The unprocessed preprotein form IAT^{C103S} of the isopenicillin N acyltransferase is transported inside peroxisomes and regulates its self-processing

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Abstract

Previous studies in Penicillium chrysogenum and Aspergillus nidulans suggested that self-processing of the isopenicillin N acyltransferase (IAT) is an important differential factor in these fungi. Expression of a mutant penDE^{C103S} gene in P. chrysogenum gave rise to an unprocessed inactive variant of IAT (IAT^{C103S}) located inside peroxisomes, which indicates that transport of the proIAT inside these organelles is not dependent on the processing state of the protein. Co-expression of the penDE^{C103S} and wild-type penDE genes in P. chrysogenum (Wis54–DE^{C103S} strain) led to a decrease in benzylenicillin levels. Changes in the wild-type IAT processing profile (β subunit formation) were observed in the Wis54–DE^{C103S} strain, suggesting a regulatory role of the unprocessed IAT^{C103S} in the processing of the wild-type IAT. This was confirmed in Escherichia coli, where a delay in the processing of IAT in presence of the unprocessable IAT^{C103S} was observed. Our results indicate that IAT is post-translationally regulated by its preprotein, which interferes with the self-processing.

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1. Introduction

Penicillium chrysogenum, Aspergillus nidulans and a few other ascomycetes (Aharonowitz et al., 1992; Laich et al., 2003) are filamentous fungi able to synthesize penicillins containing an aromatic side chain (e.g., benzylenicillin or phenoxymethylpenicillin). Penicillins are synthesized by internal cyclization of the non-ribosomal tripeptide (δ-α-aminoadipyl)-L-cysteinyl-O-valine (ACV) formed by the multienzyme (δ-α-aminoadipyl)-cysteinyl-valine synthetase (encoded by the pcbAB gene). This tripeptide is cyclized to isopenicillin N by the action of isopenicillin N synthase (encoded by the pcbC gene) (Martín and Liras, 1989). In the last step, the isopenicillin N acyltransferase (IAT; encoded by the penDE gene) synthesizes hydrophobic penicillins by substitution of the L-α-aminoadipyl side chain of isopenicillin N by aromatic acyl side chains (Álvarez et al., 1987; Martín et al., 1990). The aromatic (phenylacetic or phenoxyacetic) acid requires previous activation by a specific aryl-CoA ligase (Lamas–Maceiras et al., 2006). In P. chrysogenum, the pcbAB, pcbC and penDE genes are clustered with other ORFs forming an amplifiable DNA unit, which is present in several copies in high penicillin producing strains (Fierro et al., 2006). Each unit is bound by a conserved hexanucleotide TGTAAA/T (Fierro et al., 1995). The penDE genes, cloned from P. chrysogenum (Barredo et al., 1989) and A. nidulans (Montenegro et al., 1990; MacCabe et al., 1991), are very similar in both fungi.

The IAT is synthesized as a 40-kDa precursor (proacyltransferase, proIAT) which undergoes an autocatalytic self-processing between residues Gly102-Cys103 in P. chrysogenum. The processed protein constitutes a heterodimer with subunits α (11 kDa, corresponding to the N-terminal fragment) and β (29 kDa, corresponding to the C-terminal region) (Barredo et al., 1989; Veenstra et al., 1989; Whiteman et al., 1990; Tobin et al., 1990, 1993). Unlike the two first enzymes involved in penicillin biosynthesis, which are cytosolic, IAT is located inside the microbodies (peroxisomes) (Müller et al., 1991, 1992, 1995). Although it was initially believed that the IAT activity was located in the 11-kDa subunit (Álvarez et al., 1987), later it was shown that a higher IAT activity is observed after association of the 29-kDa and 11-kDa subunits (Tobin et al., 1990, 1993), indicating that the most active P. chrysogenum IAT is an α–β heterodimer (Whiteman et al., 1990). More recently we found that the A. nidulans proIAT remains predominantly unprocessed as a 40-kDa protein through several purification steps, whereas the P. chrysogenum enzyme is efficiently self-processed, rapidly dissociating into the 29-kDa and 11-kDa subunits (Fernández et al., 2003). In fact, only the 29-kDa subunit (but never the 40-kDa unprocessed form) is isolated from P. chrysogenum.
crude extracts and purified fractions (Álvarez et al., 1987; Fernández et al., 2003), suggesting that an efficient self-processing is associated with high rates of penicillin biosynthesis. In A. nidulans a small amount of both the 29 and 11 kDa subunits was observed but the 40 kDa unprocessed form was always found (Fernández et al., 2003). This difference in IAT processing ability between P. chrysogenum and A. nidulans might be responsible (at least in part) for the disparity in penicillin biosynthetic ability between these two fungi (wild-type strains of P. chrysogenum are able to synthesize about 30-fold higher levels of penicillin than wild-type A. nidulans).

Many fungal enzymes are formed as proenzymes (Markaryan et al., 1996) and the mechanism of self-processing is frequently a step involved in post-translational regulation of enzyme activity (Park et al., 2004). The P. chrysogenum IAT is an N-terminal nucleophile (NTN) enzyme capable of self-activation (J.F. Martín and C. García-Estrada, unpublished results), as it occurs with other NTN amidohydrolases (Brannigan et al., 1995; Seemüller et al., 1996).

One of the best known NTN enzymes is the Escherichia coli penicillin acylase (Duggleby et al., 1995). Hewitt and co-workers (2000) reported evidence indicating that in a slow-processing mutant of the E. coli penicillin proacylase, the linker peptide located between the α and β subunits, blocks the active site cleft of this enzyme.

At present it is unknown whether the self-processing IAT from P. chrysogenum works by a mechanism similar to that of other NTN enzymes and whether unprocessable proIAT or slow-processing forms, may regulate the self-processing activity of the IAT. It was, therefore, important to obtain an unprocessable mutant IAT and to construct strains in which the unprocessable IAT is synthesized together with the native processable IAT, to establish the in vivo role of unprocessed molecules on IAT activity and penicillin biosynthesis.

Tobin and co-workers (1995) showed that the recombinant IATC103S is not self-processed in E. coli and lacks acyltransferase activity. We report in this article, new insights on the IAT of P. chrysogenum including the in vivo characterization and targeting of the unprocessed IATC103S variant and the construction of P. chrysogenum strains, which simultaneously synthesize the wild-type IAT and an unprocessed variant of the protein. Results provide evidence that the unprocessed proIAT is correctly targeted to peroxisomes and affects the benzylpenicillin biosynthesis through regulation of the self-processing.

2. Materials and methods

2.1. Strains, culture conditions, plasmid constructs and transformation of P. chrysogenum protoplasts

The strains and plasmids used in this work are listed in Table 1. Fungal spores were plated on Power medium (Casqueiro et al., 1999) and grown for 5 days at 28 °C. P. chrysogenum liquid cultures were initiated by inoculating fresh spores in 100 ml of DP medium (Casqueiro et al., 1999) without phenylacetate. After incubation at 25 °C for 20 h in an orbital shaker (250 rpm), a 10 ml aliquot was inoculated in 100 ml DP medium with 1 mg/ml phenylacetate (in 500-ml flasks). Protoplasts were obtained and transformed as previously described (Cantoral et al., 1987; Diez et al., 1987). Selection of transformant clones was performed by resistance to phleomycin (30 μg/ml). E. coli JM109 (DE3) cells were used for heterologous expression of the penDE and penDEC103S genes and were grown in Luria–Bertani (LB) medium supplemented with the appropriate antibiotic.

2.2. Site-directed mutagenesis of the penDE gene

Plasmids pPenIAT (García-Estrada et al., 2007) and pPBCξς (Fernández et al., 2003) (Table 1), which contain the cDNA of the penDE gene, were used as template for site-directed mutagenesis of the Cys103 to Ser using the “QuickChange” Site-Directed Mutagenesis Kit (Stratagene La Jolla, CA, USA) following the manufacturer’s instructions. Primers used to introduce this mutation were purified by HPLC: 5′-CACCCGTGATGGCTCCACCCACGCCTAT (forward), 5′-CAAATAGCCAGCTTGAAACGCCCACCGCTG-3′ (reverse) (the triplet corresponding to the mutated Cys103 to Ser is underlined). The point mutation was confirmed by sequencing and the resulting plasmids were named pPenIATC103S and penDEC103S, respectively (Table 1).

2.3. Genomic DNA extraction and Southern blotting

DNA isolation and Southern blotting hybridisation were carried out as previously described (Lamas-Maceiras et al., 2006). A 429-bp fragment, spanning positions 727-1155 of the penDE gene (inside the third exon), was used as probe. This fragment was amplified using next primers: 5′-CCCACCCGAGTTCCCTGGC-3′ (forward)

Table 1: Fungal strains and plasmids used in this work

<table>
<thead>
<tr>
<th>Features</th>
<th>Origin</th>
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<tbody>
<tr>
<td><strong>P. chrysogenum strain</strong></td>
<td></td>
</tr>
<tr>
<td>Wis54-1255</td>
<td>Singly copy of the penicillin gene cluster</td>
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<tr>
<td>npe10-AB-C</td>
<td>Strain derived from the npe10 pyrG (Δpen). It contains the first two genes of the penicillin pathway (pCMB and pCH) integrated in the genome</td>
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<tr>
<td>npe10-AB/CDE</td>
<td>npe10-AB-C strain complemented with the penDE gene under the control of the gdh gene promoter</td>
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<tr>
<td>npe10-AB/CDE</td>
<td>npe10-AB-C strain complemented with the penDEC103S gene under the control of the gdh gene promoter</td>
</tr>
<tr>
<td>Wis54-DEC103S</td>
<td>Wis54-1255 strain complemented with the penDEC103S gene under the control of the gdh gene promoter</td>
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<tr>
<td>Plasmids</td>
<td></td>
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<tr>
<td>pPenIAT</td>
<td>Plasmid used to express the penDE gene in P. chrysogenum. It contains the cDNA of the penDE gene under the control of the gdh gene promoter</td>
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<tr>
<td>pPenIATC103S</td>
<td>Similar to pPenIAT, but by site-directed mutagenesis, the cDNA of the penDE gene has been mutated to change the Cys103 to Ser in the IAT</td>
</tr>
<tr>
<td>pPBCξς</td>
<td>Plasmid used to express the cDNA of the penDE gene in E. coli</td>
</tr>
<tr>
<td>pPBCξςC103S</td>
<td>Similar to pPBCξς, but by site-directed mutagenesis, the cDNA of the penDE gene has been mutated to change the Cys103 to Ser in the recombinant IAT</td>
</tr>
<tr>
<td>pPenIATC103S</td>
<td>Plasmid used to co-express simultaneously both the penDE and the penDEC103S genes in E. coli. The BamHI–KpnI fragment of pPBCξς, containing the pT7 and the cDNA of the penDE gene was inserted in the same restriction sites of plasmid pQE-30. The Smal–HindIII fragment of pDEC103S, including the pT7 and the cDNA of the penDEC103S gene was subcloned between the Smal and HindIII restriction sites of plasmid pQE-30</td>
</tr>
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This work
and: 5′-GATGATATCAGACAGATC-3′ (reverse). The signal provided by the Southern blotting was quantified by densitometry using the “Gel-Pro Analyzer” software (Media Cybernetics). The number of additional copies of the Pgdh-penDE103S/Tyc1 cassette was determined comparing the intensity of the 2120-bp band (including the exogenous penDE103S gene) to that of the 8000-bp band (containing the wild-type penDE gene present as a single copy in the Wis54-1255 strain (Fierro et al., 1995)).

2.4. Electron microscopy

Mycelia from P. chrysogenum Wis54-1255, P. chrysogenum npe10-AB-C and P. chrysogenum npe10-AB-C-DE103S strains were grown for 48 h under benzylpenicillin production conditions as described above. Fixation and preparation of the samples for electron microscopy was performed as described previously (Waterham et al., 1994). Ultrathin sections of unicyl-embedded (BBI International, Cardiff, UK) hyphae were immunolabeled with specific antibodies raised against IAT (1/4000 dilution) and 15 nm gold-conjugated goat-anti-rabbit antibodies (Amersham Biosciences Corp., Piscataway, NJ, USA).

2.5. Cell extracts

Penicillium chrysogenum mycelia were centrifuged for 10 min at 4400 rpm, washed three times in 0.9% (w/v) NaCl and powdered in a grinder using liquid nitrogen. The disrupted cells were resuspended in TD buffer (50 mM Tris–HCl, pH 8.0, 5 mM DTT) and stored at −80 °C. E. coli cells were harvested by centrifugation at 4400g for 10 min, washed with 0.9% (w/v) NaCl and resuspended in TD buffer. Cells were broken after sonication on ice (6 pulses of 10 s with 30 s pauses between each pulse) and centrifuged at 4400g for 10 min at 4 °C. Soluble fractions were stored at −80 °C.

2.6. IAT self-processing assessment in E. coli

Self-processing of wild-type IAT either in absence or presence of the unprocessed IATC103S was tested in competent E. coli JM109 cells. They were transformed with plasmid pPBCaβ (which expresses only the wild-type penDE gene) or with plasmid pβ-DENΔT103S (which expresses both the wild-type penDE and the penDE103S genes as indicated in Table 1). Simultaneous cultures were inoculated and induced with 0.5 mM IPTG at 37 °C for 45 min to provide enough amounts of the 40 kDa protein (only the wild-type protein able to be properly folded in cells transformed with plasmid pPBCaβ and a mixture of wild-type IAT and IATC103S variant in cells transformed with plasmid pβ-DENΔT103S). Cells were washed three times with 0.9% NaCl to remove the IPTG, resuspended in LB media and incubated at 26 °C. These conditions allow the self-processing of the preprotein. This artificial processing system provided us with a useful tool to compare the processing of IAT in absence and presence of the IATC103S. One milliliter samples were taken at different time points, centrifuged, resuspended in 100 µl of loading buffer (see below) and diluted 1000-fold. Then, they were run in SDS–PAGE gels and analyzed by Western blotting.

2.7. SDS–PAGE and Western blotting

Protein samples to be analysed by SDS–PAGE were diluted in loading buffer (60 mM Tris–HCl, pH 6.8, 2% SDS, 100 mM DTT, 10% glycerol and 0.1% bromophenol blue), boiled for 5 min, and run in a 12% acrylamide gel. The “Low Molecular Weight Calibration Kit for SDS Electrophoresis” (Amersham Biosciences Corp.) and “Precision Plus Protein All Blue Standards” (Bio-Rad, Hercules, CA, USA), were used as molecular weight marker. Proteins were stained using Coomassie Brilliant Blue R250 dying. Immunological detection of IAT (wild-type and IATC103S mutant) was performed as previously described (García-Estrada et al., 2007). Polyclonal antibodies against P. chrysogenum IAT were obtained as indicated before (Fernández et al., 2003).

2.8. In vitro IAT activity measurement

Measurement of the IAT activity was carried out using E. coli soluble extracts (see above). Briefly, 36 µl of cell extracts containing the recombinant IAT were mixed either with 36 µl of soluble extract containing the recombinant unprocessable IATC103S with 36 µl of 1/10 dilution of soluble extract containing the recombinant unprocessable IATC103S or with 36 µl of uninduced E. coli extract (as control). Extracts were pre-incubated for 1 and 5 h at 26 °C. After the pre-incubation time, 48 µl of the reaction mixture (0.1 M Tris–HCl, pH 8.0, 0.05 M DTT, 0.2 mM 6-APA and 0.2 mM phenylacetyl-CoA) were added and incubated at 26 °C for 15 min. The reaction was stopped with 120 µl of methanol, centrifuged at 13,000 rpm for 5 min and bioassayed. In vitro reactions were performed in triplicates.

2.9. RNA extraction and RT-PCR assays

RNA was extracted from cultures of P. chrysogenum at 24, 48 and 72 h using the “RNeasy Mini Kit” columns (Qiagen, Hilden, Germany), following the manufacturer’s instructions. Total RNA was treated with “RQ1 RNase-Free DNase” (Promega Corporation, Madison, WI, USA), following the manufacturer’s instructions and quantified using a NanoDrop ND-1000 spectrophotometer. RT-PCR was performed using 50 ng of total RNA and the “SuperScript One-Step RT-PCR with Platinum Taq” system (Invitrogen Corporation, Carlsbad, CA, USA), following the manufacturer’s instructions. Reverse primer was specific to the initial part of the Tyc1 terminator: 5′-GAAAAGGACGACATTCG-3′. Forward primer included the start codon of the penDE gene: 5′-ATGCTCAATCTCTGTCAAGC-3′. Annealing temperature was set to 55 °C during 35 cycles.

2.10. Bioassays and HPLC analysis

Filtrates from P. chrysogenum cultures on liquid DP medium (Casqueiro et al., 1999) or samples obtained in the in vitro acyltransferase activity experiments, were used to assess the formation of isopenicillin N or benzylpenicillin. Micrococcus luteus was used as test microorganism. It was grown at OD600 ≈ 2 in TS (Difco) and inoculated into TSA (Difco) medium at a final OD600 ≈ 0.01. Benzylpenicillin extraction and analysis by HPLC were carried out as previously indicated (García-Estrada et al., 2007).

3. Results

3.1. In vivo characterization of a tailored P. chrysogenum strain which expresses the mutant penDE103S gene encoding the unprocessable IATC103S

The change of Cys103 residue to Ser (C103S) is known to abolish self-processing and activity of IAT in P. chrysogenum (Tobin et al., 1995). In order to express the mutant penDE103S gene in P. chrysogenum, plasmid pPenIATC103S, in which the mutant penDE103S gene is expressed from the strong A. awamori gdh promoter (Table 1), was constructed as indicated in Section 2. This plasmid was introduced into the P. chrysogenum npe10-AB-C strain, which is able to synthesize isopenicillin N and lacks the penDE gene (García-Estrada et al., 2007). Transformants were selected for their ability to grow in medium with 30 µg/ml penicillin. The presence of the penDE103S
allele was tested by PCR (data not shown) and integration of the Pgdh-penDE<sup>C103S</sup>-Tcyc1 cassette was confirmed by Southern blotting (Fig. 1A) using a probe internal to the penDE gene. Transformants T22, T24 and T30 showed a 2120-bp band after the digestion of the genomic DNA with HindIII and KpnI. This band corresponds to the whole Pgdh-penDE<sup>C103S</sup>-Tcyc1 cassette. Additional bands, which are a result of the integration of fragments of this cassette, are visible in transformants T4, T9, T24 and T30. Expression of the mutant gene was tested by Western blotting using cell extracts obtained from 24-h cultures of the different transformants. As shown in Fig. 1B, the 40-kDa mutant proIAT<sup>C103S</sup> was generated only in those transformants with a full integration of the Pgdh-penDE<sup>C103S</sup>-Tcyc1 cassette (note the lack of self-processing of the IAT<sup>C103S</sup>). Transformant T22 was selected and was named P. chrysogenum npe10-AB-C-DEC103S. As internal control of these experiments, plasmid pPenIAT, which contains the cDNA of the penDE gene under the control of the strong A. awamori gdh gene promoter (García-Estrada et al., 2007), was transformed into the npe10-AB-C strain, thus generating the npe10-AB-C-DE strain. Expression of this gene generated an IAT capable of self-processing (Fig. 1B) and restored the penicillin production (data not shown), as it was previously reported (García-Estrada et al., 2007).

The ability of the mutant protein to remain unprocessed in P. chrysogenum npe10-AB-C-DEC103S was tested. As shown in Fig. 1C, only the 40-kDa band is visible throughout the culture. This band is more abundant at 36-48 h and then gradually decreases until 72 h. This shows the lack of processing of the mutant IAT<sup>C103S</sup> when it is synthesized in P. chrysogenum and indicates that no other proteolytic mechanism different from autocatalysis (absent

![Fig. 1. Expression of the penDE<sup>C103S</sup> gene and activity of the unprocessed IAT<sup>C103S</sup> in P. chrysogenum npe10-AB-C. (A) The npe10-AB-C strain was transformed with plasmid pPenIAT<sup>C103S</sup>. Different transformants (T4, T9, T22, T24 and T30) were tested by Southern blotting after digestion of the genomic DNA with HindIII and KpnI, which releases the whole Pgdh-penDE<sup>C103S</sup>-Tcyc1 cassette (2120 bp). Bands of different size indicate integration of fragments of the Pgdh-penDE<sup>C103S</sup>-Tcyc1 cassette in the transformants. Genomic DNA from the Wis54-1255 strain (Wis), which shows an 8000 bp band (internal penDE gene), was used as positive control, whereas DNA from the npe10-AB-C strain was used as negative control (C). The x-HindIII molecular weight markers are indicated as M. (B) Western blot carried out with protein extracts isolated from 24-h cultures of the different transformants and antibodies raised against IAT (they recognize the β subunit and the α–β proIAT but not the separate α subunit). Protein extracts from the npe10-AB-C strain were used as negative control (C–), whereas the npe10-AB-C-DE (C+) and Wis54-1255 (Wis) strains were used as positive controls. Molecular weight markers (lane M) are indicated on the left in kDa. Transformant T22 was selected and was named P. chrysogenum npe10-AB-C-DEC103S. (C) Western blot showing the synthesis of the unprocessed IAT<sup>C103S</sup> in the npe10-AB-C-DEC103S strain. Samples were taken at 24, 36, 48, 60 and 72 h, and hybridized with anti-IAT antibodies. (D) HPLC of filtrates taken at 72 h from cultures of the npe10-AB-C-DEC103S and the Wis54-1255 strains. As internal control a pure benzylpenicillin sample was used at a concentration of 10 µg/ml.

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in the IAT\textsuperscript{C103S} mutant) is involved in the processing of IAT in this fungus.

The benzylpenicillin biosynthetic activity of the IAT\textsuperscript{C103S} was assessed in the \textit{P. chrysogenum} npe10-AB-C-DE\textsuperscript{C103S} strain by bioassay (data not shown) and by HPLC using filtrates taken at 72 h from cultures of the npe10-AB-C-DE\textsuperscript{C103S} and the Wis54-1255 strains. Chromatograms showed the absence of benzylpenicillin in filtrates from the npe10-AB-C-DE\textsuperscript{C103S} strain (Fig. 1D), indicating that the unprocessed IAT lacks enzymatic activity in vivo.

3.2. The unprocessed IAT is transported into peroxisomes

An important question is whether the unprocessed IAT can be transported into peroxisomes. To test this, subcellular localization of the IAT\textsuperscript{C103S} was determined by immunocytochemistry as indicated in Section 2. \textit{P. chrysogenum} npe10-AB-C-DE\textsuperscript{C103S} (expressing the mutant penDE\textsuperscript{C103S} gene), Wis54-1255 (expressing the wild-type penDE gene) and npe10-AB-C (lacking the penDE gene) strains were analysed using electron microscopy. Immunolabeling with antibodies raised against IAT clearly showed that the unprocessed IAT\textsuperscript{C103S} is located on the peroxisomal matrix (Fig. 2A), as occurs with the wild-type protein (Fig. 2B). No significant labeling was obtained with the control npe10-AB-C strain (Fig. 2C) since it lacks the penDE gene and therefore, IAT is absent. These results indicate that the proIAT form (unprocessed) is able to be transported into peroxisomes, and therefore, self-processing may occur optimally under the internal peroxisomal ambient conditions.

3.3. Effect of the unprocessed IAT\textsuperscript{C103S} on penicillin biosynthesis

\textit{Penicillium chrysogenum} proIAT is very efficiently self-processed and rapidly dissociated into the 29-kDa and 11-kDa subunits. Therefore, it is very difficult to test whether the unprocessed pre-protein is able to exert some in vivo regulatory effect in this fungus. To assess this, we transformed the Wis54-1255 strain with plasmid pPenIAT\textsuperscript{C103S}, which expresses the mutant penDE\textsuperscript{C103S} gene. This gene gives rise to the unprocessed IAT\textsuperscript{C103S} protein, which is co-localized with the wild-type IAT and lacks acyltransferase activity as seen above. Positive transformants containing both the wild-type (1274-bp genomic DNA) and the mutant penDE\textsuperscript{C103S} (1071-bp intronless cDNA) genes were identified by PCR (data not shown). Integration of the Pgdh-penDE\textsuperscript{C103S}-Tyc1 cassette into the Wis54-1255 strain was confirmed by Southern blotting (Fig. 3A) in six randomly selected transformants using a fragment of the penDE gene as probe. All transformants showed the band with the internal wild-type penDE gene (8000 bp) plus a 2120-bp band, which corresponds to the whole Pgdh-penDE\textsuperscript{C103S}-Tyc1 cassette. Additional bands, which are a result of the integration of fragments of this cassette, are visible in transformants T7 and T20. Densitometric analysis of the Southern blotting revealed that a different number of copies of the whole cassette had been integrated in the genome of these transformants; 1–2 copies in transformants T4, T7, T21 and T29, and 3–4 copies in transformants T16 and T20. Expression of the penDE\textsuperscript{C103S} gene was tested by RT-PCR (Fig. 3B). In order to discriminate between the endogenous wild-type penDE transcripts and the exogenous penDE\textsuperscript{C103S} mRNAs generated from the Pgdh-penDE\textsuperscript{C103S}-Tyc1 cassette, retrotranscription was performed using a reverse primer specific to the 5’ region of the Tyc1 terminator. An oligonucleotide including the start codon of the penDE gene was used as forward primer. All transformants were able to express the penDE\textsuperscript{C103S} gene, and were named \textit{P. chrysogenum} Wis54-DE\textsuperscript{C103S}. Transformants containing more copies of the Pgdh-penDE\textsuperscript{C103S}-Tyc1 cassette (T16 and T20), gave rise to higher steady-state mRNA levels along the culture, especially at 48 and 72 h. The synthesis of both the wild-type IAT and the unprocessable IAT\textsuperscript{C103S} variant was confirmed by Western blotting using protein extracts taken from 24-h cultures of the different transformants (Fig. 3C). Benzylpenicillin specific production was analysed at different time points in the Wis54-1255 strain and transformants of the Wis54-DE\textsuperscript{C103S} strain. As shown in Fig. 3D, the Wis54-DE\textsuperscript{C103S} transformants produced lower amounts of benzylpenicillin than the Wis54-1255 strain. The decrease in the β-lactam production was especially remarkable in transformants T16 and T20. This difference in penicillin production among the transformants is likely dependent on the integration site of plasmid pPenIAT\textsuperscript{C103S} in the \textit{P. chrysogenum} genome that may facilitate its expression and may also be due to differences in the penDE\textsuperscript{C103S} gene copy number. In transformants T16 and T20, there are more copies of the penDE\textsuperscript{C103S} gene (Fig. 3A) and levels of the mutant penDE transcript were highest (Fig. 3B), which indicates that the biosynthesis of the antibiotic is affected by the presence of the IAT\textsuperscript{C103S} protein.

3.4. The β subunit formation pattern is affected by the IAT\textsuperscript{C103S} in \textit{P. chrysogenum}

In order to find out the mechanism by which the unprocessed IAT\textsuperscript{C103S} is affecting the biosynthesis of benzylpenicillin, the β sub-unit formation pattern of wild-type IAT was studied in presence (transformants T16 and T20 of the Wis54-DE\textsuperscript{C103S} strain) and absence (Wis54-1255 strain) of the mutant IAT\textsuperscript{C103S}. Thus, protein extracts taken from cultures of these strains, were analysed by
Western blotting. The same amount of protein was loaded in each lane. In the parental Wis54-1255 strain, the β subunit (evidencing rapid proIAT self-processing) is more abundant at 36–48 h, decreasing thereafter (Fig. 4). However, when the unprocessed IATC103S was present, the pattern of β subunit formation was altered in transformants T16 and T20 (Fig. 4). In these transformants, the 40-kDa protein band is present throughout the culture time. However, the β subunit, originated after processing of the wild-type IAT, is only detected during the initial 24–48 h of culture. Since the antibodies cannot distinguish between the IATC103S and the wild-type IAT (when it is in the unprocessed state as proIAT), the 40-kDa band may contain a mixture of both proteins. These results suggest that the unprocessed IATC103S exerts a regulatory role on wild-type IAT processing, probably affecting the self-processing. Therefore, transformants T16 and T20, which synthesize the IATC103S during the culture time, have a deficient self-processing of the wild-type IAT, giving rise to lower amounts of the β subunit and to a low benzylpenicillin production.
3.5. The unprocessed IAT\textsuperscript{C103S} affects the self-processing of the wild-type IAT in the heterologous host E. coli

In light of the results obtained in P. chrysogenum, the effect of the proIAT\textsuperscript{C103S} on the processing of the wild-type IAT was studied in E. coli. Therefore, plasmid p\textsuperscript{β−DE}\textsuperscript{C103S}, which is able to co-express both the penDE\textsuperscript{C103S} and the penDE gene, was constructed as indicated in Table 1. Co-expression was performed at 26 °C for 2 h and the formation of the β (29 kDa) subunit was used as the criterion of processing. SDS–PAGE analysis (Fig. 5A) showed the presence of the three bands, which correspond to the unprocessed mutant IAT\textsuperscript{C103S} (40-kDa) and to the β and α subunits (29 kDa and 11 kDa, respectively), both generated after self-processing of the wild-type IAT. This result indicates that simultaneous co-expression of both genes in E. coli is a suitable system to study the regulation of self-processing of IAT. To test whether the IAT\textsuperscript{C103S} exerts a partial effect on IAT maturation (i.e. generation of slow-processing molecules), IAT self-processing was assessed in absence or presence of the IAT\textsuperscript{C103S} as indicated in Section 2. Western blot analyses (Fig. 5B) showed that the wild-type preprotein is processed into the β and α subunits. This processing was practically complete after 5 h at 26 °C (only traces of the 40 kDa protein remain at this time). However, when the penDE\textsuperscript{C103S} gene was co-expressed with the wild-type penDE gene, the 40-kDa band (corresponding to the mutant protein and the wild-type IAT that remained unprocessed) was present throughout the experiment. Only after 5 h at 26 °C, the 29-kDa band (corresponding to the β subunit formed by processing of wild-type IAT) was visible. Therefore, the unprocessed protein interferes with the in vivo processing of the recombinant IAT in the E. coli heterologous expression system. These results indicate that IAT is post-translationally regulated by its preprotein, the excess of which is able to delay the self-processing generating slow-processing molecules.

3.6. The IAT\textsuperscript{C103S} does not inhibit the in vitro acyltransferase activity of previously processed IAT

In order to assess whether the lower benzylpenicillin production of the strain which co-expresses both the penDE\textsuperscript{C103S} and the penDE genes (P. chrysogenum Ws54-DE\textsuperscript{C103S}) is a consequence of an inhibition of the acyltransferase activity exerted by the unprocessed IAT\textsuperscript{C103S}, in vitro experiments were performed. penDE and penDE\textsuperscript{C103S} genes were overexpressed in separate E. coli cultures at 26 °C for 3 h. Soluble fractions containing the active recombinant α−β heterodimer were mixed with soluble fractions containing the unprocessed IAT\textsuperscript{C103S}. After a pre-incubation period of 1 h or 5 h at 26 °C (to allow protein–protein interaction), 6-APA and phenylacetyl-CoA were added to the reaction (see Section 2). The in vitro acyltransferase activity (benzylpenicillin forming) of the previously processed IAT was not affected by the presence of the unprocessed IAT\textsuperscript{C103S} (Fig. 6). Therefore, the in vivo decrease in the benzylpenicillin biosynthesis observed in the Ws54-DE\textsuperscript{C103S} strain is unlikely due to an inhibitory effect exerted by the IAT\textsuperscript{C103S} on the acyltransferase activity of the previously processed enzyme (see Section 4).

4. Discussion

Proteolytic processing of many proteins (proenzymes) is a general mechanism of activation of enzymes that are synthesized as inactive proenzymes. The IAT, encoded by the penDE gene, is self-processed forming an α−β heterodimer (Barredo et al., 1989; Montenegro et al., 1990; Veenstra et al., 1989; Tobin et al., 1990, 1993). When the self-processing ability of the IATs of P. chrysogenum and A. nidulans were compared to each other we observed that, whereas the P. chrysogenum enzyme is processed very efficiently, the A. nidulans proIAT was slowly processed. Although the processing site Gly102/Cys103 was identical in IATs of both fungi, the structure of the enzyme showed differences in about 25% of the amino acids, a fact that may explain the slow self-processing of the A. nidulans IAT. It seems that P. chrysogenum has evolved a mechanism which allows this fungus to perform a very efficient self-processing of the IAT avoiding the accumulation of proIAT, whereas this regulatory mechanism still persists in A. nidulans that accumulates a significant amount of unprocessed IAT (Fernández et al., 2003). The immunolocalization of the proIAT\textsuperscript{C103S} inside peroxisomes clearly indicates that the proIAT is targeted to those organelles as reported for the mature enzyme (Müller et al., 1992). In addition, the proIAT may be transported into the peroxisomal matrix before processing. The high concentration of enzyme in these organelles...
and the adequate ambient pH value are likely to be essential for efficient self-processing.

In this work we observed that \textit{P. chrysogenum} transformants of the npe10-\textit{AB-C-DEC103S} strain (which contains the first two genes of the penicillin pathway and the mutant \textit{penDEC103S} gene encoding the unprocessable \textit{IATC103S}) lack acyltransferase activity. Therefore, they are unable to synthesize benzylpenicillin despite the proper peroxisomal location of the variant protein; i.e. the processing of \textit{proIAT} in vivo is absolutely required for functionality of this enzyme.

The \textit{IAT} appears to be a NTN (N-terminal nucleophile) enzyme (Brannigan et al., 1995) where the Cys103 is the N-terminal residue acting as nucleophile. This amino acid is essential for processing and therefore, we used the \textit{IATC103S} protein variant to study the

**Fig. 5.** Effect of the \textit{IATC103S} on the processing of \textit{IAT} in \textit{E. coli}. (A) Coomassie blue stained SDS–PAGE of samples obtained from IPTG-induced (2 h at 26 °C) cells transformed with plasmid pPBC\(_{\alpha\beta}\) (which expresses the \textit{penDE} gene) or with plasmid p\(_{\alpha\beta}\)-\textit{DEC103S} (expressing both the \textit{penDEC103S} and the \textit{penDE} genes). Uninduced cells (lane -I) were used as control. Molecular weight markers (lane M) are shown on the left. (B) Western blotting (using anti-IAT antibodies) showing the processing of the recombinant \textit{IAT} at 26 °C into the \(\beta\) subunit, either in absence (upper panel), or presence (lower panel) of the unprocessed \textit{IATC103S} form. Induction of protein synthesis was achieved at 37 °C for 45 min with IPTG. Then, cells were washed to remove the IPTG and incubated at 26 °C to allow self-processing. Samples were taken after 30, 60, 120 and 300 min, diluted and analysed by Western blotting. Samples from uninduced cells are indicated as (-I). Molecular weight markers (lane M) are shown on the left in kDa.
role of this protein (unprocessed heterodimer) in the processing activity of the wild-type IAT. We have observed that transformants of the Wis54-DECrownS strain, which express simultaneously the penDE and the penDECrownS genes, show a decrease in penicillin production with respect to the parental Wis54-1255 strain (Fig. 3). This result supports the hypothesis that the presence of the 40-kDa proIAT affects the synthesis of benzylpenicillin. We have observed that the biosynthesis of IATCrownS in the Wis54-DECrownS strain (transformants T16 and T20) leads to a reduction of the β-subunit formed by self-cleavage of normal IAT, which is only detected in the early hours of culture (Fig. 4). In vitro experiments using previously processed (mature) IAT (Fig. 6) proved that the acyltransferase activity of mature IAT was not affected by the presence of the preprotein. This indicates that the decrease in benzylpenicillin biosynthesis observed in transformants T16 and T20 of the Wis54-DECrownS strain, was not simply due to an inhibitory effect of the proIATCrownS on the activity of the mature α-β heterodimer. One explanation of these results is that the unprocessed 40-kDa preprotein, when accumulated, exerts a regulatory role affecting the processing of the endogenous IAT, as reported for other NTN enzymes (Seemüller et al., 1996; Böck et al., 1983; Choi et al., 1992), thus decreasing the amount of the active form. The effect of the unprocessed proIATCrownS on the wild-type IAT processing was confirmed in E. coli, where synthesis of the variant IATCrownS was easily induced (Fig. 5). The results in E. coli indicated that the presence of the unprocessable protein does not completely prevent the self-processing of the wild-type IAT, but caused a delay in this process generating slow-processing molecules. In the P. chrysogenum strain, which co-express both the penDE and the penDECrownS gene, the IATCrownS gene acts to delay the processing of the wild-type IAT, thus interfering with the generation of the α-β heterodimer. Our results suggest that the IAT forms homo-oligomers and that processing occurs in the context of those oligomers.

A similar regulatory phenomenon by the unprocessed precursor protein was observed in the 20S proteasome, another NTN enzyme (Seemüller et al., 1996). Our results support the hypothesis that processing of these enzymes may be intermolecular, i.e., one IAT molecule serving as substrate of another IAT molecule. An excess of unprocessed IATCrownS could block the processing sites of the active form. A similar mechanism has been reported for the penicillin acylase from E. coli (Böck et al., 1983; Choi et al., 1992), a NTN enzyme that catalyzes hydrolysis of the amide bond of benzylpenicillin releasing 6-APA and phenylacetic acid (Valle et al., 1991; McVey et al., 2001; Kasche et al., 1987, 1999). The E. coli penicillin acylase is synthesized as an inactive precursor and cleaved initially between amino acids 263–264 releasing the β subunit (residues 264–820). The N-terminal fragment is subsequently processed into a linker region (residues 209–263) and the small N-terminal A subunit. The protein is inactive until the first cleavage, which exposes the Ser264 nucleophile (Kasche et al., 1999).

In summary, taking into account the results reported in this article, it is concluded that the P. chrysogenum IAT is very rapidly and efficiently self-processed into α and β subunits. Therefore, it escapes inhibition of self-processing since the preprotein is never accumulated, unlike what occurs in A. nidulans. Since the preproteins variant is well transported into microbodies, the lack of transport can not be the reason for the slow processing of the A. nidulans IAT.

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