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Purification and characterization of the isopenicillin N epimerase from *Nocardia lactamdurans*

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Isopenicillin N (IPN) epimerase, an enzyme involved in cephalosporin and cephamycin biosynthesis that converts IPN into penicillin N, was extracted from *Nocardia lactamdurans* and purified 88-fold. The enzyme was unstable but could be partially stabilized by addition of pyridoxal phosphate. The purified enzyme did not require ATP for activity in contrast to other amino acid racemases. The enzyme had an M_r of 59000 as determined by gel filtration; IPN epimerase from *Streptomyces clavuligerus* had an M_r of 63000. A protein band of M_r 59000 was found to be enriched in SDS-PAGE of active fractions from *N. lactamdurans*. The optimal temperature of the epimerase was 25 °C and the optimal pH 7·0. The apparent K_m for IPN was 270 μM. Fe^{2+}, Cu^{2+}, Hg^{2+} and Zn^{2+} strongly inhibited enzyme activity. α-Aminoadipic acid, valine, glutamine, glycine, aspartic acid and glutathione do not affect enzyme activity, whereas ammonium sulphate was inhibitory. The epimerase activity was partially inhibited by several thiol-specific reagents.

Introduction

The biosynthesis of cephalosporins by *Cephalosporium acremonium* (syn. *Acremonium chrysogenum*) and cephamycins by *Nocardia lactamdurans* (syn. *Streptomyces lactamdurans*) and *Streptomyces clavuligerus* takes place through similar biosynthetic pathways (reviewed by Martín & Liras, 1985). In both cases, the first step involves the formation of the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) by the ACV synthetase complex, followed by cyclization of ACV to isopenicillin N (IPN). Conversion of IPN to deacetoxycephalosporin C (the first intermediate carrying the six-membered dihydrothiazine ring characteristic of the cephalosporin molecules) is catalysed by two enzymes: IPN epimerase, which epimerizes IPN to penicillin N and deacetoxycephalosporin C synthase (expandase) which expands the five-membered thiazolidine ring into the six-membered dihydrothiazine ring. Deacetoxycephalosporin C is later converted into either cephalosporin C or cephamycins by a series of enzymic reactions (Martín *et al.*, 1986; Martín & Liras, 1989).

IPN epimerase, which converts IPN into penicillin N by changing the L-α-aminoadipic acid side chain to the D-configuration (Fig. 1) was first detected in *C. acremonium* (Konomi *et al.*, 1979; Sawada *et al.*, 1980). The enzyme was so labile that it could not be characterized further (Lübbe *et al.*, 1986). The extreme lability of the epimerase of *C. acremonium* has been confirmed by other groups (Baldwin *et al.*, 1981; Jayatilake *et al.*, 1981) who found activity only in freshly prepared cell-free extracts. The epimerase of *S. clavuligerus* appears to be more stable and could be partially purified, although the protein band corresponding to the theoretical M_r was barely detectable in polyacrylamide gels (Jensen *et al.*, 1983). It seems, therefore, that IPN epimerases from other cephamycin-producing micro-organisms might possess properties (including higher stability) that may allow a complete characterization of this enzyme.

Little is known about the molecular mechanism of amino acid epimerization during antibiotic biosynthesis. Some of the best-known epimerases involved in peptide antibiotic biosynthesis require ATP as an activating cofactor (Takahashi *et al.*, 1971).

The availability of purified IPN synthases (Ramos *et al.*, 1985; Jensen *et al.*, 1986; Castro *et al.*, 1988) and deacetoxycephalosporin C synthases (Kupka *et al.*, 1983; Dotzlaf & Yeh, 1987; Cortés *et al.*, 1987) from different species of fungi and *Streptomyces* has created a great deal of interest in the utilization of these enzymes to obtain new antibiotics from modified substrates (Jensen

Abbreviations: ACV, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine; IPN, isopenicillin N; PP, pyridoxal phosphate.

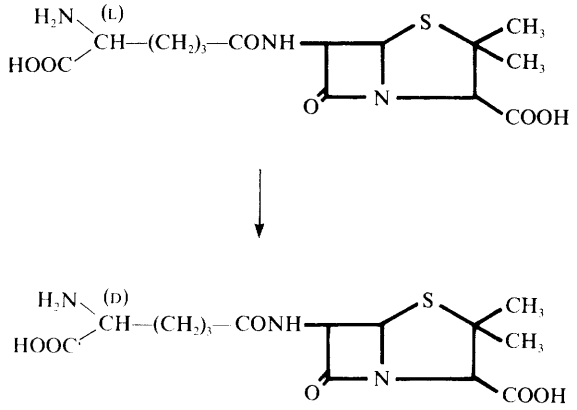

Fig. 1. Reaction catalysed by IPN epimerase. Note the change from the L- to the D-configuration in carbon-2 of the α-aminoadipyl side chain.

et al., 1984; Luengo *et al.*, 1986; Castro *et al.*, 1986) or in the use of immobilized biosynthetic enzymes (Wolfe *et al.*, 1984). The possibility of utilizing the epimerase coupled to other biosynthetic enzymes in enzyme reactors illustrates the need to characterize this enzyme from different cephamycin-producing actinomycetes in order to find one with better stability.

We report in this paper the purification and characterization of the epimerase of *N. lactamdurans*, a cephamycin producer in which two other biosynthetic enzymes, IPN synthase and deacetoxycephalosporin C synthase, have been studied (Castro *et al.*, 1985; Cortés *et al.*, 1986) and purified to near homogeneity (Cortés *et al.*, 1987; Castro *et al.*, 1988).

Methods

Micro-organisms and antibiotic assays. N. lactamdurans var. JC1843, a stable aerial-mycelium-forming (*amy*$^+$) strain that produces higher levels of cephamycin C than the wild-type strain *N. lactamdurans* NRRL 3802 (Castro *et al.*, 1986) was used in this work. *Micrococcus luteus* ATCC 9341 and *Escherichia coli* Ess 22-31 were used for the routine differential determination of IPN and penicillin N. *E. coli* Ess 22-31 is very sensitive to penicillin N but is not inhibited by IPN at the concentrations routinely used as substrate. *S. clavuligerus* NRRL 3585 was used as a source of IPN epimerase for comparative studies. The culture was grown and the cells disrupted as described by Jensen *et al.* (1983).

IPN and penicillin N were assayed as described previously (Castro *et al.*, 1985).

Cell-free extracts. Mycelium of *N. lactamdurans* grown for 72 h in NYG medium (Castro *et al.*, 1985) supplemented with 4 mg $MgCl_2$ ml^{-1} was collected by centrifugation at 10000 *g* for 10 min, washed twice with sterile saline (0·85% NaCl) and suspended in TPDP buffer [containing 50 mM-Tris/HCl, pH 7·0, 1 mM-PMSF, 1 mM-DTT and 0·05 mM-pyridoxal phosphate (PP)]. Cells were resuspended to 1/40th of the initial volume (final cell density about 320 mg wet cell wt ml^{-1}), and disrupted by sonication with a Branson Sonifier B-12 in an ice-bath for 15 s periods with 30 s intervals for a total time of 3 min, or in a cooled French press (Aminco). Cell extracts were centrifuged at 15000 *g* for 15 min in a refrigerated centrifuge (Sorvall RC-5B) and then at 100000 *g* for 60 min in a Beckman L8-70 ultracentrifuge. The supernatant (S100) was collected and used as crude cell-free extract.

IPN epimerase assay. The reaction mixture contained, in a final volume of 100 µl: IPN, 0·3 to 2 mM; cell-free extract, 30 to 70 µl (0·02 to 0·4 mg of protein depending on the purification stage); and TPDP buffer. The reaction was allowed to proceed at 20 °C for 60 min (unless otherwise stated) and then stopped either by quick freezing or by addition of 100 µl methanol. The reaction product (penicillin N) was determined by bioassay against *E. coli* Ess 22-31. When the residual untransformed substrate was higher than 0·27 mM it gave an inhibition zone against *E. coli*. This was avoided by diluting the reaction mixtures as appropriate (and the control without enzyme) after the reaction was completed, so that the residual IPN was in all cases below the detection level. The activity of the enzyme is expressed as pkat (mg protein)$^{-1}$ [pmol penicillin N formed s^{-1} (mg protein)$^{-1}$].

Purification of the IPN epimerase

The steps used in the purification of IPN epimerase are listed in Table 3; they were done at 4 °C as follows.

Precipitation of nucleic acids and fractionation of the enzyme activities. A 10 mg ml^{-1} solution of protamine sulphate (Sigma) in TPDP buffer was added slowly with gentle stirring to the S100 (81 ml) extract to a final concentration of 2 mg ml^{-1}. The suspension was kept at 4 °C for 15 min and then centrifuged (17000 *g*, 15 min). Solid ammonium sulphate (22·5 g; Sigma, enzyme grade) was added slowly with stirring to the supernatant, keeping the pH adjusted to 7·0, to give a final concentration of 30% of saturation. The solution was centrifuged (17000 *g*, 15 min, 4 °C) and the pellet was discarded. Additional ammonium sulphate was added to the supernatant to give a final concentration of 50% of saturation and the new precipitate was collected by centrifugation as before. The pellet was dissolved in 1 ml of TPDP buffer.

Gel filtration. Two ml of the 30 to 50% ammonium sulphate fraction was applied to a Sephadex G-75 column (Pharmacia; 600 × 26 mm) equilibrated with TPDP buffer. The epimerase activity was eluted with the same buffer using a flow of 10 ml h^{-1}. The column had been previously calibrated with commercial proteins of known M_r: cytochrome *c* (12400); carbonic anhydrase (29000); ovalbumin (43000); and BSA (66000).

DEAE-HPLC chromatography. The active fractions obtained by Sephadex G-75 filtration were pooled (total volume 19·2 ml) and applied by repeated injections to a Mono-Q HR 5/5 (Pharmacia) column (50 × 5 mm), equilibrated with 25 mM-Tris/HCl buffer, pH 7·0, containing 0·05 mM-PP and 0·1 mM-DTT (buffer I), attached to a Varian 5000 HPLC apparatus. The epimerase was eluted using a linear gradient of increasing ionic strength in the same buffer [from buffer I (25 mM-Tris/HCl) to buffer II (250 mM-Tris/HCl)] over a period of 60 min with a flow of 0·5 ml min^{-1}. The active fractions (1·1 ml) were pooled, diluted 10-fold, applied to the same column and eluted with a flow of 0·5 ml min^{-1} of a discontinous gradient according to the following programme: time 0 min, 10% buffer II; time 15 min, 75% buffer II; time 20 min, 80% buffer II; time 30 min, 85% buffer II; time 60 min, 100% buffer II.

Superose 12 FPLC filtration. The active fractions from the Mono-Q HR column were pooled, applied to a Superose 12 HR 10/30 column and eluted with TPDP buffer with a flow of 0·5 ml min^{-1}.

SDS-PAGE and isoelectric focusing. These were done in a Phast system (Pharmacia) using the commercial Phast-Gel gradients 10–15 and 8–25, with Phast-Gel SDS buffer strips. Alternatively, classical SDS-PAGE was done in an LKB Protean 16 cm vertical slab cell as described previously (Castro *et al.*, 1988). The isoelectric point was

obtained by the O'Farrell technique modified as indicated by Phillips (1988), using ampholytes 3–10 or 2–5·5 (Pharmacia). Isoelectric focusing was done in a Bio-Rad tube-cell cuvette (model 165) using 110 × 1·5 mm gels. The voltage applied increased from 250 V at zero time to 300 V (15 min), 350 V (30 min) and 500 V (60 min), and then was kept constant for 17 h. The second dimension was run in SDS-PAGE with 10% acrylamide as indicated before. Low-M_r and pI protein calibration kits from Pharmacia were used.

Chemicals. Pure cephamycin C was provided by D. Hendlin (Rahway, NJ, USA). IPN (62% purity) and ACV (dimer form) were a gift from P. Van Dijck (Delft, The Netherlands). Penicillin N was obtained from H. H. Peter, Basel, Switzerland. Sephadex G-75, Mono-Q HR 5/5 and the proteins cytochrome *c*, carbonic anhydrase, ovalbumin and BSA were obtained from Pharmacia. All other reagents were from Sigma.

Table 1. *Effect of putative cofactors on IPN epimerase activity*

Cofactor(s) added	Concn (mM)	IPN epimerase activity† (% of control)
None	–	100
2-Oxoglutarate	0·1	74
2-Oxoglutarate	1·0	74
ATP	0·5	82
EDTA	1·0	78
Ascorbate + ATP	2·8 + 2	74
$FeSO_4$ + Ascorbate	0·135 + 2·8	0
$FeSO_4$ + Ascorbate + DTT	0·135 + 2·8 + 2	0
$(NH_4)_2SO_4$	100	53
No added PP*	–	85

* IPN epimerase activity assayed in TPD buffer, i.e. without added PP. The other assays were done in TPDP buffer, which contains 0·05 mM-PP.

† The activity value equivalent to 100% was 0·12 pKat (mg protein)$^{-1}$.

Results

Cofactor requirement of IPN epimerase

Highly purified IPN epimerase catalysed the epimerization reaction without addition of cofactors other than PP (Table 1). The reaction required only IPN and enzyme in Tris/HCl buffer. No stimulation of enzyme activity was observed when the cofactors normally required by IPN synthase or deacetoxycephalosporin C synthase (2-oxoglutarate, 0·1 mM; ascorbic acid, 2·8 mM; Fe^{2+}, 0·135 mM) were added, or when oxygen was excluded from the reaction mixture by bubbling nitrogen through it. Fe^{2+} completely inhibited the IPN epimerase activity. Ammonium sulphate (100 mM) inhibited enzyme activity although at this concentration (1·3%) no protein precipitation occurred.

Role of pyridoxal phosphate

IPN epimerase activity was demonstrated in crude extracts (S100) without supplementation with PP. However, PP (0·05 mM) was routinely added to the extracts because it stabilizes the enzyme during purification (see below). Removal of PP from the reaction buffer decreased the enzyme activity slightly. To study the role of PP, an ammonium sulphate preparation of the enzyme was repeatedly washed with TPD (Tris/HCl, PMSF, DTT) buffer by centrifugation for 10 min at 6000 r.p.m. in an Ultrafree MC 10000 PTGC polysulphone filter (Millipore) to eliminate the PP that could remain loosely bound to the enzyme, and then incubated for 30 min at 25 °C with sodium borohydride (which inhibits PP-dependent enzymes due to reduction of the enzyme–

Table 2. *Effect of sodium borohydride,* D-*cycloserine and isoniazid on IPN epimerase activity*

The enzyme was pretreated for 30 min at 25 °C and washed by centrifugation in Ultrafree MC. IPN epimerase activity was measured in TPD buffer with or without supplementation with PP, and the indicated additions. Values in parentheses are the activity expressed as a percentage.

Pretreatment	Addition during the reaction	IPN epimerase activity [pkat (mg protein)$^{-1}$]	
		−PP	+PP
None	None	0·160 (100)	0·186 (116)
None	2·5 mM-Isoniazid	–	0·180 (111)
None	2·5 mM-D-Cycloserine	0·160 (100)	0·186 (116)
1 mM-Borohydride	None	0·085 (53)	0·120 (75)
5 mM-Borohydride	None	0·016 (10)	0·070 (44)
2·5 mM-Borohydride	None	0·044 (28)	0·070 (44)
2·5 mM-Borohydride	2·5 mM-Isoniazid	–	0·090 (62)

cofactor complex; Snell & Di Mari, 1970). This reagent was eliminated by washing three times through Ultrafree filters as indicated above, and the remaining activity was assayed in TPD buffer with or without supplementation with PP. The effects of isoniazid and other PP antagonists was tested by adding these compounds to the reaction mixture. Results are shown in Table 2. After pretreatment with 1 mM-borohydride for 30 min, 53·5% of enzyme activity remained; this increased to 75·6% when PP was added to the enzyme reaction. Pretreatment with 5 mM-borohydride produced an almost complete loss of enzyme activity but 44·2% activity was restored when the reaction mixture was supplemented with PP; when higher concentrations of borohydride were used activity was completely lost and could not be restored by addition of PP. D-Cycloserine and isoniazid (2·5 mM) had no significant effect on activity. These results suggest that PP is tightly bound to the enzyme since it is inactivated by borohydride but is not significantly antagonized by isoniazid or D-cycloserine. The partial reversal of the borohydride inhibition by PP suggests that this compound may act as a cofactor of IPN epimerase.

Separation of epimerase from IPN synthase and deacetoxycephalosporin C synthase

Ammonium sulphate fractionation gave a good separation of the epimerase from the IPN synthase and the deacetoxycephalosporin C synthase; the epimerase precipitated in the 30 to 50% of ammonium sulphate saturation fraction that contains little protein, while the two other enzymes required a 50 to 80% concentration of ammonium sulphate to precipitate. Only a small increase in specific activity was obtained in this step (Table 3). A large part of the enzyme activity was lost during ammonium sulphate precipitation. Since no significant epimerase activity precipitated in the 50 to 80% ammonium sulphate fraction, it seems that this reduction in total activity is due to the inhibition of enzyme activity by excess ammonium sulphate, as activity was recovered after removal of the salt by gel filtration. As an alternative method, proteins were fractionated by ultrafiltration using an Amicon P-30 membrane, which gave a 4-fold purification. The viscosity of the S100 extract made this step difficult.

Purification of IPN epimerase

The elution profile of *N. lactamdurans* epimerase from the Sephadex G-75 column is shown in Fig. 2(*a*); for comparison the elution profile of IPN epimerase from *S. clavuligerus* is shown in Fig. 2(*b*). The enzyme activity eluted with an V_e/V_0 of 1·209 ($K_{av.}$ 0·095), which corresponds to an M_r of 59000 ± 1000. Part of the enzyme activity that was masked after ammonium sulphate fractionation was recovered after this gel-filtration step, after which the total cumulative purification of enzyme activity was 7·1-fold.

The epimerase eluted together with several other contaminant proteins (as shown by SDS-PAGE) after ion exchange through the Mono-Q column. The purification after this step was 22-fold. A second filtration through the same column using a discontinuous gradient (Fig. 3; see also Methods) gave a highly purified (88-fold) preparation (Table 3). The epimerase eluted from this

Table 3. *Purification of the IPN epimerase of N. lactamdurans*

Purification step	Activity (pkat)	Protein (mg)	Specific activity [pkat (mg protein)$^{-1}$]	Recovery (%)	Purification (-fold)
Crude extract (S100)	242·3	1757·7	0·13	–	1
Protamine sulphate supernatant	260·3	1720·9	0·15	107·4	1·1
Ammonium sulphate precipitate (30–50%)	13·6	80·0	0·17	5·6	1·2
Sephadex G-75	57·4	58·4	0·98*	23·7	7·1
HPLC ion exchange gradient (1)	23·7	7·8	3·03*	9·8	22·0
HPLC ion exchange gradient (2)	10·3	0·85	12·11*	4·3	87·9
Further purification on Superose led to a high enrichment in the epimerase protein with loss of most of the enzyme activity (see text)					

* Specific activities are the values of pooled active fractions.

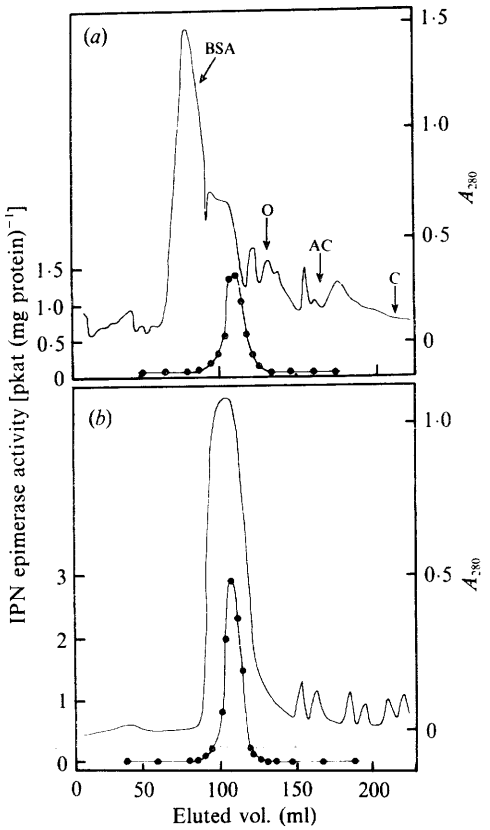

Fig. 2. Elution profile of the IPN epimerase after filtration through Sephadex G-75. (*a*) IPN epimerase from *N. lactamdurans*; (*b*) IPN epimerase from *S. clavuligerus*. ——, A_{280}; ●, IPN epimerase activity. BSA, bovine serum albumin (M_r 66000); O, ovalbumin (43000); AC, carbonic anhydrase (29000); C, cytochrome *c* (12400).

column at a buffer II concentration of 89%, corresponding to 225 mM-Tris/HCl.

A further purification of the epimerase protein was obtained by using FPLC gel-filtration through Superose 12 HR 10/30. The epimerase activity eluted early (retention time 14 min; V_e 7 ml) but the protein content and the activity of this fraction were barely detectable suggesting that the enzyme lost most of its activity at this high-purification step.

Purification of the IPN epimerase through the different steps was monitored by SDS-PAGE (Fig. 4). The epimerase appeared as a band of M_r 59000 with two

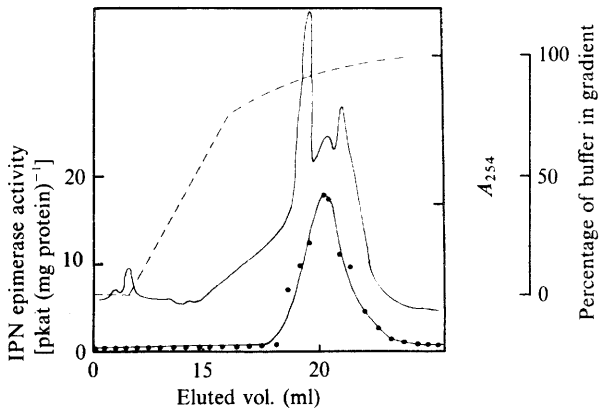

Fig. 3. Elution profile of the IPN epimerase after the second HPLC step through Mono-Q HR 5/5 using a discontinuous Tris/HCl buffer gradient (---) as indicated in Methods. ——, A_{254}; ●, IPN epimerase activity.

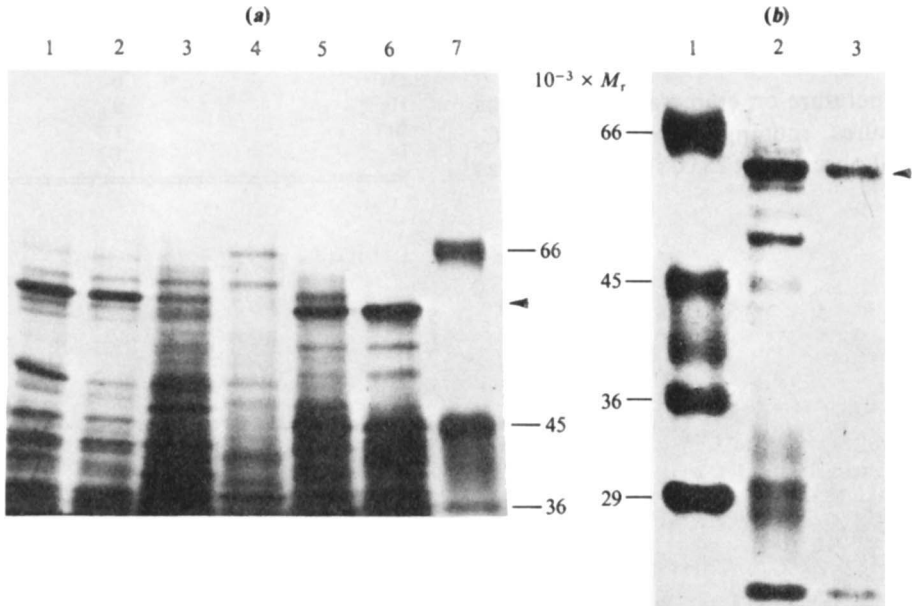

Fig. 4. SDS-PAGE of fractions from different stages of the purification of IPN epimerase. Gel (*a*) (10% acrylamide): lane 1, crude extract; 2, protamine sulphate precipitate; 3, ammonium sulphate precipitate (30–50%); 4, Sephadex G-75; 5, first HPLC (Mono-Q HR 5/5); 6, second Mono-Q HPLC; 7, M_r standards. Gel (*b*) (8% acrylamide): lane 1, M_r standards; 2, second Mono-Q HPLC; 3, Superose 12 active fractions. The M_r 59000 protein bands (IPN epimerase) are arrowed.

other contaminant bands in the purest preparation obtained after Superose 12 FPLC gel-filtration (Fig. 4*b*).

M_r and isoelectric point

The M_r of IPN epimerase from *N. lactamdurans*, as established by Sephadex G-75 gel filtration was 59000 ± 1000. IPN epimerase from *S. clavuligerus* eluted from the same column with an V_e/V_0 of 1·14, corresponding to an M_r of 63000 ± 1000. Enzyme activity was lost when applied to a chromatofocusing Mono-P column eluted with a decreasing pH gradient from 8·3 to 5·0. Bidimensional O'Farrel electrophoresis of the purest samples of epimerase gave two spots with isoelectric points of 5·5 and 5·8 for the protein of M_r 59000 (Fig. 5) (see Discussion).

Kinetic characteristics of IPN epimerase

The epimerase reaction was linear for at least 80 min under the assay conditions used, and then decreased, probably due to the fact that the reaction is reversible. To establish if penicillin N, the product of the reaction, could also serve as substrate for the enzyme, the differential assays of IPN and penicillin N against *E. coli* Ess 2231 and *M. luteus* were optimized. The forward reaction was clearly shown to proceed quantitatively (up to 80% conversion in 100 min). The reverse reaction was also observed using pure preparations of penicillin N as substrate. A total of 8 μg IPN was formed from 100 μg of substrate after 100 min incubation, indicating that the forward reaction is more effective than the reverse reaction.

The effect of temperature on epimerase activity was tested at temperatures ranging from 5 to 45 °C. Maximal activity was observed at 25 °C; the activity was slightly lower at 20 and 30 °C. At temperatures below 20 °C or above 30 °C enzyme activity was reduced. Both penicillin N and IPN were stable in the PP-containing reaction buffer at temperatures between 20 and 40 °C for 120 min. The epimerase showed maximal activity at pH 7·0 (using two different buffers) with a slightly higher activity when MOPS buffer was used. A drastic reduction of activity was found when the pH was above pH 8·0 or below pH 6·5.

The epimerase activity was proportional to the protein concentration in the range 0·5 to 10 mg protein ml^{-1}. The apparent K_m for IPN was 270 μM.

Effect of ions, amino acids and thiol-specific reagents

The effect of different anions and cations on the IPN epimerase activity was tested at 1 mM concentration (Table 4). Neither F^-, Cl^-, NO_3^-, $H_2PO_4^-$, AsO_4^{3-} or SO_4^{2-} (sodium salts) affected the activity. Br^- was slightly inhibitory (15%) and I^- inhibited activity by 27%. Some

Table 4. *Effect of some ions on the activity of IPN epimerase*

All other ions tested (see text) had no effect on enzyme activity.

Ion (1 mM)	Specific activity [pkat (mg protein)$^{-1}$]	Inhibition (%)
None	1·65	0
Zn^{2+}	0·82	50
Co^{2+}	1·38	16
Mn^{2+}	1·38	16
Cu^{2+}	0	100
Hg^{2+}	0	100
Br^-	1·4	15
I^-	1·2	27

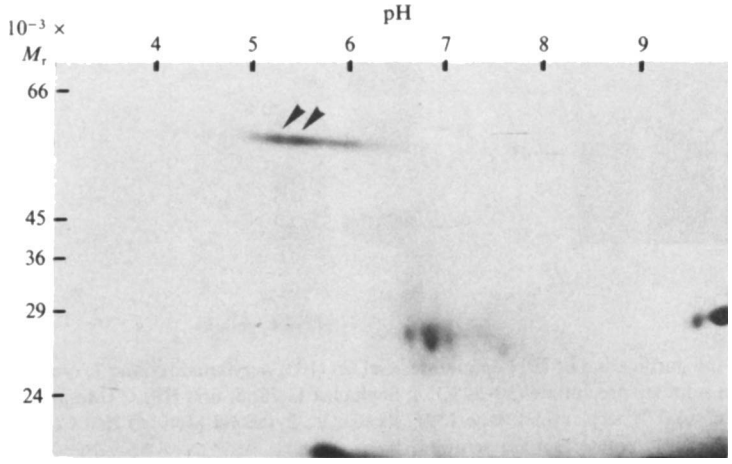

Fig. 5. Bidimensional electrophoresis of IPN epimerase (30 μg protein) visualized by the silver staining technique. Isoelectric focusing was run in the first (horizontal) direction using ampholytes 3–10, followed by SDS-PAGE in the second (vertical) direction (see Methods). Two main spots corresponding to the IPN epimerase (M_r 59000) are indicated by arrows.

divalent cations severely affected the enzyme activity: Fe^{2+}, Cu^{2+} and Hg^{2+} completely inactivated the enzyme and Zn^{2+} inhibited activity by 50%; Na^+, K^+, Mg^{2+} and Ca^{2+} (sulphate salts) did not have any effect on enzyme activity (Table 4).

The effect on the IPN epimerase of several amino acids that are direct precursors of cephamycin or affect indirectly the formation of cephamycin C in fermentation (Castro *et al.*, 1985) was also tested. Neither α-aminoadipic acid, valine, glutamine, glycine, aspartic acid or glutathione (1 mM) affected the activity of the enzyme *in vitro*.

Several compounds that react with the –SH groups of proteins inhibited the IPN epimerase activity. *p*-Chloromercuribenzoate (0·1 mM) inhibited activity by 36%, N-ethylmaleimide (0·01 to 0·1 mM) by 30 to 33% and iodoacetamide (0·01 to 0·1 mM) by 37·5 to 40%. Hg^{2+} ions (as the chloride salt) inhibited epimerase activity by 26% at 0·01 mM and by 100% at 0·1 mM concentration.

Enzyme stability

The IPN epimerase of *N. lactamdurans* was found to be unstable, as are other IPN epimerases. Preparations containing ammonium sulphate were much more stable when frozen than the corresponding desalted preparations. Dialysis of an ammonium sulphate enzyme preparation [activity 0·148 pkat (mg protein)$^{-1}$] for 6 h at 4 °C against TPDP buffer led to complete loss of activity; the same preparation lost only about 50% of its activity when kept at 4 °C (without desalting) for the same time. In addition, PP greatly stabilized the enzyme. In the absence of PP, activity was undetectable after 3 d at −70 °C. However, in the presence of 0·05 mM-PP the enzyme was active after 17 d at −70 °C. Increasing the concentration of PP up to 0·3 mM or adding PMSF (1 mM) did not stabilize epimerase activity further.

When the stability of the enzyme was tested at different pH values, the same activity was found (after incubating for 3 h at 4 °C) at pH values ranging from 4·5 to 9. Similar results were found when the extract was incubated at different pH values at 20 °C, although the enzyme lost about 45% of its activity in all the preparations due to the higher temperature. The epimerase from *S. clavuligerus*, purified using the same procedure, was less stable than the enzyme from *N. lactamdurans*.

Discussion

D-Amino acids are widely found as structural components of peptide antibiotics (Martín & Liras, 1981; Kleinkauf & von Döhren, 1987). However, very little is known about enzymes that isomerize L- to D-amino acids in the antibiotic biosynthetic pathways.

IPN epimerase from *N. lactamdurans* converts IPN to penicillin N by changing the configuration of the chiral centre at carbon-2 of the L-α-aminoadipic acid component of IPN to the D-configuration. The cofactors for IPN synthase (Fe^{2+}, ascorbic acid, DTT) (Castro *et al.*, 1988) and expandase (Fe^{2+}, 2-oxoglutarate) (Cortés *et al.*, 1987) do not stimulate the IPN epimerase activity *in vitro*. Cofactor studies with the partially purified epimerase of *S. clavuligerus* (Jensen *et al.*, 1983) gave similar results. The reaction appears to be reversible although the forward reaction (IPN to penicillin N) is favoured under our optimal experimental conditions.

The epimerase of *N. lactamdurans* is more stable than the corresponding enzymes of *C. acremonium* and *S. clavuligerus*. The extreme lability of the *C. acremonium* enzyme has been confirmed by Lübbe *et al.* (1986) and has prevented purification of this enzyme. The stability of the enzyme from *N. lactamdurans* is increased in the presence of PP. It was not clear whether this compound is required as a cofactor since only slightly lower enzyme activity (85%) was found in reaction mixtures without added PP. Jensen *et al.* (1983) suggested that PP associates tightly with epimerase protecting the enzyme from inactivation. Sodium borohydride, an inhibitor of PP-requiring enzymes, inhibited IPN epimerase activity; this inhibition was partially reversed by addition of PP. These results suggest that PP is a cofactor of IPN epimerase and binds tightly to the enzyme.

The strong inhibitory effect of divalent cations such as Cu^{2+}, Hg^{2+} or Zn^{2+} is similar to the effect that these cations exert on IPN synthase (Castro *et al.*, 1988; Ramos *et al.*, 1985) and deacetoxycephalosporin C synthase (Cortés *et al.*, 1987). However Fe^{2+}, which is a cofactor of oxygenases such as IPN synthases and deacetoxycephalosporin C synthase has a strong inhitory effect on IPN epimerase. The molecular mechanisms by which those ions affect enzyme activity are unclear but they may affect interaction of the substrate with thiol-containing active centres. Compounds that react with –SH groups also partially inhibited IPN epimerase.

The M_r of the epimerase from *N. lactamdurans*, as established by gel filtration, is 59000 ± 1000. The M_r of the protein shown by SDS-PAGE to be enriched during purification was also 59000. An M_r of 63000 ± 1000 was obtained for the IPN epimerase of *S. clavuligerus*, which agrees with the value reported by Jensen *et al.* (1983). The M_r of the *C. acremonium* enzyme has not been established.

The IPN epimerase of *N. lactamdurans*, after isoelectric focusing (see Fig. 5) gave two spots with close pI values (5·5 and 5·8), both of which correspond to the

59 kDa protein; this phenomenon is frequent in isoelectric focusing of purified proteins (Hare *et al.*, 1978).

Two enzymes involved in peptide antibiotic biosynthesis, gramicidin synthetase I (GS1, M_r 100000; Kanda *et al.*, 1978) and tyrocidine synthetase I (TY1, M_r 100000), can catalyse the racemization of activated L-phenylalanine to the D-configuration. In etamycin biosynthesis *trans*-4-hydroxy-L-proline is isomerized to *cis*-4-hydroxy-D-proline (Katz *et al.*, 1979). Racemization of phenylalanine during the formation of gramicidin S and tyrocidine takes place by an ATP-dependent mechanism after activation of L-phenylalanine on an enzyme-bound thioester-linked intermediate (Takahashi *et al.*, 1971). This racemization reaction is not dependent on the addition of cofactors such as FAD or PP, nor is it affected by known inhibitors of PP-containing enzymes (Yamada & Kurahashi, 1969). A similar mechanism is probably involved in the reaction catalysed by the IPN epimerase, although no ATP is required.

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