conditions in which there was an efficient conversion of P6C into α-aminoadipic acid. Both the P5C dehydrogenase of *S. coelicolor* [13] and that of *S. clavuligerus* (this study) require $NADP^+$ as cofactor, whereas the P6C dehydrogenase of *S. clavuligerus* uses NAD^+.

In addition the P5C dehydrogenase of *S. coelicolor* is a tetramer with a molecular mass of 265 kDa [13], whereas the P6C dehydrogenase of *S. clavuligerus* is a monomer of 56.2 kDa. Given the structural similarity between P65 and P5C, it is somewhat surprising that two different enzymes have evolved in actinomycetes for primary and secondary metabolism.

The time course of P6C dehydrogenase formation indicates that the synthesis of this enzyme follows that of LAT, suggesting that P6C dehydrogenase is likely to be expressed from a promoter different from that of the *lat–pcb*AB–*pcb*C or the late genes.

The formation of P6C dehydrogenase is strongly repressed, but not inhibited, by high ammonium concentrations in the culture medium. Ammonium ions are well-known repressors of cephamycin biosynthesis in *S. clavuligerus* [21] and *N. lactamdurans* [22]. It is interesting that several enzymes, including isopenicillin N synthase, isopenicillin N isomerase and deacetoxycephalosporin C synthetase in addition to P6C dehydrogenase and LAT, which are now known to be expressed from different promoters are strongly regulated by a common effector such as ammonium ions.

The repression effect *in vivo* of pipecolic acid (a saturated structural analogue of P6C) on cephamycin biosynthesis seems to be due specifically to an effect on the formation of the P6C dehydrogenase *in vivo* because pipecolic acid did not inhibit P6C dehydrogenase activity *in vitro*. It is likely that P6C accumulated by the action of LAT might act as an inducer of the P6C dehydrogenase gene and pipecolic acid might repress that induction.

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