Purification and Characterization of the Isopenicillin N Synthase of
Streptomyces lactamdurans

By JOSÉ M. CASTRO, PALOMA LIRAS, LEONILA LAÍZ, JESÚS CORTÉS
AND JUAN F. MARTÍN*

Departamento de Microbiologia, Facultad de Biologia, Universidad de León, León, Spain

(Received 24 April 1987; revised 10 July 1987)

The isopenicillin N synthase (cyclase) of Streptomyces lactamdurans (syn. Nocardia lactamdur-
ans) has been purified to near homogeneity as judged by SDS-PAGE and isoelectric focusing.
This enzyme catalyses the oxidative cyclization of the tripeptide δ-(L-α-aminoadipyl)-L-
cysteinyl-D-valine to isopenicillin N. The enzyme required DTT, Fe²⁺ and oxygen and it was
greatly stimulated by ascorbic acid. It was strongly inhibited by Co²⁺, Zn²⁺ and Mn²⁺. Optimal
pH and temperature were 7.0 and 25 °C (with the assay conditions used), respectively. The
apparent $K_m$ of isopenicillin N synthase for δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine was
0.18 mM. The enzyme is a monomer with an $M_r$ of 26500 ± 1000 and a pI of 6.55.

INTRODUCTION

Formation of isopenicillin N (IPN) from δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) is a
common step in the biosynthetic pathways of penicillins, cephalosporins and cephamycins (Fig.
1) (Abraham, 1985; Demain, 1983; Martin & Liras, 1985). The cyclization of the linear
tripeptide ACV to a molecule of IPN with antibiotic activity is of great interest since it opens the
way for the direct enzymic synthesis of penicillins (Luengo et al., 1986; Wolfe et al., 1984). The
cyclization reaction in the fungi Penicillium chrysogenum and Acroemium chrysogenum (syn.
Cephalosporium acremonium) is catalysed by the enzyme isopenicillin N synthase (IPNS)
(cyclase). It proceeds with the removal of four hydrogen atoms from the cysteine and valine
residues of the tripeptide ACV to form the β-lactam and thiazolidine rings of penicillins or the
β-
lactam-dihydrothiazine nucleus of cephalosporins (Baldwin et al., 1984).

The IPNS of A. chrysogenum has been studied in some detail (Baldwin et al., 1985; Hollander
et al., 1984; Konomi et al., 1979; Pang et al., 1984). It requires oxygen, Fe²⁺ and ascorbate and
was reported to have an $M_r$ of 41 000 (Hollander et al., 1984) or 38 000 (Baldwin et al., 1984). The
gene coding for the cyclase has been cloned recently; it encodes a protein consisting of 338
amino acids with an $M_r$ of 38416 (Samson et al., 1985). Similarly, the IPNS of P. chrysogenum
has been purified to near homogeneity (Ramos et al., 1985). It has an $M_r$ of 39000 and shows
similar requirements to the cyclase of A. chrysogenum. The gene of P. chrysogenum AS-P-78
coding for this enzyme has also been cloned (Carr et al., 1986; J. L. Barredo & J. F. Martin,
unpublished results) and shown to be related to the gene of A. chrysogenum.

The production of β-lactam antibiotics is not restricted to filamentous fungi. The ability to
synthesize cephamycins is widely distributed among actinomycetes. At least 26 different species
of Streptomyces are known to produce cephamycins (Martin, 1981). Recently, the cyclase of
Streptomyces clavuligerus has been purified to homogeneity (Jensen et al., 1986). This enzyme
has an $M_r$ of 33000 and appears to have requirements similar to the fungal cyclases (Wolfe et al.,
1984).

Abbreviations: ACV, δ-L-(α-aminoadipyl)-L-cysteinyl-D-valine; IPN, isopenicillin N; IPNS, isopenicillin N
synthase.

0001-4152 © 1988 SGM
Fig. 1. Cyclization of \(d-L-(\alpha\text{-aminoacidyl})-L\text{-cysteinyl}-D\text{-valine}\) to IPN by IPNS. The reaction proceeds with removal of four hydrogen atoms to form the four-membered \(\beta\text{-lactam}\) and the five-membered thiazolidine ring.

**Streptomyces lactamdurans** (syn. *Nocardia lactamdurans*) is a cephamycin-producing strain. Biosynthesis of cephamycin C in this strain and its regulation by carbon and nitrogen catabolism have been previously studied (Castro et al., 1985; Cortes et al., 1986). In some aspects, the cephamycin biosynthetic enzymes of *S. lactamdurans* appear to be different from those of the filamentous fungi and *S. clavuligerus* (Martin et al., 1986) and, therefore, the purification and characterization of these enzymes was of utmost interest. We describe in this work the purification to homogeneity and the characterization of the IPNS of *S. lactamdurans*, a functionally similar enzyme having a smaller \(M_r\) than the cyclases previously known.

**METHODS**

**Micro-organisms.** *S. lactamdurans* (syn. *Nocardia lactamdurans*) NRRL 3802 var. JC1843, a stable aerial-mycelium positive (Amy⁺) mutant (Castro et al., 1985) that produces higher levels of cephamycin C than the wild-type strain *S. lactamdurans* NRRL 3802 (Stapley et al., 1972) was used in this work. Single clones were isolated and tested for antibiotic production before use. Mycelia were kept frozen at -20 °C in 20% (v/v) glycerol.

**Antibiotic assays.** IPN was assayed as described previously (Castro et al., 1985) using *Micrococcus luteus* ATCC 9341 as test strain. Penicillin N, cephamycin C and deacetoxycephalosporin C were determined by bioassays and HPLC as described previously (Castro et al., 1985).

**Cell-free extracts.** Cultures of *S. lactamdurans* var. JC1843 were grown in NYG medium (Ginther, 1979) containing 0.4% MgCl₂ at 28 °C in an orbital incubator (New Brunswick, G10) at 220 r.p.m. The mycelium [approximately 5 mg cell dry weight (ml culture)-¹] was collected at 72 h when the IPNS activity was maximal, by centrifugation at 10000 g for 10 min, washed twice with sterile saline solution (0.85% NaCl) and suspended in TPD buffer [50 mM-Tris/HCl, pH 7.0, containing 0.1 mM-DTT and 1 mM-phenylmethylsulphonyl fluoride (PMSF)]. Cells were resuspended in 1/30th of the initial volume and disrupted by sonication with a Branson Sonifier B-12 in an ice bath for periods of 15 s with 0.5 min intervals for a total time of 3 min. Cell extracts were centrifuged at 15000 g for 15 min in a refrigerated centrifuge (RC-5B Sorvall) and then at 100000 g for 60 min in a Beckman L8-70 ultracentrifuge. The supernatant was collected and used as crude (S100) cell-free extract.

**IPNS.** The reaction mixture contained in a final volume of 200 μl the following compounds: bis-\(d-(\alpha\text{-aminoacidyl})\)-L-cysteinyl-D-valine (bis-ACV), 0.19 mM; ascorbic acid, 2.8 mM; DTT, 2 mM; FeSO₄, 0.135 mM; Tris/HCl buffer, 50 mM, pH 7.0; cell-free extract, containing 0.8 mg protein. A higher concentration (0.45 mM) of FeSO₄ was required when crude enzyme extracts were used (see Results). The reaction was done at 20 °C for 60 min and then stopped either by rapid freezing or by addition of 200 μl methanol. The reaction product was routinely determined by bioassay against *M. luteus*. Total protein was determined by the Lowry method. The specific activity of the enzyme is expressed as pkat (g protein)⁻¹ [pmol IPN formed s⁻¹ (g protein)⁻¹].

**Purification of IPNS.** The steps used are summarized in Table 1 and were as follows.

Precipitation of nucleic acids and fractionation of the extract. A 1% (w/v) solution of protamine sulphate in TPD buffer was slowly added to the S100 extract (49 ml, containing a total of 1063 mg protein) at 4 °C with gentle...
Table 1. Purification steps of the IPNS of Streptomyces lactamdurans

<table>
<thead>
<tr>
<th>Purification step</th>
<th>IPNS activity (pktat)</th>
<th>Protein (mg)</th>
<th>Specific activity [pktat (g protein)(^{-1})]</th>
<th>Recovery (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S100 extract</td>
<td>94.7</td>
<td>1063.3</td>
<td>89.1</td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>Protamine sulphate precipitate</td>
<td>119.5</td>
<td>1018.4</td>
<td>117.3</td>
<td>126.1</td>
<td>1.3</td>
</tr>
<tr>
<td>Ammonium sulphate precipitate*</td>
<td>28.9</td>
<td>83.6</td>
<td>345.7</td>
<td>30.5</td>
<td>3.9</td>
</tr>
<tr>
<td>Sephadex G-75 filtration†</td>
<td>81.0</td>
<td>14.4</td>
<td>5625.0</td>
<td>85.5</td>
<td>63.1</td>
</tr>
<tr>
<td>DEAE-HPLC ionic exchange†</td>
<td>3.4</td>
<td>0.48</td>
<td>7083.3</td>
<td>3.6</td>
<td>79.5</td>
</tr>
</tbody>
</table>

* The decrease in total IPNS activity during ammonium sulphate precipitation is only apparent since the enzyme activity was regained in the following step. The enzyme is inhibited by the high residual salt concentration.

† The total IPNS activity after Sephadex G-75 filtration and DEAE-HPLC ionic exchange corresponds to the values obtained when all the enzyme obtained from the ammonium sulphate precipitation step (2.2 ml) was used.

Stirring, to a final concentration of 0.1%. The suspension was kept at 4 °C for 15 min and then centrifuged at 20000 g for 15 min. Solid ammonium sulphate (15 g, enzyme grade) was added slowly, with stirring, to the supernatant to give a final concentration of 50% saturation. The solution was centrifuged at 20000 g for 15 min at 4 °C and the pellet (that contained the isopenicillin N epimerase activity) was discarded. Additional ammonium sulphate (10.4 g) was added to the supernatant to obtain a final concentration of 80% saturation and the new precipitate was centrifuged as above. The pellet was dissolved in 2.2 ml TPD buffer and desalted by passage through Sephadex G-25.

**Gel filtration.** The desalted extract (1 ml, containing 38 mg protein) was applied to a Sephadex G-75 column (650 × 20 mm, Pharmacia) equilibrated with buffer I [MgSO₄, 5 mM; KCl, 5 mM; sucrose, 5% (w/v); in Tris/HCl, 25 mM, pH 7-0] (Hollander et al., 1984). The IPNS was eluted with the same buffer at a flow rate of 4 ml h⁻¹. The column had been previously calibrated with proteins of known Mᵦ; cytochrome c (12400), carbonic anhydrase (29000), ovalbumin (45000) and BSA (66000). The filtration was repeated using the remaining 1.2 ml of the desalted extract.

**DEAE-HPLC ionic exchange.** All the active fractions obtained by gel filtration (14.4 mg protein in 4 ml) were applied to a DEAE-5PW anionic column (Waters Protein Pack) previously equilibrated with buffer I using a Varian 5000 HPLC chromatograph equipped with a UV (254 nm) detector. The proteins were eluted with a gradient of buffer II (MgSO₄, 10 mM; KCl, 10 mM; sucrose, 10%; in Tris/HCl, 250 mM, pH 7-0) programmed as follows: at time 0, 10% (v/v) buffer II; at 25 min, 40%; at 30 min, 42%; at 41 min, 60%; at 61 min, 60% and at 85 min, 100% buffer II.

**PAGE.** Purification of the IPNS was followed by 10% (w/v) PAGE as described by Laemmli (1970). Samples of the enzyme were mixed with 0.33 vol. sample buffer [10% (w/v) SDS, 1-0 ml; glycerol, 1-0 ml; 2-mercaptoethanol, 0-1 ml; 0.1% bromophenol blue, 0-2 ml; 0.5 M-Tris/HCl buffer, pH 6-8, 1-0 ml] (total volume of 8 ml) and heated at 100 °C for 10 min. The gel was developed at 120 V for 6 h in a BioRad Protein 16 cm vertical slab cell with circulating water at 4 °C. The proteins were stained with Coomassie blue R 250 or with silver nitrate (Oakley et al., 1980). The following Mᵦ, standards were used as controls: bovine lactalbumin (14200), trypsin inhibitor (20100), trypsinogen (24000), carbonic anhydrase (29000), glyceraldehyde-3-phosphate dehydrogenase (36000), ovalbumin (45000) and BSA (66000).

**Isoelectric focusing.** This was done in 1 mm polyacrylamide gels bound to glass plates (230 × 115 mm) using a Pharmacia flat-bed apparatus FBE 3000 and a Shandon Vokam 2000-300-150 constant-power source according to LKB instructions modified as below. Gels contained, in a total volume of 25.6 ml: acrylamide, 1-76 g; bis-acrylamide, 0-09 g; Pharmalyte, pH 3-10, 1-9 ml; glycerol, 1-9 ml. The cross-linking reaction was initiated by adding ammonium persulphate, 3-84 mg; riboflavin, 0-06 mg; and TEMED, 7 µl. Aspartic acid, 0-04 M, and NaOH, 1 M, were used as anolyte and catholyte, respectively. Gels were cooled to 6 °C with circulating iced water. Prefocusing was obtained in a total time of 90 min after increasing gradually the voltage to 1800 V. Samples were loaded onto the gels and focused for 60 min at 1600 V. Proteins of known PI in the range 3.5-9.3 (Broad PI calibration kit, Pharmacia) were used as controls.

**Substrates and chemicals.** Pure cephamycin C was provided by D. Hendlin (Merck, Sharp and Dohme). IPN (62% purity) and bis-ACV (dimer form) were a gift of P. van Dijck (Gist Brocades); bis-ACV was reduced to the monomeric form by addition of DTT. Phenylacetyl-L-cysteinyl-D-valine was kindly provided by J. M. Luengo and F. Sávio (University of Leon, Spain). Sephadex G-75, Sephadex G-100, Pharmalyte (pH 3-10) and the PI standard proteins were from Pharmacia. All other chemicals, including the Mᵦ, marker proteins, were from Sigma.
RESULTS

Purification of IPNS

The IPNS of *S. lactamdurans* is an intracellular soluble enzyme that was found in the S100 cell-free extract (see Methods) but not in the culture broth. Removal of nucleic acids and nucleoproteins from the S100 extract by precipitation with protamine sulphate led to an increase (26%) in the total enzyme activity of the extracts, suggesting the presence of an enzyme inhibitor(s) in the crude extract that was removed (Table 1).

After ammonium sulphate precipitation (50–80% saturation), the IPNS and deacetoxy-cephalosporin C synthase activities in the extract were recovered in the precipitate, but the isopenicillin N epimerase was removed. The specific activity of IPNS increased approximately 3.9-fold in this step. Although there was an apparent loss in the recovery of the enzyme, this was not the case since the enzyme was regained in the following step after gel filtration (or dialysis). Apparently, the enzyme activity is inhibited by the high salt concentration remaining after ammonium sulphate precipitation.

Gel filtration through Sephadex G-75 led to a large increase in purification (63.1-fold) with a good recovery. A single peak of enzyme activity was obtained (Fig. 2a).

The IPNS was further purified by ion exchange HPLC through a DEAE column and was eluted as a single narrow peak in the gradient when the high-strength buffer II reached a 54% concentration (Fig. 2b). No other peak of activity was found when fractions from the entire gradient were assayed. This step was very convenient for removing contaminating proteins as
concluded from PAGE studies (see below), although it improved only slightly the specific activity since there was a severe loss in the activity of the enzyme. Apparently, the enzyme activity is unable to withstand, at high pressure, the ionic strength required to elute the enzyme.

A total purification of 79.5-fold and a specific activity of 7083.3 pkat (g protein)$^{-1}$ were obtained after these purification steps, after which the enzyme was used for further kinetic studies. It converted ACV into IPN but did not catalyse the conversion of IPN to penicillin N, deacetoxycephalosporin C or cephamycin C. The purified enzyme was stable at $-70\, ^\circ$C in the presence of 5% sucrose for at least one month.

$M_r$ determination

The elution profile of an enzyme preparation filtered through a Sephadex G-75 column (Fig. 2a) showed that the cyclase activity eluted with a $V_e/V_o$ of 2:18 ($K_v$, 0-39), corresponding to an $M_r$ of 26500 $\pm$ 1000. This was identical to the value obtained on filtration in Sephadex G-100 and to the value calculated for the cyclase protein band obtained on PAGE.

PAGE and isoelectric point

Samples from all the purification steps were routinely analysed by PAGE in the presence of SDS. A band that migrated just ahead of the carbonic anhydrase marker ($M_r$ 29000) was enriched in all the purification steps (Fig. 3). This band was barely detectable before the ammonium sulphate fractionation but became the major protein after gel filtration and DEAE-HPLC chromatography (Fig. 3a). As concluded from PAGE studies, the IPNS protein was highly purified by DEAE-HPLC (purity increased from about 20% in lane 5 to more than 95% in lane 6). However, there was no parallel increase in specific activity during this step, supporting our observation that although DEAE-HPLC results in a good purification of the IPNS protein, it leads to a loss of enzyme activity (Table 1). A purity of more than 95% was estimated from lane 7 of PAGE (Fig. 3a). The IPNS was freed from most of the residual contaminants by ultrafiltration through an Amicon P-30 membrane. The cyclase purified in this form remained active and showed a single band on SDS-PAGE (Fig. 3a). The $M_r$ of the SDS-denatured enzyme, calculated from expanded SDS-PAGE gels using additional low-$M_r$ markers of 20100 and 24000, was 26500 (Fig. 3b), which agrees well with the $M_r$ estimated by
gel filtration. The protein present as a single band in this preparation electrofocused in thin polyacrylamide gels at a pH of 6·55.

Cofactors required for IPNS activity

Different components of the reaction mixture were tested, singly or in combinations, to study their effect on IPNS activity.

DTT (or a sulphhydryl-containing reducing agent) was always required for enzyme activity (Fig. 4a). The enzymic activity was stimulated by DTT concentrations from 0·25 to 2 mM.

A requirement of Fe\(^{2+}\) for IPNS activity was observed (Fig. 4b). However, the concentration of this ion required for optimal IPNS activity changed according to the degree of purification of the enzyme. IPNS preparations after ammonium sulphate fractionation required 450 μM-Fe\(^{2+}\), whereas IPNS purified by gel filtration and subsequent purifications steps required only 100 μM for optimal activity (Fig. 4b).

Ascorbic acid up to 2·5–3 mM stimulated IPNS activity. In the absence of ascorbic acid, less than 8% of the maximal activity was retained (Fig. 4c). ATP was not required for IPNS activity, and therefore the enzyme should be named IPN synthase rather than IPN synthetase.

Optimal pH, temperature and protein concentration

IPNS activity increased linearly with protein concentrations between 0·6 and 12 mg ml\(^{-1}\). pH studies were done using enzyme extracts purified by gel filtration and dialysed against distilled water for 2 h at 4 °C to remove the interference effect of the pH of the elution buffer on the enzyme activity. The effect of pH on IPNS activity was determined using 100 mM-MOPS buffer in the range from pH 5·8 to 7·0, and 100 mM Tris/HCl buffer between pH 7·0 and 9·0. Optimal activity was found at pH 7·0 in both buffers.

A good IPNS activity was observed at temperatures between 20 and 30 °C with optimal activity at 25 °C with the assay system used. A considerable reduction of activity was observed at 15 and 35 °C.

Oxygen requirement

Oxygen was required for IPNS activity. When oxygen was removed by bubbling N\(_2\) through the reaction mixture and the reaction occurred in closed tubes under a N\(_2\) atmosphere, there was a 97% reduction in activity. Increasing the oxygen transfer by agitating the open tubes at 250 r.p.m. raised the activity by only 5%. Pure oxygen inactivated the enzyme.
Isopenicillin N synthase of *S. lactamdurans*

**Influence of ions**

None of the anions tested (F\(^-\), I\(^-\), Br\(^-\), Cl\(^-\), NO\(_3\), H\(_2\)PO\(_4\), AsO\(_4\)\(^-\), SO\(_4\)\(^2-\)) affected IPNS activity at 1 mM concentration. Among the cations tested, Co\(^2+\), Zn\(^2+\) and Mn\(^2+\) severely inhibited IPNS (50%, 38% and 23%, respectively, at 1 mM) whereas Cu\(^2+\) had a slight inhibitory effect (10%) and Hg\(^2+\) was inactive (see Discussion). Fe\(^3+\) (as compared to Fe\(^2+\)) had no effect on enzyme activity. Na\(^+\), K\(^+\), Mg\(^2+\) and Ca\(^2+\) (1 mM) did not affect IPNS activity.

**Effect of regulatory effectors and cephamycin C precursors**

NH\(_3\), which strongly repress IPNS formation *in vivo* (Castro et al., 1985), had no effect on IPNS activity *in vitro* in the range 10–40 mM. The enzyme activity was inhibited 83% by 1 mM glucose 6-phosphate and slightly by pyruvate (13%), but other sugar phosphates were not inhibitory (Cortes et al., 1986).

The amino acids α-aminoadipic acid, cysteine, valine (precursors of cephamycin C); glutamic acid, glycine (precursors of glutathione); aspartic acid and glutamine had no effect on IPNS activity at a concentration of 1 mM. Glutathione showed a slight inhibitory effect (14%) on IPNS activity at the same concentration.

**Kinetics of the reaction**

The kinetics of IPNS, determined using a sample of the purified enzyme containing 4 mg protein ml\(^-1\) at saturation levels of the cofactors, indicated a \(K_m\) for ACV of 1.8 × 10\(^{-4}\) M. The affinity of the enzyme for the cofactors DTT, Fe\(^2+\) and ascorbic acid is shown in Fig. 4. The (DTT) \(V_{0.5}\) was 1.0 mM and (ascorbic acid) \(V_{0.5}\) was 0.55 mM, whereas \(V_{0.5}\) for Fe\(^2+\) was about 13.0 \(\mu\)M.

**DISCUSSION**

The oxidative cyclization of ACV to IPN is a new type of enzymic conversion existing only in β-lactam-producing micro-organisms (Abraham, 1985). The conversion by the IPNS of *S. lactamdurans* is similar in many respects to the cyclization reactions catalysed by the cyclases of *P. chrysogenum* (Ramos et al., 1985), *A. chrysogenum* (Hollander et al., 1984; Pang et al., 1984) and *S. clavuligerus* (Jensen et al., 1982; 1986).

The IPNS of *S. lactamdurans* has an absolute requirement for oxygen, Fe\(^2+\) and DTT and is greatly stimulated by ascorbic acid (a two-electron donor), i.e. it behaves as an oxygenase, although it does not require 2-oxoglutarate. In this respect, the IPNS of *S. lactamdurans* is similar to the cyclases of the other β-lactam producers known so far. However, the \(M_r\) of the IPNS of *S. lactamdurans* (26500) is clearly smaller than that of the cyclases of *P. chrysogenum* (39000) (Ramos et al., 1985), *A. chrysogenum* (38416) (Samson et al., 1985) and *S. clavuligerus* (33000) (Jensen et al., 1986). The optimal pH and temperature of the cyclase of *S. lactamdurans* are slightly different from those of the fungal cyclases. Another difference of the IPNS of *S. lactamdurans* is the isoelectric point of this protein. The PI of the cyclase of *A. chrysogenum* is 5.05 (Baldwin et al., 1985) whereas the IPNS of *S. lactamdurans* has a pl of 6.55. The pl values of the cyclases of *Penicillium* and *S. clavuligerus* have not been reported.

The IPNS of *S. lactamdurans* appears to be a monomer since the \(M_r\) of the natural (non-denatured) form calculated by gel filtration is identical to the \(M_r\) of SDS-denatured protein as estimated by PAGE. The cyclase of *A. chrysogenum* is also a monomer (Baldwin et al., 1985).

The requirement of DTT to achieve optimal enzyme activity (2 mM) is well in excess of the concentration required to reduce the bis-ACV as substrate (0.19 mM). This result suggests that DTT may play an additional role in protecting the adequate configuration of the enzyme, as reported also for the cyclase of *P. chrysogenum* (Ramos et al., 1985). The cyclase of *A. chrysogenum* contains two cysteine residues that are required for enzyme activity (Samson et al., 1987). DTT may, therefore, play an important role in interconverting the two forms of the enzyme by reducing an accessible intramolecular disulphide bridge (Baldwin et al., 1985).

The strong inhibitory effect of Co\(^2+\), Zn\(^2+\), Mn\(^2+\) and, to a lesser extent, Cu\(^2+\) on the IPNS of *S. lactamdurans* is similar to their effect on the cyclase of *P. chrysogenum* (Ramos et al., 1985) and appears to be due to competition with the Fe\(^2+\) requirement.
The purified enzyme showed an apparent $K_a$ for ACV of 0.18 mM, similar to the $K_a$ of the cyclase of \textit{P. chrysogenum} (0.13 mM) \cite{Ramos1985} and slightly lower than the reported value for \textit{A. chrysogenum} (0.3 mM) \cite{Kupka1983} and \textit{S. clavuligerus} (0.32 mM) \cite{Jensen1986}. The IPNS of \textit{S. lactamdurans} converted phenylacetyl-L-cysteinyl-d-valine (PCV), a structural analogue of the natural substrate, into penicillin G, but the affinity of the enzyme for PCV was about 20 times lower than its affinity for ACV. The cyclization of PCV has the same cofactor requirement as the conversion of ACV into IPN \cite{Castro1986}. The substrate specificity of IPNS is rather high although some modifications in the a-aminoadipyl residue of ACV are tolerated \cite{Baldwin1984, Jensen1984, Luengo1986}. Modification of the substrate affinity and specificity for direct enzymic synthesis of new $\beta$-lactam antibiotics is one of the goals we have in mind once the genes have been cloned. For this purpose, the IPNS of \textit{S. lactamdurans} is of great interest since the molecular genetics of \textit{Streptomyces} is more developed than the cloning systems in $\beta$-lactam-producing filamentous fungi. Moreover, the availability of different IPNSs offers a convenient choice of the more adequate ones for enzymic synthesis of new antibiotic molecules.

\textbf{REFERENCES}


\textbf{Konomi, T., Hershen, S., Baldwin, J. E., Yoshida, M., Hunt, N. & Demain, A. L. (1979).} Cell free conversion of $\delta$(L-$\alpha$-aminoadipyl)-L-cysteinyl-D-valine into an antibiotic with the properties of isopenicillin N in \textit{Cephalosporium acremonium}. \textit{Biochemical Journal} 184, 427-430.


This research was supported by grants of the CAICYT, Madrid to J. F. M., and of the Diputación de León to P. L. We are indebted to Miss Mª Paz Puertas and Mr Bernabé Martín for their excellent technical assistance.


