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ACV Synthetase: Expression of Amino Acid Activating Domains of the *Penicillium chrysogenum* Enzyme in *Aspergillus nidulans*

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Fragments of ACV synthetase of *Penicillium chrysogenum* carrying partial activities of amino acid activation were expressed under the *alcA* promoter in an *acvA*-deletion mutant of *Aspergillus nidulans*. The 210 kDa domain A-β-galactosidase fusion protein was partially cleaved to fragments of 200 and 97 kDa. The domain A fragment and the 312 kDa domain BC construct were identified by peptide specific antibodies and shown to catalyze α-aminoadipate-, cysteine-, and valine-dependent ATP/[^{32}P]PPi exchange activity. Substrate specificities were investigated using amino acid analogues. Unexpectedly neither α-aminoadipate nor valine activation was exclusive, implying possible misactivations and proof reading functions. Both fragments were only expressed in limited amounts and found to be unstable.

Peptide antibiotics formed non-ribosomally are assembled by multifunctional modular enzymes termed peptide synthetases [1,2]. Modules of high similarity were first identified by Smith et al. [3] after the cloning and sequencing of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) synthetase. Three highly conserved regions identified in the ACV synthetase (ACVS) have been termed domains A, B and C. It has been assumed that the domains will sequentially activate the amino acids: α-aminoadipate, cysteine and valine, based on the thiotemplate mechanism [2,4]. In the first catalytic steps ACVS activates the amino acids as acyladenylates, which are subsequently thioesterified to the enzyme, followed by formation of peptide bond and epimerization of the tripeptide [5], which is released by an internal thioesterase. ACV is a key intermediate in the biosynthesis of penicillins and cephalosporins [6].

ACVS has been partially characterized [5,7-11], but neither the sequence of reactions [12] nor the functional specificities of the activating domains have been established. To assign these functions the dissection both by limited proteolysis [13,14] and fragment expression have been applied [15,16]. However, expression of domains of eukaryotic peptide synthetases in bacterial systems has generally led to inclusion bodies requiring solubilization [15] and lack of post-translational modifications essential for catalysis, particularly the loading of apo-enzymes with the cofactor 4′-phosphopantetheine [17]. Homologous expression of fragments in *Aspergillus nidulans* should overcome these problems.

MATERIALS AND METHODS

Materials. The peptides ^{458}DSSKFPAHNLDD^{469} and ^{3766}CATIKEHLARY^{3776}, segments of ACVS from *Penicillium chrysogenum* were produced at SmithKline Beecham (Brockham Park, UK), and a C-terminal cysteine was added to the first to facilitate the attachment to BSA. Genetic constructs containing partial *acvA* genes were expressed in a deletion strain of *A.nidulans* (ΔACVS). The *alcA* promoter of *A.nidulans* [18] was used for the expression of all the gene fragments. for the construction of the domain A expression strain. The *acvA* gene was contained in pPEN510 (SmithKline Beecham), the *alcA*-promoter region from pTAwtSn (Allelix), and the *lacZ/argB* frames from pAN923-42B [19]. For construction of the domain BC-expression strain, pAL4 containing the *alcA* promoter and *pyr4* was used. The strains used in this work were JKAβ2 and JKBC12 [18]. Tetrasodium [^{32}P]PPi (16.06 Ci/mmol) was purchased from Du Pont-New England Nuclear (Bad Homburg, Germany). All amino acids used in the assays were in the L-form, ATP and dithioerythritol (DTE) were purchased from Sigma.

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Enzyme linked immunosorbent assay (ELISA). The peptides were attached to BSA using the reagent sulfosuccinidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate [SMCC] as a crosslinker [20]. Rabbit antibodies were produced by hyperimmunization of a New Zealand white rabbit. Test blood was collected before the first injection and 7 days after each inoculation. A three week interval after each inoculation was observed. After five injections and confirmation of an immunization plateau, blood (20-30 ml) was collected. ELISA was carried out in triplicates on microtiter plates. Peptide-BSA (2-5 (g/ml) and BSA (control) were adsorbed at 4 °C, overnight, following standard ELISA procedures [20]. Uncoated sites in the plate were blocked with 3% BSA in 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.05% tween 80 [TST] buffer. Primary and secondary antibodies diluted in TST with 1% added BSA were sequentially incubated on each plate well for 60 min, at 37 °C. The secondary antibody, goat anti-rabbit IgG labeled with horseradish peroxidase was used. Plates were developed spectrophotometrically at 490 nm using o-phenylenediamine (OPD) in a microtiter plate reader from Milenia Kinetic Analyzer, Diagnostic Products Corporation (DPC).

Protein preparations and analysis. A.nidulans was grown from seed cultures for 43 hours at 27 °C and 250 rpm [21]. The mycelia were harvested by filtration, washed with 0.5 M NaCl, squeezed to remove excess water and freeze dried. An ammonium sulfate saturation of 50% was used to precipitate the ACVS constructs [7]. All operations were performed at 4 °C. The buffers employed were, buffer A [100 mM Tris-HCl, pH 7.5, 50% glycerol (w/v), 1 mM EDTA, 10 mM DTE] and buffer B [50 mM Tris-HCl, pH 7.5, 10% glycerol, 0.1 mM EDTA, 1mM DTE]. Protein concentration was measured by the method of Bradford [22], using bovine serum albumin as the standard. Electrophoresis and Western blots were performed according to Laemmli [23] and Towbin et al. [24] with protein samples prepared from trichloro acetic acid (TCA) precipitates. Membranes were sequentially stained with Ponceau red for visualization of proteins, blocked with 5% BSA, and incubated with antibodies at 1:1,000 dilution. The secondary antibody (Goat anti-rabbit IgG/alkaline phosphatase conjugate from Promega Biotec, Madison, WI) was used and the membranes were developed with nitro blue tetrazolium-5-bromo-4-chloro-3-indolyl-phosphate (Sigma) [20].

The domain A fragment was purified starting with 20 g of freeze dried *A.nidulans* cells; the resulting ammonium sulfate pellet was resuspended in 1.5 ml of buffer B [7], dialysed against buffer B, and centrifuged for 15 minutes at 16,000 rpm (JA20 rotor, Beckmann RCL-5). 3 ml of the final 3.75 ml were loaded onto a 250 ml Ultrogel ACA-34 column equilibrated with buffer B, and eluted at a flow rate of 20 ml per hour. A selected fraction (3.9 ml) was treated with ammonium sulfate (powder) up to 1.2 M and loaded onto a 1ml Butyl-Sepharose column, previously equilibrated with 1.2 M ammonium sulphate in Buffer B. The column was eluted in steps with decreasing concentrations of ammonium sulphate, the most active fraction eluting with 0.5M ammonium sulphate in buffer B. Column fractions were assayed for ATP/[^{32}P]PPi exchange after dialysis against 50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA and 1mM DTE, and concentrated on Microcon concentrators from Amicon, with a membrane cut off of 10 kDa. An identical procedure was employed to obtain the partially purified construct of domain BC.

ATP/[^{32}P]PPi exchange. Amino acid dependent ATP/PPi exchange was measured essentially as described [7]. Each assay mix contained 20μg of protein in a final volume of 200ml for column fractions or 100ml for the final enzyme preparation. Reactions were performed at 28 °C for 40 minutes in a mixture containing, 1mM ATP, 5mM MgCl2, 1mM DTE, 0.1mM EDTA, 50mM Tris-HCl, pH 8.0, 2.5mM amino acid, 0.1mM Na_4PPi and [^{32}P]PPi (1.5×105 cpm).

RESULTS AND DISCUSSION

The plasmid pJK1 was constructed by cloning the 3.2 kb *Nco* I fragment containing domain A into pTAwtSn to produce an in frame fusion with the *alcA* promoter. A 3.2kb *Bgl* II fragment containing the *alcA* promoter-domain A construct was then cloned into pAN923-42B digested with *Bam* HI to produce pJK1. This plasmid contains a fusion gene consisting of a domain A fragment of *acvs* encoding amino acids 48 to 988 fused to an Escherichia coli β-galactosidase gene all under the control of the *alcA* promoter. The plasmid for the expression of the domains B and C was constructed by cloning the 9kb *Bgl* II-*Spe* I fragment from pPEN510 into pAL4 to create pJKBC7. Sequencing revealed that this plasmid did not contain an in frame fusion between the alcA promoter and the *acvA* gene fragment due to the presence of an extra base in the sequence of pAL4. The frameshift was corrected by digestion with *Psp* AI, digestion of the 4 base overhangs and religation to produce pJKBC7.7. This plasmid contains the 3′ end of the *acvA* gene encoding the C-terminal part of ACVS beginning at amino acid 989 (domains BC) under the control of the *alcA* promoter.

The *A. nidulans* arginine autotroph and *acvA* deletion strain JK6 was transformed with plasmid pJK1 to arginine prototrophy. Integration of single copies of pJK1 at the *argB* locus were confirmed by Southern blot and hybridisation (details will be published elsewhere, see [18]). The strain obtained (JKAβ2) was grown in 50 mM glucose, then transferred into carbon free medium and expression induced with 10 mM cyclopentanone. Expression can be monitored by β-galactosidase activity (results not shown). The *A. nidulans* uridine auxotroph and *acvA* deletion strain JK2 was transformed with pJKBC7.7 to uridine prototrophy. The presence of an undisrupted copy of the domain BC region was confirmed by Southern blotting and hybridisation. The selected strain JKBC12 was grown in fermentation medium with the addition of 10 mM cyclopentanone.

In order to confirm the expression of fragments peptide-specific rabbit polyclonal antibodies were employed. For domain A the peptide ^{458}DSSKFPAHNLDD^{469} was selected by a search program for antigenic peptides [25]. Three major bands immunoreactive to anti-domain A antibodies were detected in the JKAβ2 strain, of approximately 210 kDa, 200 kDa and 97 kDa (Figure 1A). The 210 kDa protein corresponds to the expected size of the fusion protein, while the others should originate from endogenous proteolysis. A fragment of identical size to the 97 kDa protein, also immunoreactive to anti-domain A antibodies was detected in crude preparations of ACV synthetase of *P.chrysogenum* [26]. The respective cleavage site can be positioned between the first carrier domain and the first elongation domain. This fragment was further purified by ammonium sulfate precipitation, Ultrogel AcA-34 gel-filtration, and a butyl-Sepharose column. Adenylate formation was then assayed employing the amino acid dependent ATP-PPi exchange reaction (Table 1).

Not only L-α-aminoadipic acid was found to be substrate but also, among others isoleucine and valine.

To detect the domain BC construct, antibodies directed against the C-terminal peptide ^{3766}CATIKEHLARY^{3776} were used [27]. An immunoreactive protein of over 300 kDa, not present in the ΔACVS strain, confirmed the expression of domain BC (Fig. 1B). For this BC construct no activation of α-aminoadipate was observed. A high activation for α-amino butyric acid relative to the values obtained for the substrates valine and cysteine was observed (Table 1), and also activation of iso-leucine and leucine was detected.

When the partial enzymatic activity of the *P.chrysogenum* ACVS constructs was compared to the values obtained with the integral ACVS purified from *Acremonium chrysogenum* [26] (Table 1), surprisingly, the activity of the constructs was higher than the enzyme itself for activation of α-aminoadipate and valine, respectively. For both constructs cysteine activation was very low when compared to the integral ACVS. The results obtained support the hypothesis that α-amino adipic acid is activated at domain A. In addition, according to our data, both cysteine and valine seem to be activated preferentially by the C-terminal region of ACVS (domain BC construct). The instability and low yields of of the expressed fragments has restrained further enzymatic characterization, such as a more extended analysis of the ATP/PPi exchange reactions, and aminoacylation studies. In a separate work, the authors were able to identify the site of acylation for cysteine in ACV synthetase of *Cephalosporium acremonium* employing a quench-flow approach following to limited proteolysis and N-terminal sequence analysis [28]. The results indicated that cysteine is covalently bound to domain B of ACVS after short time incubations in the presence of the other amino acids.

TABLE 1

Formation of [^{32}P]ATP Catalysed by ACVS-Fragments

Amino acid	A-domain % activation relative to Aad [ACVS]	BC-domain % activation relative to Cys [ACVS]
Aminoadipic acid (Aad)	100 [142]	—
allo-Isoleucine	40	nd
Isoleucine	—	27
Valine	36 [43]	94
a-Aminobutyric acid	28	113
Cysteine	25 [1]	100 [5]
Carboxymethyl-cysteine	21	nd
Glutamic acid	19	nd
Leucine	nd	15

nd, not determined; [ACVS] activity was calculated relative to the values obtained with ACV synthetase from *Cephalosporium acremonium*.

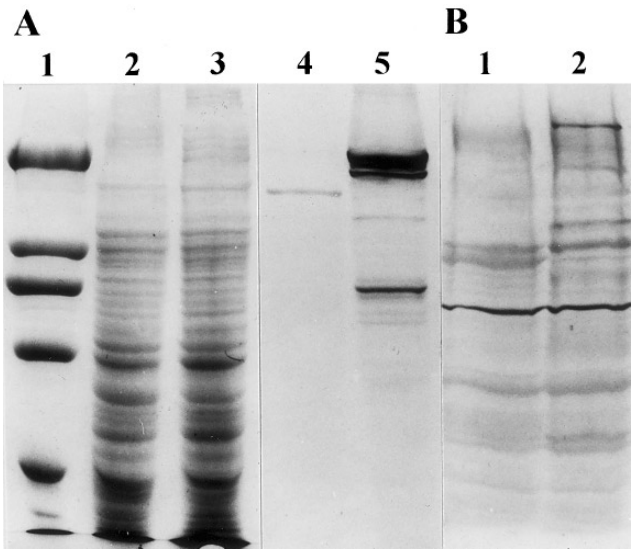

FIG. 1. Expression of the domain A-β-galactosidase fusion protein and the domain BC construct. (A) SDS-PAGE: Lane (1), molecular markers 205, 116, 97, 66, and 45 kDa; lanes (2) and (3), 50% ammonium sulfate pellet of the strains ΔACVS and JKAβ2, respectively. Lanes (4) and (5), western blot of the corresponding gel probed with anti-domain A-peptide specific antibodies. (B) Western blot of the domain BC construct. Membrane probed with anti-C-terminal antibodies. Lane (1), 50% ammonium sulfate pellet of the deletion strain; lane (2), 50% ammonium sulfate pellet of the strain carrying the domain BC construct.

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