New type of hexameric ornithine carbamoyltransferase with arginase activity in the cephamycin producers *Streptomyces clavuligerus* and *Nocardia lactamdurans*

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The ornithine carbamoyltransferases (OTCases) from the β-lactam-producing actinomycetes *Streptomyces clavuligerus* and *Nocardia lactamdurans* have been purified to near-homogeneity by δ-*N*-phosphonoacetylornithine-Sepharose 4B affinity chromatography. The *S. clavuligerus* and *N. lactamdurans* OTCase monomers had a molecular mass of 37 kDa. The native OTCases of *S. clavuligerus*, *N. lactamdurans* and *Streptomyces coelicolor* had molecular masses of 248, 251 and 247 kDa respectively, which correspond to a hexameric structure. The apparent K_m values for ornithine and carbamoylphosphate of the *S. clavuligerus* enzyme were respectively 2.3 and 6.0 mM at pH 8.0. The enzyme showed a reverse activity on citrulline and used lysine and putrescine as substrates. The hexameric complex showed coupled arginase–OTCase activities and was able to convert arginine into citrulline in a carbamoylphosphate-dependent manner. The requirement for carbamoylphosphate might prevent the arginase–OTCase complex from carrying out a futile cycle of arginine biosynthesis and degradation.

INTRODUCTION

The biosynthesis of arginine proceeds in all organisms from glutamic acid in eight enzymic steps. The sixth step leads to the formation of citrulline from ornithine and carbamoylphosphate mediated by the enzyme ornithine carbamoyltransferase (OTCase) [EC 2.1.3.3]. Anabolic OTCases have been purified to homogeneity from *Escherichia coli*, *Mycobacterium bovis* and bovine and rat liver [1–3]. Most native anabolic OTCases have a molecular mass of approx. 110 kDa and are trimeric structures composed of identical subunits of approx. 37 kDa. Their monomers frequently show association/dissociation phenomena that are pH-dependent. The amino acid sequence of the OTCase monomer has been deduced from the *arg*F/*arg*I genes of *E. coli* and from the cloned *arg*F genes of *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*, *Halobacterium halobium* and other organisms [4–7]. In some organisms, separate catabolic OTCases catalyse the phosphorolysis of citrulline to ornithine and have higher molecular masses (normally above 200 kDa).

Arginine is a precursor of many secondary metabolites, including clavulanic acid and streptomycin, produced by several species of the genus *Streptomyces* [8–10]. Addition of arginine or ornithine to *Streptomyces clavuligerus* cultures produces a large increase in clavulanic acid production, whereas addition of ornithine (but not arginine) decreases cephamycin C production [11]. Therefore amplification of the genes of the arginine pathway in *Streptomyces clavuligerus* is a goal of great interest. Feedback regulation of enzymes in the arginine pathway alters the accumulation of intermediates of the pathway, which in turn might affect the production of secondary metabolites.

Here we report the purification to near-homogeneity and the biochemical characterization of two OTCases from the β-lactam antibiotic-producing actinomycetes, *Streptomyces clavuligerus* and *N. lactamdurans*.

EXPERIMENTAL

Strains and culture

Streptomyces clavuligerus NRRL 3585, the producer of clavulanic acid, was grown in trypticase soy broth (TSB) medium (30 g/l). Portions (25 ml) from a 48 h TSB culture were used to inoculate 100 ml of either defined GSPG medium [12] or TSB medium in 500 ml triple-baffled flasks. *Nocardia lactamdurans* LC411, the producer of cephamycin C, was inoculated into NYG medium (8 g/l nutrient broth, 10 g/l glucose, 2 g/l yeast extract, 9 g/l $MgCl_2$,$10H_2O$) and 5 ml of a 48 h inoculum culture was used to seed baffled flasks containing 100 ml of NYG medium [13].

OTCase assays

OTCase forward activity was assayed in extracts of *Streptomyces clavuligerus* or *N. lactamdurans* desalted through PD-10 columns (Pharmacia, Uppsala, Sweden) or in purified fractions (see the Results section). The assay contained 25 mM L-ornithine, 25 mM lithium carbamoylphosphate, 250 mM Tris/HCl buffer, pH 8.0, and 20 μl of enzyme preparation (0.2–400 μg of protein) in a final volume of 80 μl. After incubation at 30 °C for 10 min the citrulline formed was quantified as described by Flint and Kemp [14]. One unit of OTCase was defined as the activity that catalyses formation of one μmol of citrulline per min. The specific activity is given as units per mg of protein.

The reverse OTCase activity was measured with citrulline as substrate coupled with the degradation of the carbamoylphosphate formed in the reaction with carbamate kinase. The reaction contained, in a 50 μl final volume, 0.05 mM L-[*carbamoyl*-^{14}C]citrulline (0.2 μCi; specific radioactivity 2 GBq/mmol; Amersham, U.K.), 20 mM KH_2PO_4/K_2HPO_4 ions as substrate for the carbamate kinase, 0.25 unit of carbamate kinase from *Streptococcus faecalis* (Sigma), 0.02 unit of δ-*N*-phosphono-

Abbreviations used: DTT, dithiothreitol; OTCase, ornithine carbamoyltransferase; PALO, δ-*N*-phosphonoacetylornithine; TSB, tripticase soy broth.

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acetylornithine (PALO)-Sepharose purified OTCase (direct activity) and 0.6 M imidazole/HCl buffer, pH 6.8. The reaction was started by addition of the OTCase in closed 1.5 ml Eppendorf tubes. The $^{14}CO_2$ formed from the carbamoylphosphate by the carbamate kinase was recovered in 6 mm cellulose AA discs (Whatman) saturated with $Ba(OH)_2$ held by a pin above the reaction. The assay was run for 30 min at 30 °C and stopped by the addition of 50 μl of 12 M H_2SO_4. After incubation for a further 30 min at 30 °C to allow carbon dioxide to react with the $Ba(OH)_2$, the disc paper was dried and immersed in 2,5-diphenyloxazole/1,4-bis-(5-phenyloxazol-2-yl)benzene (PPO/POPOP) scintillation liquid and the radioactivity was counted in a Beckman LS-6000TA scintillation counter.

Assays to test arginine, lysine, putrescine, cadaverine, *N*-acetylornithine or *N*-acetylarginine as substrates at 25 mM concentration were made in a 60 μl volume in the presence of 0.125 μCi of [^{14}C]carbamoylphosphate (0.27 GBq/mmol, DuPont, Les Ulis, France). The reaction was stopped with 60 μl of methanol; after centrifugation of the mixtures to eliminate the protein pellet, 10 μl of each was spotted on the Kieselgel 60 aluminium sheets (Merck, Darmstadt) and the TLC developed in butanol/acetic acid/water (6:2:1, by vol). The labelled products were detected with an Instant Imager (Packard Instrument) scanner.

Arginase assay

The standard arginase assay contained 0.2 μCi of L-[U-^{14}C]arginine/HCl (11 GBq/mmol; Amersham), 20 μl of enzyme purified by PALO-Sepharose chromatography (0.22 μg of protein) and 300 mM Tris/HCl buffer, pH 8.0, in a final volume of 60 μl. After 60 min the reaction was stopped by adding 60 μl of methanol and after centrifugation at 15000 ***g*** for 5 min, 10 μl was spotted on Kieselgel 60 plates and the products were separated by TLC developed in NH_4OH/n-propanol (3:7, by vol). Under these conditions the R_F values of arginine and citrulline were 0.2 and 0.54 respectively. Alternatively, L-[*ureido*-^{14}C]arginine (15 GBq/mmol; Amersham) was used in the arginase assay to confirm the removal of the ureido group.

Intracellular amino acid concentration

Total amino acids were extracted from the cells with ethanol as described by Gouesbet et al. [15]. Citrulline and homocitrulline were determined colorimetrically by using the BUN acid reagent [14]. Alternatively, citrulline and ornithine were separated by TLC on Kieselgel 60 aluminium sheets with butanol/acetic acid/water (6:2:1, by vol). Under these conditions the R_F of citrulline is 0.18, of ornithine is 0.10, of carbamoylphosphate is 0.10 and of putrescine is 0.13. The amino acids were detected with ninhydrin and the spots were quantified with a Howtek SM3 digitalyser with Diversity One® software and pure standards of citrulline and ornithine.

Preparation of PALO and PALO-Sepharose 4B

The citrulline structural analogue PALO was prepared by the method of Hoogenraad [16] as modified by Martinis et al. [17]. To prepare PALO-Sepharose 4B the epoxy-activated Sepharose 4B resin (Sigma, St. Louis, MO, U.S.A.) was treated and coupled with PALO in accordance with the manufacturer's instructions.

OTCase purification

Cells from cultures 48 h old were centrifuged at 5000 ***g*** for 30 min, washed with 0.9 % NaCl and resuspended in 20 ml of buffer A [50 mM Tris/HCl (pH 8.0)/0.2 mM dithiothreitol (DTT)/1 mM EDTA/5 % (v/v) glycerol/2 mM lithium carbamoylphosphate]. The cells were broken in a french press (Aminco) at 4 °C and the cell-free extract was centrifuged at 10000 ***g*** for 30 min. Proteins in the supernatant were precipitated by adding solid ammonium sulphate to 75 % saturation and the protein pellet obtained after centrifugation was dissolved in 20 ml of buffer A, concentrated through a Diaflo YM-100 membrane (Amicon) to 1 ml, diluted 1:5 in Tris/HCl to a final concentration of 10 mM and applied to a 0.5 ml PALO-Sepharose 4B affinity column equilibrated with 10 mM Tris/HCl. pH 8.0, containing 0.2 mM DTT. The column was washed with 40 vol of buffer B [10 mM Tris/HCl (pH 8.0)/0.2 mM DTT/70 mM NaCl] with a flow rate of 0.1 ml/min. The OTCase was eluted stepwise with buffer B containing 80 and 100 mM NaCl.

SDS/PAGE

Proteins were precipitated with 2 vol of cold acetone and centrifuged at 10000 ***g*** for 30 min; the pellet was dried in a Speed-Vac (Savant) centrifuge and resuspended in 5 μl of loading buffer. After being boiled for 5 min at 100 °C the proteins were subjected to SDS/PAGE [10 % (w/v) gel] [18] on a Mini-Protean II apparatus (Bio-Rad). Protein bands in the gels were revealed by the silver nitrate method.

Electrofocusing

Purified OTCase preparations were subjected to electrophoresis in a Rotofor cell (Bio-Rad); the enzyme was loaded in 50 ml of 50 mM Mops/HCl buffer, pH 7.5, containing 5 % glycerol, and electrofocused in 1 % Bio-Lyte 3-10 at 12 W for 5 h at 4 °C. Samples from the electrofocusing (2 ml) were desalted through PD-10 (Pharmacia) columns equilibrated with the same buffer and the OTCase activity was measured as described above.

RESULTS

Regulation of *Streptomyces clavuligerus* OTCase by arginine

To discover whether ornithine (a substrate for OTCase), arginine (the final product of the pathway) or uracil (a well-known effector of the carbamoylphosphate synthetase required for carbamoylphosphate synthesis) have any effect on the OTCase

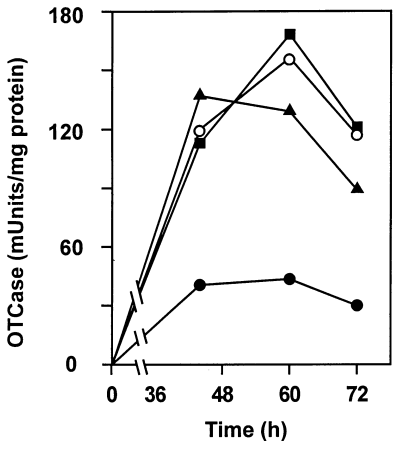

Figure 1 Influence of amino acids and bases related to the arginine pathway on OTCase activity of *Streptomyces clavuligerus*

Compounds were added at 10 mM concentration to *Streptomyces clavuligerus* grown in GSPG medium. Symbols: ○, control; ■, uracil; ▲, ornithine; ●, arginine.

Table 1 Purification of the OTCases of *Streptomyces clavuligerus* and *N. lactamdurans*

One enzyme unit is defined as the activity that catalyses the formation of 1 μmol of citrulline per min.

Organism	Purification step	Protein (mg)	Activity (units/ml)	Specific activity (units/mg of protein)	Yield (%)	Purification (fold)
Streptomyces clavuligerus	Cell-free extract	200	19.4	0.097	100	1
	Ammonium sulphate (75%)	125	14.7	0.117	76	1.2
	YM-100 ultrafiltration	46	10.5	0.232	54	2.4
	PALO-Sepharose affinity chromatography	0.11	8.32	79.22	43	816
N. lactamdurans	Cell-free extract	164	24.5	0.149	100	1
	Ammonium sulphate (75%)	100	19.84	0.198	81	1.3
	YM-100 ultrafiltration	35	15.55	0.44	63.5	2.95
	PALO-Sepharose affinity chromatography	0.07	12.32	176	50.3	1181

levels in *Streptomyces clavuligerus*, the specific activity of this enzyme was measured in extracts of *Streptomyces clavuligerus* grown in defined GSPG medium with or without supplementation with L-arginine, L-ornithine or uracil at 10 mM concentration (Figure 1). The addition of uracil or L-ornithine exerted a very small effect on OTCase levels but supplementation with L-arginine resulted in a clear decrease (approx. 80 %) in the specific activity of OTCase throughout the culture. This decrease was not due to inhibition of the OTCase activity by arginine (which was absent from the desalted cell-free extracts).

The intracellular pools of citrulline and ornithine were measured in cells 72 h old by using TLC or by the citrulline colorimetric method. Both methods showed that the intracellular citrulline concentration increased 2–3-fold in cells grown in the presence of ornithine or arginine in relation to control unsupplemented cells (50 μmol of citrulline/mg dry weight). Ornithine was accumulated intracellularly in ornithine-grown cells, whereas it was undetectable in control unsupplemented cells.

Purification of OTCase

The OTCase of *Streptomyces clavuligerus* was purified to near-homogeneity in three steps, including affinity chromatography through PALO-Sepharose 4B as shown in Table 1. After 816-fold purification a yield of 43 % was obtained. A major protein band of molecular mass 37 kDa observed in the purest preparations of the *Streptomyces clavuligerus* OTCase (Figure 2A, lane 5), corresponds to the OTCase monomer according to the correlation between the enzyme activity and the intensity of this band in different fractions from both PALO-Sepharose 4B and DEAE-Sepharose chromatography (results not shown). Ion-exchange chromatography through DEAE-Sephadex, however, produced a marked decrease in the enzyme activity and was not used for the final purification. A second minor protein band (molecular mass 44.5 kDa) was eluted from the column in all fractions of *Streptomyces clavuligerus* extracts independently of the salt concentration; the nature of this band has not been studied further. The pI calculated by electrofocusing ($\pm$S.E.M.; $n = 3$) for the native OTCase was 4.2 ± 0.1.

Molecular mass of the *Streptomyces clavuligerus* OTCase

The molecular mass of the native *Streptomyces clavuligerus* OTCase was estimated by gel-filtration chromatography through Sephacryl S-300 column (2.5 cm $\times$ 40 cm) equilibrated with buffer A. The column was calibrated with ovalbumin (43 kDa), BSA (67 kDa), aldolase (158 kDa) and catalase (232 kDa). Both crude and purified OTCase samples were used to determine its molecular mass. The *Streptomyces clavuligerus* OTCase was always eluted just ahead of the catalase, with a K_{av} of 0.285, corresponding to a molecular mass of 248 ± 10 kDa for the native enzyme (Table 2). This indicates that in the buffer used for gel

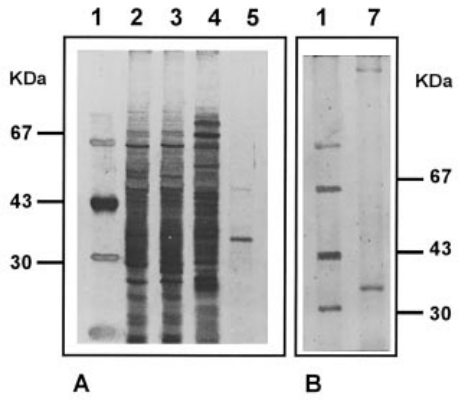

Figure 2 SDS/PAGE of the different steps of purification of the OTCases

(**A**) *Streptomyces clavuligerus*, (**B**) *N. lactamdurans*. Lane 1, molecular mass standards; lane 2, desalted cell-free extracts; lane 3, ammonium sulphate precipitate; lane 4, ultrafiltration through YM-100 Amicon membrane; lanes 5 and 7, affinity chromatography in PALO-Sepharose 4B. KDa, kDa.

Table 2 Molecular masses of OTCases deduced from Sephacryl S-300 gel filtration

Strain	K_{av}	Molecular mass§
*Streptomyces clavuligerus**	0.285	248 ± 15 kDa
Streptomyces coelicolor†	0.293	247 ± 15 kDa
*N. lactamdurans**	0.281	251 ± 15 kDa
Streptococcus faecalis‡	0.274	260 ± 15 kDa
E. coli†	0.410	115 ± 15 kDa

* Pure enzymes.
† Cell-free extracts.
‡ Commercial preparation (Sigma).
§ Values given $\pm$S.E.M.; $n = 3$.

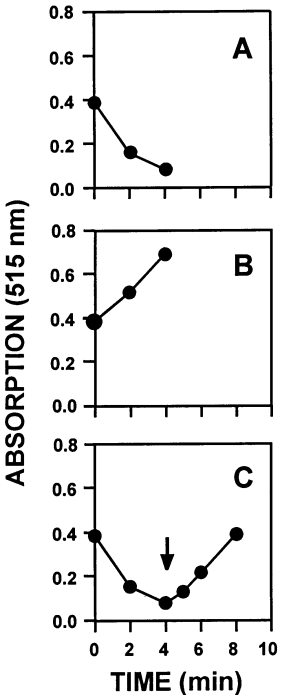

Figure 3 Sequential binding of carbamoylphosphate to the OTCase

Desalted cell-free extracts were incubated with the following substrates at 25 mM concentration: (**A**) carbamoylphosphate; (**B**) carbamoylphosphate and ornithine; (**C**) carbamoylphosphate with the addition of ornithine after 4 min of incubation. The absorbance at 515 nm due to carbamoyl groups was detected colorimetrically [14].

filtration (50 mM Tris/HCl, pH 8.0) the native protein behaves as a hexamer formed of 37.4 ± 0.3 kDa monomers. This size of the monomer is in good correlation with the molecular mass of the monomer estimated by SDS/PAGE to be 37 kDa (see above). This result was unexpected because known anabolic OTCases (most of them from Gram-negative bacteria) do not show a hexameric structure [19]. To test whether the pH of the buffer affected the aggregation of the monomers, the molecular mass was also estimated by gel filtration in the same column (Sephacryl S-300) equilibrated with buffer C [50 mM Mops/HCl (pH 6.0)/0.2 mM DTT/5% glycerol/1 mM EDTA]. At this lower pH approx. 80% of the OTCase activity was lost but the calculated molecular mass of the enzyme (248 kDa) remained the same as that at pH 8.0.

Different actinomycetes show OTCases with a hexameric structure

To test whether the hexameric organization was common to other actinomycetes' OTCases, their molecular masses were tested in desalted cell-free extracts from *Streptomyces coelicolor* and in the purest preparation (Figure 2B, lane 6) of *N. lactamdurans*, with controls consisting of the hexameric OTCase of *Streptococcus faecalis* (Sigma) and the *E. coli* DH5α OTCase (reported as trimeric). The elution volume for the catalase (used as molecular mass standard) was confirmed by the formation of free oxygen from H_2O_2. As shown in Table 2 the actinomycetes and the *Streptococcus faecalis* OTCases showed molecular masses of 248, 247, 251 and 260 kDa respectively, consistent with a hexameric organization, whereas the *E. coli* OTCase showed a molecular mass of 115 kDa, which corresponds to a trimeric structure.

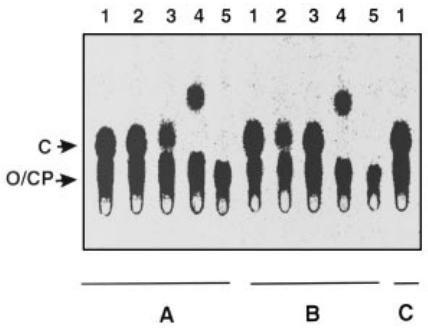

Figure 4 TLC of products formed by OTCase in the presence of different substrates

Assays of OTCase in the presence of different substrates and [^{14}C]carbamoylphosphate were spotted (10 μl) and TLC plates were developed in butanol/acetic acid/water (6:2:1, by vol). (**A**) *Streptomyces clavuligerus* OTCase, (**B**) *N. lactamdurans* OTCase, (**C**) *Streptococcus faecalis* OTCase. The substrates used were: lanes 1, ornithine; lanes 2, arginine; lanes 3, lysine; lanes 4, putrescine; lanes 5, none. Arrows indicate carbamoylphosphate (CP), ornithine (O) and citrulline (C).

Stability and substrate kinetics of *Streptomyces clavuligerus* OTCase

The *Streptomyces clavuligerus* OTCase was 100% stable for 4 days at 4 °C in 50 mM Tris/HCl buffer, pH 8.0, and remained 100% stable for longer periods in the presence of carbamoylphosphate (1 mM). Heat treatment at 65 °C for 5 min did not affect the activity. The enzyme can be conserved at -20 °C in 50 mM Tris/HCl, pH 8.0, containing 0.2 mM DTT and 50% glycerol for up to 4 months without significant loss of activity.

The pH dependence of the *Streptomyces clavuligerus* OTCase at 30 °C was determined by using 250 mM Tris/HCl buffer (pH 7.0–9.0) and 250 mM Mops/HCl buffer (pH 6.0–8.5). The optimal pH was 7.7 in the former and 8.0 in the latter. The enzyme activity decreased almost linearly (to 40% of the initial activity) at increasing NaCl concentrations (between 0.1 and 0.8 M).

The *Streptomyces clavuligerus* OTCase binds carbamoylphosphate very tightly in the absence of ornithine; this is shown by the decrease in absorption of the 515 nm peak of carbamoylphosphate owing to the trapping of this substrate by the enzyme (Figure 3A). Addition of ornithine to the carbamoylphosphate–enzyme complex resulted in an immediate increase in absorption at 515 nm owing to the formation of citrulline (Figures 3B and 3C). The addition of aspartate instead of ornithine to the OTCase–carbamoylphosphate complex did not result in an increased absorption at 515 nm, as might be caused by the formation of carbamoylaspartate by the aspartate carbamoyltransferase present in the crude extracts (result not shown); this indicates that once the carbamoylphosphate is bound by the OTCase it cannot be used by the competing aspartate carbamoyltransferase.

Pure *Streptomyces clavuligerus* OTCase shows Michaelis–Menten kinetics for both substrates in 250 mM Tris/HCl buffer, pH 8.0. The affinity for ornithine at saturating carbamoylphosphate concentration (50 mM) was pH-dependent in the pH range 7.5–8.7. At pH 7.5 or 8.7 the apparent K_m values for ornithine were 0.7 mM increasing to 2.3 at pH 8.0. At 25 mM ornithine saturation the K_m ($\pm$S.E.M.; $n = 4$) for carbamoylphosphate was 6.06 ± 0.1 mM.

A clear reverse activity of the OTCase was found under the assay conditions indicated in the Experimental section. The

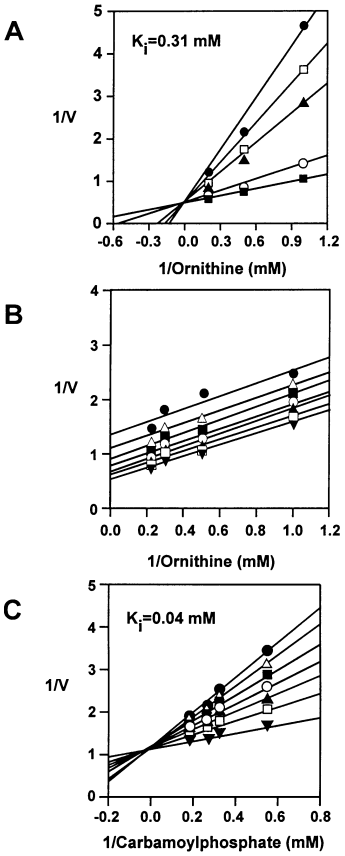

Figure 5 Kinetics of the inhibition by norvaline and PALO of the *Streptomyces clavuligerus* OTCase

(**A**) Norvaline in the presence of 1, 2 or 5 mM ornithine. Norvaline concentrations were: 0 (■), 0.2 (○), 1 (▲), 1.5 (□) and 2 mM (●). (**B**) PALO in the presence of 1.85, 3.75, 5 and 10 mM carbamoylphosphate. The PALO concentrations used were: 0 (▼), 20 (□), 40 (▲), 60 (○), 80 (■), 100 (△) and 120 μM (●). (**C**) PALO in the presence of 0.4, 1, 2 and 5 mM ornithine. The concentrations of PALO were as in (**B**).

apparent K_m value of the reverse activity for citrulline was 1.37 mM. Several compounds (ornithine, arginine, lysine, putrescine, cadaverine, *N*-acetylornithine and *N*-acetylarginine) were tested as substrates for the *Streptomyces clavuligerus* OTCase by using a purified preparation (79.2 units/mg of protein) of the enzyme. [^{14}C]Carbamoylphosphate was used as co-substrate and the formation of labelled carbamoylated products was detected by TLC. Additional spots that did not correspond to the carbamoylphosphate mark (control in Figure 4, lane 5) were found when ornithine or arginine were used as substrates (Figure 4, lanes 1 and 2). The spot with R_F 0.16 was identified as citrulline. New products with R_F values of 0.20 and 0.28 (Figure 4, lanes 3 and 4 respectively) were formed from lysine (presumably *N*-homocitrulline) and the five-carbon diamine putrescine (probably *N*-carbamoylputrescine). No detectable amounts of carbamoylated products were formed from the six-carbon diamine cadaverine, or from the acetylated amino acids *N*-acetylornithine or *N*-acetylarginine (results not shown).

Allosteric and competitive inhibitors

The effects of several nucleotides as allosteric inhibitors of the enzyme activity were tested. None of GTP, CTP, ADP or AMP had any effect on OTCase activity but UTP and ATP inhibited

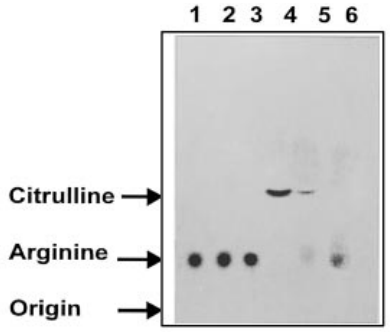

Figure 6 TLC of the reaction products of the arginase and OTCase activities present in *Streptomyces clavuligerus* OTCase

Lane 1, arginase assay without enzyme; lanes 2 and 3, arginase reaction using pure OTCase; lane 4, OTCase reaction with ornithine and 0.125 μCi of [^{14}C]carbamoylphosphate as substrates; lane 5, arginase reaction supplemented with 5.5 mM carbamoylphosphate; lane 6, arginase reaction supplemented with 5.5 mM carbamoylphosphate in which 0.5 mM L-[*ureido*-^{14}C]arginine/HCl (0.2 μCi) was used as substrate.

the enzyme activity by up to 15% at 2 mM concentration. Arginine inhibited OTCase activity (15–40%) at non-physiological concentrations (50–200 mM). Norvaline was a strong inhibitor of the enzyme, showing competitive-type inhibition (Figure 5A). The K_i for norvaline was 0.31 mM. PALO, known to be a bisubstrate analogue for the enzyme [20], showed competitive inhibitor behaviour with respect to carbamoylphosphate (Figure 5C) with a K_i of 0.04 mM; this indicates a high affinity of PALO for *Streptomyces clavuligerus* OTCase, approx. 150-fold higher than for the rat liver OTCase. With respect to ornithine, PALO behaves as a non-competitive inhibitor (Figure 5B). Zn^{2+} ions (1–3 mM) did not affect the affinity of the OTCase for ornithine, a difference from the *Bacillus subtilis* enzyme [21].

Characteristics of the *N. lactamdurans* OTCase

To elucidate whether the same multimeric structure of native *Streptomyces clavuligerus* OTCase occurs also in other actinomycetes, the OTCase of *N. lactamdurans* was purified to homogeneity with the same protocol (Table 1). A 1181-fold purification was obtained with a 50.3% yield. After affinity chromatography the OTCase monomer in *N. lactamdurans* showed a protein band with a molecular mass of 37 kDa according to SDS/PAGE (Figure 2B, lane 7). When the native *N. lactamdurans* enzyme was filtered through Sephacryl S-300 it was eluted ahead of the catalase, with a K_{av} of 0.28, which corresponds to a molecular mass of 251 kDa. The OTCase of *N. lactamdurans* showed Michaelis–Menten kinetics for both substrates with K_m values of 0.14 mM for ornithine and 3.5 mM for carbamoylphosphate. The reverse activity of *N. lactamdurans* OTCase was of the same order as that found in *Streptomyces clavuligerus* OTCase. Arginine, lysine and putrescine were also used as substrates by the *N. lactamdurans* OTCase. Antiserum to native rat and chicken OTCases did not cross-react with OTCase monomers of *N. lactamdurans* or *Streptomyces clavuligerus*.

The purified OTCases show carbamoylphosphate-dependent arginase activity

The efficient formation of citrulline from carbamoylphosphate and arginine (instead of ornithine) as substrate (Figure 4, lane 2) indicated that arginine is split into ornithine and urea by the

OTCase of *Streptomyces clavuligerus* or *N. lactamdurans*. The same results were obtained when a partly or highly purified preparation of the OTCase was used. To confirm the arginase activity of the hexameric OTCase complex, L-[U-^{14}C]arginine was incubated with highly purified enzyme with and without addition of carbamoylphosphate. The results (Figure 6) showed unequivocally that [U-^{14}C]arginine was converted into [U-^{14}C]-citrulline when carbamoylphosphate was added to the reaction (Figure 6, lane 5) but not in its absence (Figure 6, lanes 2 and 3). No products of arginase were observed in controls without enzyme (Figure 6, lane 1). The product formed in the presence of carbamoylphosphate by the arginase activity co-migrated with an authentic sample of labelled citrulline freshly prepared from ornithine and [^{14}C]carbamoylphosphate (Figure 6, lane 4). The presence of arginase activity in purified OTCase preparations was confirmed by incubation of L-[*ureido*-^{14}C]arginine with the enzyme. In the presence of carbamoylphosphate the guanidino group of arginine was removed and this amino acid was converted to unlabelled citrulline (Figure 6, lane 6).

DISCUSSION

Anabolic OTCases involved in the biosynthesis of citrulline from ornithine and carbamoylphosphate are trimeric molecules in *Saccharomyces cerevisiae* [22], *E. coli* [1] and other Gram-negative bacteria. This type of structure is shared with aspartate carbamoyltransferases. However, in *Streptococcus faecalis* the OTCase is hexameric [23] and in *B. subtilis* it exists as a mixture of dimeric, trimeric and hexameric forms [24]. In contrast, catabolic OTCases that degrade citrulline to ornithine are large proteins of 3, 6, 8 or 9 subunits [1,25,26]. Aggregation of the OTCase monomers is frequently related to pH and affects the allosteric properties of OTCases. Organisms able to perform phosphorolysis of citrulline possess both anabolic and catabolic OTCases. In those organisms, antibodies against catabolic and anabolic OTCases do not cross-react.

The monomer molecular mass of the *Streptomyces clavuligerus* and *N. lactamdurans* OTCases is 37 kDa; this figure agrees well with the known sizes of OTCase monomers and the molecular mass deduced from different *arg*F genes [4], although the *arg*F gene has not yet been cloned from any actinomycete. We have found that the native OTCases of *Streptomyces clavuligerus*, *N. lactamdurans* and *Streptomyces coelicolor* have a hexameric structure; pH variations do not affect the multimeric organization. The hexameric organization seems to be common to most Gram-positive bacteria, although *Mycobacterium bovis* OTCase has been described as a trimer [2].

Streptomyces clavuligerus OTCase binds carbamoylphosphate and ornithine sequentially, as occurs also with *Streptococcus faecalis* OTCase [23], but it lacks the ornithine co-operative inhibition found in the *B. subtilis* OTCases [21]. The kinetics of OTCase inhibition by PALO in relation to ornithine and carbamoylphosphate are in agreement with those described for other OTCases [16].

The substrate specificity of the actinomycete OTCases is interesting. Although ornithine is the best substrate (K_m 0.7 mM at pH 7.5), lysine and putrescine are also carbamoylated. The lack of detectable carbamoylation of cadaverine, a known substrate for the putrescine carbamoyltransferase [23], confirms that the purified enzyme is indeed an OTCase and not a diamine carbamoyltransferase.

The reactions catalysed by OTCase can participate in either the biosynthesis or the catabolism of arginine [19]. *E. coli*, *Pseudomonas*, *Aeromonas*, *Proteus* and other Gram-negative bacteria were shown to possess two distinct OTCases [1]. The anabolic enzyme catalyses the carbamoylation of ornithine to yield citrulline, whereas the phosphorolysis of citrulline is catalysed by the catabolic enzyme. Our results suggest that in actinomycetes a single hexameric OTCase with arginase activity catalyses both the biosynthetic and catabolic processes because no second peak of OTCase activity was observed in any purification step. This result is identical with that reported in *Streptococcus faecalis*, which possesses a hexameric OTCase with a native molecular mass of 250 kDa [23]. We have confirmed these results by gel filtration; the *Streptococcus faecalis* enzyme in our hands showed a molecular mass of 260 kDa. However, in contrast with *Streptococcus faecalis*, which is able to grow on arginine, *Streptomyces clavuligerus* and *N. lactamdurans* can deaminate arginine but cannot use it as sole carbon source. *Streptococcus faecalis* possesses a separate diamine carbamoyltransferase that carbamoylates putrescine and several other diamines (diaminopropane, spermine, spermidine and cadaverine). The purified *Streptomyces clavuligerus* OTCase is unable to carbamoylate cadaverine and it is therefore different from the *Streptococcus faecalis* diamine carbamoyltransferase.

The formation of labelled citrulline when arginine is used as substrate suggests that the OTCase possesses arginase activity. Interestingly, the arginase activity was found to be dependent on the presence of carbamoylphosphate. This result suggests that interaction of carbamoylphosphate with the enzyme modifies the substrate specificity, allowing the OTCase to degrade arginine. By this mechanism the cell prevents a futile cycle of arginine biosynthesis and degradation by the OTCase/arginase activities of the protein. Arginases produce ornithine from arginine and have been found to be associated with OTCases in *Saccharomyces cerevisiae* and *B. subtilis* [21,22] forming hexameric structures. Association of both enzymes results in an 'epi-enzymic' control of the arginine cycle in yeast. Because in these two organisms both the OTCase and the arginase are trimeric proteins of similar molecular masses, we cannot exclude the possibility that almost identical trimers of both enzymes are associated in actinomycetes, to form a hexameric complex. However, the lack of arginase activity in the absence of carbamoylphosphate and the homogeneous protein band in SDS/PAGE suggest that the enzyme is composed of six identical OTCase subunits. Cloning and characterization of the corresponding gene(s) will clarify this point.

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