Domain Structure Characterization of the Multifunctional α-Aminoadipate Reductase from *Penicillium chrysogenum* by Limited Proteolysis

ACTIVATION OF α-AMINOADIPATE DOES NOT REQUIRE THE PEPTIDYL CARRIER PROTEIN BOX OR THE REDUCTION DOMAIN*

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Maria José Hijarrubia‡, Jesús F. Aparicio‡, and Juan F. Martín‡

From the 1Instituto de Biotecnología, Parque Científico de León, Avenida del Real no. 1, León 24006 and the 2Universidad de León, Facultad de Ciencias Biotológicas y Ambientales, Área de Microbiología, León 24071, Spain

The α-aminoadipate reductase (α-AAR) of *Penicillium chrysogenum*, an enzyme that activates the α-aminoadipic acid by forming an α-aminoadipyl adenylate and reduces the activated intermediate to α-aminoadipyl semialdehyde, was purified to homogeneity by immuno-affinity techniques, and the kinetics for α-aminoadipic acid, ATP, and NADPH were determined. Sequencing of the N-terminal end confirmed the 10 first amino acids deduced from the nucleotide sequence. Its domain structure has been investigated using limited proteolysis and active site labeling. Trypsin and elastase were used to cleave the multi-enzyme, and the location of fragments within the primary structure was established by N-terminal sequence analysis. Initial proteolysis generated two fragments: an N-terminal fragment housing the adenylation and the peptidyl carrier protein (PCP) domains (116 kDa) and a second fragment containing most of the reductive domain (28 kDa). Under harsher conditions the adenylation domain (about 64 kDa) and the PCP domain (30 kDa) become separated. Time-dependent acylation of α-AAR and of fragments containing the adenylation domain with tritiated NADPH to the labeled α-AAR released most of the radioactive substrate. A fragment containing the adenylation domain was labeled even in absence of the PCP box. The labeling of this fragment (lacking PCP) was always weaker than that observed in the di-domain (adenylation and PCP) fragment suggesting that the PCP domain plays a role in the stability of the acyl intermediate. Low intensity direct acylation of the PCP box has also been observed. A domain structure of this multi-enzyme is proposed.

In fungi, lysine derives from α-ketoglutarate via the so-called α-aminoadipate (α-AA)1 pathway, a biosynthetic route that starts with the condensation of acetyl-CoA and α-keto-glutarate to form homocitrate (3), which is later subjected to isomerization (4),2 oxidative decarboxylation and amination to yield α-AA. This intermediate is then converted into α-AA-δ-semialdehyde by the action of the α-aminoadipate reductase (α-AAR, EC 1.2.1.31) encoded by the *lys2* and *lys5* genes. The product of the *lys2* gene constitutes the apoenzyme, whereas the *lys5* product appears to be a specific phosphopantetheinyl transferase for post-translational modification of Lys2 (5, 6).

The α-AAR, also called α-aminoadipate semialdehyde dehydrogenase, first activates the α-AA δ-carboxyl group by an ATP-dependent process through the formation of an α-AA-adenylate, a unique step among amino acid biosynthetic pathways, that is then reduced by the reduction domain using NADPH to yield α-AA-δ-semialdehyde and AMP.

In filamentous fungi, α-AA is not only an essential intermediate in lysine biosynthesis but also a well-known precursor in the biosynthesis of β-lactam antibiotics (7–9). It constitutes the branching point for lysine and penicillin or cephalosporin biosynthesis, where it is condensed with L-valine and L-cysteine to form the tripeptide L-(α-aminoadipyl)-L-cysteinyl-D-valine (ACV) by the ACV synthetase (10–12) a member of the large family of non-ribosomal peptide synthetases (13, 14).

The domain structure of *Penicillium chrysogenum* α-AAR has been predicted (15) on the basis of sequence comparison analyses with other α-AARs (16–19). The α-AAR also shows a striking similarity with non-ribosomal peptide synthetases in the N-terminal two-thirds of the protein and NAD-dependent dehydrogenases in the last third of the enzyme. However, direct evidence for the topology and domain structure of native α-AAR enzymes has never been achieved, in part due to the lack of efficient purification protocols. Some purification attempts have been described for *Saccharomyces cerevisiae* (20) and *P. chrysogenum* (21) native enzymes; however, purification to homogeneity has never been previously attained. Limited proteolysis (22, 23) has been used in this report, in combination with specific active site radiolabeling, to study the native α-AAR of *P. chrysogenum* and to provide the first structural information on any fungal α-AAR. This work provides structural and functional evidence of the domains of the α-AAR in comparison with the domains occurring in the α-AA activating domains of the five known ACV synthetases.

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1 The abbreviations used are: α-AA, α-aminoadipate; α-AAR, α-aminoadipate reductase; ACV, δ-L-(α-aminoadipyl)-L-cysteinyl-D-valine; PCP, peptidyl carrier protein; PBS, phosphate-buffered saline; MES, 4-morpholineethanesulfonic acid.

2 P. Teves, J. Casqueiro, and J. F. Martín, unpublished results.
**EXPERIMENTAL PROCEDURES**

**Materials**

1-t-1-Tosylamido-2-phenylethyl chloromethyl ketone-treated bovine trypsin, elastase, thryoglobulinic acid (mercaptoacetic acid), and 3-cyclohexylaminol-1-propanesulfonic acid buffer were purchased from Sigma; dl-o-[3H]aminoacidic acid (38 Ci mmol-1) was synthesized by Amersham Biosciences. The expression vector pGEX-2T and Sephadex G-25, protein A-Sepharose, phenyl-Sepharose, Sephacryl S-300, DEAE-Sepharose, Superose 6, and Superose 12 columns and high range molecular weight standards for SDS-PAGE were from Amersham Biosciences; the immunoaffinity column was prepared using the Affi-Gel Hz system (Bio-Rad Laboratories); ProBlott polyvinylidine difluoride membranes and sequencing reagents were from Applied Biosystems Inc. All other reagents were from commercial sources and of analytical grade.

**Organisms and Culture Conditions**

*P. chrysogenum* AMA, a strain derived from *P. chrysogenum* Wis 54-1255 pyrG, which overexpresses lys2 in an autonomous replicating plasmid was used as α-AAR source to purify this enzyme. Sporulation in PW medium and growth in DPM medium was performed as described elsewhere (24).

**Recombinant DNA Methods**

Standard genetic techniques with Escherichia coli and in vitro DNA manipulations were as described by Sambrook and Russell (25). Polymerase chain reaction were performed using Pfu DNA polymerase as described by the enzyme supplier (Stratagene). DNA sequencing was accomplished by the dideoxynucleotide chain-termination method using the enzyme supplier (Stratagene). DNA sequencing was performed by the dideoxynucleotide chain-termination method using the Finch software. Inhibitors were added to the reaction mixtures at specified concentrations.

**Determination of α-AAR Activity**

Crude enzyme preparations were obtained by grinding the cells in liquid nitrogen and suspending them in 10 mM Tris-Cl, pH 8.0. The extract was then dialyzed (cellulose membranes, 12,000) against the same buffer at 4°C to remove possible low molecular weight enzyme inhibitors. The α-AAR activity was assayed by the procedure of Sagisaka and Shimura (26), as described by Suvarna et al. (18). The standard reaction mixture contained 12.5 mM α-AA, 15 mM ATP, 10 mM MgCl2, 1 mM reduced glutathione, 0.625 mM β-NADPH, 250 mM Tris-Cl, pH 9.0, and an appropriate amount of enzyme solution, in a total volume of 1 ml. Reaction mixtures lacking α-AA were used as controls. Reactions were incubated at 30°C for up to 1 h and terminated by the addition of 1 ml of 2% dithiothreitol. The enzyme activity was then quantified by measuring the absorbance at 460 nm. The enzyme activity was determined by quantifying the formation of α-aminoadipic acid deaminase in 2-methoxyethanol. The enzyme activity was then determined by quantifying the formation of α-aminoadipic acid deaminase as previously described (19). The α-AA-d-semialdehyde forms a yellow complex with 12-aminobenzenearsonic acid or ammonium sulfate precipitation. After complete incubation, the reaction mixture was centrifuged for 10 min at 3500 × g and the supernatant was used as the source of enzyme.

**Preparation of an Immunoaffinity Column with Anti-α-AAR Antibodies**

The Affi-Gel Hz (Bio-Rad Laboratories) system was used for the preparation of an immunoaffinity column. Binding of the α-α-AAR antibodies to the matrix was performed according to the supplier’s instructions. Purified IgG (12.6 mg) was used to react with 5 ml of the matrix yielding 46% efficiency in the binding reaction.

**Enzyme Purification**

All purification procedures were performed at 4°C. *P. chrysogenum* mycelium (40 g of wet weight) was washed and ground in liquid nitrogen. The disrupted mycelium was resuspended in buffer A (25 mM Tris-Cl, pH 8.0, 10 mM MgCl2, 1 mM 1,4-dithiothreitol) and ultracentrifuged at 20,000 × g for 1 h. The supernatant was then used as the source of enzyme.

**Step 1: Axion-exchange Chromatography** — The mycelial extract (2085 mg of protein and 5525 units of enzyme) was applied to a DEAE-Sepharose Fast Flow column (3.4 × 11 cm, 100 ml) equilibrated with buffer A (flow rate, 1.2 ml/min). The column was washed with 200 ml of this buffer before elution with a 600 ml of linear gradient of 0–0.5 M NaCl in buffer A. Fractions (6 ml) were collected, and those enriched in α-AAR were combined (60 ml) and designated as the α-α-AAR pool (98.5 mg of protein, 5418 units).

**Step 2: Sephacryl S-300 Chromatography** — The DEAE-Sepharose pool was concentrated to 4 ml in Centriprep YM-50 concentrators (Miltipore), dialyzed 20-fold in buffer A containing 75 mM NaCl, and again concentrated to 4 ml. This preparation was then applied (flow rate, 1.4 ml/min) to a Sephacryl S-300 column (2.6 × 90 cm; 478 ml) equilibrated with buffer A containing 75 mM NaCl. Fractions (7 ml) were collected and assessed for the presence of α-AAR by SDS-PAGE and immunodetection.

**Step 3: Phenyl-Sepharose Chromatography** — The Sephacryl S-300 pool (22 ml, 23 mg of protein, 1620 units) was diluted 3-fold in buffer A containing 1.5 M (NH4)2SO4 (to achieve a final concentration of 1 M (NH4)2SO4 and applied (flow rate 1.1 ml/min) to a phenyl-Sepharose 6 Fast Flow column (1.5 × 6.4 cm; 11 ml) previously equilibrated with buffer A containing 1 M (NH4)2SO4. Bound proteins were eluted with a linear gradient of 1 to 0 M (NH4)2SO4 in buffer A and subsequently the enzyme was eluted with H2O. The final volume of the pooled active fractions was 50 ml (2 mg of protein, 339 units).

**Step 4: Immunoaffinity Chromatography** — The phenyl-Sepharose pool was dissolved in buffer A (flow rate 0.3 ml/min) to an immunoaffinity column (5 ml, 5.67 mg of IgG anti-α-AAR) previously equilibrated with buffer A. The column was washed with 5 volumes of this buffer containing 0.5 M NaCl and with 5 volumes of buffer A before elution with a solution of 100 mM glycine-HCl buffer, pH 2.5. Immediately after elution, 100 µl of 1 M Tris-Cl, pH 8.0, was added to each fraction (1 ml) containing the enzyme to avoid protein denaturation. The enzyme solution was concentrated in a Centriprep YM-50 concentrator, dialyzed against buffer A containing 50% (v/v) glycerol, and preserved at –20°C.

**Limited Proteolysis of α-AAR**

Purified α-AAR was incubated with 1-t-1-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin at an enzyme/substrate ratio of 1/20 (w/w) or with elastase at an enzyme/substrate ratio of 1/10 (w/w) in distilled water. Reactions were performed at 30°C for various times and terminated by heating at 100°C for 5 min in electrophores sample buffer (unless otherwise indicated). The reaction products were separated by SDS-PAGE, and after electrophoresis, gels were stained either with Coomassie Brilliant Blue R-250 or transferred to ProBlott membranes for N-terminal sequencing.

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*F. J. Casqueiro and J. F. Martin, unpublished data.*
**Acylation of α-AAR with Labeled [d-α-[3H]]Haminoadipic Acid**

Labeling was performed at 30 °C, before or after limited proteolysis, using [d-α-[3H]]Haminoadipic acid in the absence of NADPH. Reactions (25 μl) contained 250 mM Tris-HCl, pH 9.0, 21 mM MgCl2, 32 mM ATP, 2.2 mM reduced glutathione, 11.4 μM [d-α-[3H]]HAA, and 1.6 μg α-AAR. The preparations were incubated for 10 min, terminated by rapid freezing on dry ice/acetone, and stored at −80 °C. The trichloroacetic acid precipitation assay was performed with 10% trichloroacetic acid in the presence of 200 μg of bovine serum albumin. Precipitated proteins were washed twice with 5% trichloroacetic acid and resuspended in 100 μl of 1 M Tris base, and the amount of incorporated [d-α-[3H]]HAA was quantified by liquid scintillation counting. For fluorography, gels were soaked for 30 min in 1× sodium salicylate (pH 6.0) solution and dried before exposure to Hyperfilm-MP (Amersham Biosciences); otherwise the radioimmunoassay for determination of peptide acylation involved gel slicing, protein extraction with 30% H2O2 (3 h at 60 °C), and [3H]-label quantification by liquid scintillation counting.

**Analytical Methods**

Protein concentrations were estimated using the Bradford method (28) or by measuring the absorbance of column eluates at 280 nm. SDS-PAGE was performed in 7, 10, or 12% polyacrylamide gels by standard procedures. After electrophoresis, gels were stained either with Coomassie Brilliant Blue R-250 or with silver. Alternatively gels were transferred to ProBlott polyvinylidene difluoride membranes according to Matsudaira (29) and subjected to Edman degradation on an Applied Biosystems 477A pulsed-liquid protein sequencer. The molecular mass of the enzyme was estimated to be 171 kDa, whereas analysis on Superose 12 resulted in a value of 169 ± 10 kDa. Taken together, these results suggest that the native enzyme has a monomeric nature.

**RESULTS**

**Purification of P. chrysogenum α-AAR and Determination of the N-terminal Sequence**—The enzyme was purified from mycelium grown for 24 h (early phase of growth) in DPM medium. A summary of the specific activity and recovery of the enzyme during the purification procedure is given in Table I. Each purification step was also analyzed by SDS-PAGE followed by silver staining (Fig. 1). The purified enzyme showed a homogenous protein band on SDS-PAGE. The enzyme was purified 117-fold with a yield of 5.5% from the cell extract. The purified enzyme rapidly lost activity, even in 50% (v/v) glycerol at −20 °C, probably due to denaturation and degradation of this large protein as deduced by immunoblot analysis of old preparations (not shown). The N-terminal sequence of the α-AAR protein obtained from the immunooaffinity column was Met-Ala-Val-Gly-Thr-Ala-Ser-Leu-Gln-Asp, which fully agrees with the sequence deduced from the Tyg2 gene starting at the first ATG codon of the open reading frame.

**Table I**

| Pool          | Protein | mg | a-AAR activity | Specific activity | Yield | Purification |
|---------------|---------|----|----------------|------------------|-------|-------------|
| Extract       |         | 2085| 5525           | 2.6              | 100   | 1           |
| DEAE-Sepharose|         | 96.5| 5418.5         | 56.2             | 98    | 21.2        |
| Sephacyrl S-300|        | 23 | 1619           | 70.4             | 29.3  | 27          |
| Phenyl-Sepharose|       | 2  | 339            | 169.5            | 6.1   | 65          |
| Immunoaffinity|         | 1  | 305            | 305              | 5.5   | 117         |

**Optimal Conditions for Activity**—In a standard 60-min reaction the optimal temperature was found to be in the range of 25–29 °C. Above 30 °C the enzyme activity was rapidly lost. Within this temperature range, the period of linear phase of reaction lasted for 30 min, and the enzyme remained active for at least 2 h.

The α-AAR stability was assessed by measuring activity after a previous treatment (5–60 min) at −20 °C, 4 °C, 30 °C, or 37 °C. Whereas at −20 °C or 4 °C the enzyme remained stable during the whole treatment, and at 30 °C there was a 20% loss in activity after 1 h of incubation. This activity loss was particularly evident at 37 °C where only 50% activity remained after 15 min of incubation.

The pH dependence of enzyme activity on α-AAR was measured in 100 or 250 mM MES-NaOH, pH 5.5–7.5, 100 or 250 mM Tris-HCl, pH 7.0–9.0, and 100 or 250 mM glycine-NaOH, pH 8.5–11.0, buffers. The enzyme activity was favored by using buffers with the higher ionic strength and became apparent throughout the pH range 7.5–9.5 with a peak at pH 9.0 (not shown).

The analysis of the effect of divalent cations on activity showed that Mg2+ is absolutely required for activity; the optimal concentration was 10 mM. Mn2+ could replace Mg2+ to some extent (optimal concentration 5 mM), yielding 60% of the maximum activity attained with Mg2+. The ions Ca2+, Co2+, Hg2+, and Cu2+ could not substitute for Mg2+. The addition of chelating agents such as EDTA (5 mM) to the reaction mixture produced a 75% inhibition of the activity, further supporting the requirement of Mg2+ for the reaction.

**Kinetics of α-AA Conversion to α-AA-δ-Semialdehyde**—The formation of α-AA-δ-semialdehyde by reduction of α-AA was monitored spectrophotometrically by monitoring the cyclized form of α-AA-δ-semialdehyde, δ1-piperidine carboxylate, which reacts with p-dimethylnobenzaldehyde giving a yellow complex (λmax = 460 nm). Using this assay, the K_m of P.
**Domain Structure of P. chrysogenum α-Aminoadipate Reductase**

**A**

**B**

**Fig. 2. Predicted domain organization and tryptic fragmentation pattern of the P. chrysogenum α-aminoadipate reductase.** A, conserved motifs in the α-aminoadipate reductases (numbers 1–12; see Refs. 15 and 19 for the amino acid sequences of the 12 motifs) and organization of the predicted adenylation, PCP, and reduction domains of this protein. Motif number 10 corresponds to the sequence LGGGSHS containing the serine to which the phosphopantetheine is bound (PCP domain). B, limited proteolysis fragments (T1 to T9) of the α-aminoadipate reductase obtained by increasing the duration of the tryptic treatment. The determined N-terminal amino acid sequence of each tryptic fragment is indicated on its left end. The number before the amino acid sequence indicates the position of the first amino acid of each fragment in the protein.

*P. chrysogenum* Lys2 for its substrate α-AA was determined to be 1.4 ± 0.1 mM, and the *k*ₐ was 66 ± 5 min⁻¹. In a similar way, the *k*ₐ for ATP was 1.3 ± 0.0 mM, whereas for NADPH it was 160 ± 5 μM.

**Substrate Specificity of the Enzyme—**Several analogs of the Lys2 substrate, α-AA, were assayed in the reaction mixture at the same concentration, including adipic acid, DL-diaminopimelic acid, L-glutamate, and S-carboxymethyl-L-cysteine. Only S-carboxymethyl-L-cysteine could be accepted as substrate by *P. chrysogenum* α-AAR, which yielded, however, 20-fold lower activity than with its natural substrate.

**Limited Proteolysis of α-AAR Generated Fragments Containing the Adenylation and the Different Domains—**Lys2 was digested with either trypsin or elastase at various molar ratios at 30 °C as described under “Experimental Procedures” for various lengths of time, and the resulting fragmentation pattern is shown in Fig. 2. Fragment patterns were unaltered when SDS-PAGE was performed in the presence of dithiothreitol or 2-mercaptoethanol. Limited proteolysis was also attempted using chymotrypsin, but no stable fragments were observed.

The fragmentation pattern generated by trypsin or elastase cleavage was similar. Fig. 2 shows the fragment pattern generated by trypsin. The initial cleavage introduced by trypsin resulted in the release of a 116-kDa fragment (T1) and a 28-kDa fragment (T2) as the first stable products (see Fig. 2B). The size on SDS-PAGE and the N-terminal sequence of T1 showed that it comprises the adenylation domain, the PCP, and the NADPH binding site (19). Similarly, the N-terminal sequence of T2 indicated that this fragment includes most of the reduction domain, excluding the nucleotide binding site. As the hydrolysis continued, the C-terminal piece of T1 was removed leading to the generation of a 105-kDa fragment (T3) containing the adenylation and PCP domains. Subsequently, this fragment loses its N-terminal end resulting in a 76-kDa protein fragment (T4).

As the time of incubation was increased, further fragments were generated concurrent with the disappearance of T3 fragment. Fragments T5 (30 kDa) and T6 (64 kDa) have the same N terminus as T3, and both have lost the PCP domain. Finally, other fragments generated were T7 (32 kDa), T8 (30 kDa), and T9 (29 kDa), which contain the C-terminal end of the adenylation domain (Fig. 2B). T8 also contains the whole PCP domain (see below). Under harsher conditions, the fragments become unstructured and get degraded.

As summarized in Fig. 2, the primary cleavage site is located at the N-terminal portion of the reductive domain (N-terminal end of fragment T2 (Ile¹¹¹)), dividing the molecule into two independent parts, namely the di-domain comprising the adenylation and PCP domains, including the NADPH-binding region located C-terminal of the PCP, and a fragment containing most of the reductive domain excluding the NADPH-binding site.

**Time-dependent Acylation of α-AAR Occurs in the Absence of NADPH, and the Label Is Released by Cofactor Addition—**A trichloroacetic acid-mediated protein precipitation assay was used to assess the dl-α-[³H]AA time-course acylation of *P. chrysogenum* α-AAR in the absence of NADPH (Fig. 3). Under such conditions the aminoacyl adenylate cannot be reduced and remains bound to the enzyme (5). NADPH addition after 10 min of incubation released most of the radioactivity, indicating that in the presence of NADPH the full reaction is resumed, which is consistent with the reductive cleavage of the α-AA-PCP acyl enzyme to form the α-AA-⃞-semialdehyde.

**α-AAR Fragments Containing the Adenylation Domain Were Labeled Even in the Absence of the PCP Box—**When the α-AA protein was labeled in the absence of NADPH and then subjected to SDS-PAGE and fluorography, the α-[^³H]AA radioactivity was found associated with the Lys2 polypeptide (Fig. 4B, lane 1).

To determine which domains were being acylated, various proteolytic digests of α-AAR were labeled as indicated above. Alternatively, the protein was first labeled and subsequently proteolysed. In both cases, the pattern of labeled bands was identical (Fig. 4), finding radioactivity bound to fragments T1, T3, T4, and T6 but not to other small fragments. This indicates that any fragment containing the adenylation domain (Fig. 2A) (30) can be labeled. The labeling observed in T6 is significant, because this fragment lacks the PCP domain. This labeling was always weaker than that obtained in other fragments (see below), indicating that the PCP domain might play a role in the stability of the acyl intermediate.

These results were corroborated by liquid scintillation counting of peptide-bound radioactivity in slices of the polyacrylamide gel (see “Experimental Procedures”). Again, no significant difference was found between labeling before or after proteolysis, indicating that the adenylation domain functions as an independent entity and that there is no need for collaboration with other domains to load its substrate. Interestingly,
Domain Structure of P. chrysogenum α-Aminoadipate Reductase

The relatively high $K_m$ value of the purified enzyme for α-AA might be the reflection of a metabolic bias toward penicillin biosynthesis in the strain used for enzyme purification. The strain used derives from P. chrysogenum Wis 54-1255, which shows partial lysine requirement in minimal medium and an improved penicillin production when compared with the wild type. The relatively low affinity of α-AAR toward its substrate α-AA would lead to an increase in the α-AA intracellular levels, which favor the formation of the tripeptide δ-l-(α-aminoadipyl)-l-cysteinyl-d-valine, and hence penicillin formation (21). We have previously correlated high penicillin-producing strains with increased intracellular α-AA pools (15). The low affinity of α-AAR toward α-AA may, therefore, direct the α-AA pool to ACV formation.

The purification to homogeneity of the α-AAR from P. chrysogenum has provided the first opportunity to explore the structure of this multifunctional key enzyme for lysine and β-lactam biosynthesis. In general, multifunctional proteins are composed of independently folded, compact domains connected by linker regions (33, 34), which are readily cleavable by limited treatment with proteolytic enzymes (35). From x-ray diffraction studies, it has been demonstrated (36) that segments of the polypeptide chain of high flexibility are correlated with those regions that are vulnerable to limited proteolysis. Many interdomain loops are located at the surface of the protein and therefore adopt accessible conformations that facilitate their interaction with proteases.

Proteolytic studies on α-AAR were performed using two different proteinases, chosen for their relatively broad primary specificity, so that the accessibility to potential target sites, rather than the specificity of the protease itself, is the factor likely to determine the sites of cleavage. The similar pattern of digestion obtained with both proteases reassures this hypothesis. N-terminal sequencing of the fragments generated allowed them to be placed within the context of the known primary structure of Lys2 (15). The initial cleavages observed indicated that the multifunctional protein (155 kDa) could be divided into two fragments of 116 and 28 kDa as estimated by SDS-PAGE, with a cutting site at Arg¹¹⁴⁰-Ile¹¹⁴¹. The large fragment comprises the adenylation domain, the PCP domain, and the NADPH-binding site (19), whereas the small one includes the rest of the protein. The same pattern of degradation was observed upon digestion with elastase or after long term storage of the protein. The main point of cleavage (Arg¹¹⁴⁰-Ile¹¹⁴¹) is located just at the N-terminal end of a region rich in aspartic and glutamic acids (¹¹⁴⁰-EDDDME¹¹⁵⁰), which are thought to provide flexibility together with alanine and proline (37). In the same way, the C-terminal end of fragments T₃, T₄, and T₈, and hence the boundary between the PCP and the NADPH-binding domains, is likely to lie at another putative linker region (⁹⁵⁴AANEPDDE⁹⁶¹). This result was unexpected considering the putative individual domains predicted by using sequence comparisons (15, 19), where the NADPH binding site was believed to be included within the reductive domain, and suggests that the nucleotide binding site could belong to an individual domain that under the proteolytic conditions used in this study is associated to the PCP domain. Indeed, under harsher conditions this nucleotide-binding box is never found as an independent fragment and is degraded, which suggests a less structured organization than that of other domains. This behavior is not unprecedented and has been observed with structural domains of other multidomain systems (35, 38).

The remaining fragments identified belong to different regions of the adenylation domain, including or not the PCP.

### DISCUSSION

This report represents the first purification to homogeneity of any natural α-AAR. Some previous attempts for purification of α-AAR have been described for the S. cerevisiae enzyme (20) and for the P. chrysogenum enzyme (21, 32), but in both cases the purification protocols yielded enzyme preparations only partially pure. Recently, the Lys2 enzyme has been cloned from S. cerevisiae DNA, overexpressed in E. coli, and purified (5).

The purification of P. chrysogenum α-AAR has been greatly facilitated by (i) the use of mycelium from the strain P. chrysogenum AMA, a strain that shows α-AAR yields 10-fold higher than its parental strain P. chrysogenum Wis 54-1255 and (ii) obtaining anti-Lys2 antibodies, which turned out to be highly specific for P. chrysogenum α-AAR (24), thus allowing the use of an efficient immunoaffinity column. The native enzyme has been shown to be a monomer, and its N terminus corresponds exactly to that proposed from the cloned gene (15).
in agreement with (Fig. 3) and its subsequent release upon addition of NADPH are the time-dependent accumulation of radioactivity observed to prevent reductive cleavage of a putative acyl-adenylate (5).

Selective release, of labeled -AA is bound to the enzyme through a thioester linkage, NADPH binding site; DUF, unknown function domain (see text for details).

Interestingly, fragment T5 corresponds to the N-terminal portion of the Lys2 protein (~270 residues), a region presently unassigned for function. Data base searches with this region revealed a significant degree of identity with DUF4 domains of nocardiopeptide synthetases (NosA proteins), other Lys2 proteins, peptide synthetases (e.g. P. chrysogenum ACV synthetase, Bacillus subtilis surfactin synthetase 2 and 3, and Bacillus brevis gramicidin synthetase) or CoA ligases, among others. The function of this D4F4 domain remains unknown, although its conservation in a wide range of multifunctional proteins suggests a structural role, perhaps in maintaining the integrity of the protein.

Acylation of α-AAR was performed in the absence of NADPH to prevent reductive cleavage of a putative acyl-adenylate (5). The time-dependent accumulation of radioactivity observed (Fig. 3) and its subsequent release upon addition of NADPH are in agreement with α-[3H]AA being presumably activated in the adenylation domain and then transferred to the PCP domain, where it forms a transient acyl-thioether intermediate previous to its hydrolysis by the reductase domain (Fig. 5) (5). The selective release, of labeled α-AA from the acylated α-AAR by performic acid but not by formic acid treatment, indicates that the α-AA is bound to the enzyme through a thioester linkage, supporting the involvement of the phosphopantetheine arm on the transfer of the activated substrate to the reduction domain (R in Fig. 5).

Formation of thioester intermediates has been shown also in the activation of phenylalanine in the tyrocidine synthase 1 of B. brevis (40). This enzyme catalyzes the activation, thioesterification, and epimerization of the α-phenylalanine component of tyrocidine. The same mechanism occurs therefore in α-aminoacidopropionate reductases and non-ribosomal peptide synthetases.

The finding that, after trypsin digestion, all fragments containing both the adenylation active site and the PCP domain (T1, T3, and T4) were labeled by α-[3H]AA provided evidence that both domains retain activity after proteolysis and supports the former model, where the PCP domain would covalently tether the acyl-thioether. The radioactivity observed in fragment T6 (which lacks the PCP domain) goes beyond, suggesting that the adenylation domain is catalytically competent even in the absence of its C-terminal region and supporting the assumption that such domain functions in the loading of free α-AA (5, 15, 19). Similar PCP-independent non-covalent substrate binding of an adenylation domain has been reported for the phenylalanine-activating domain of the gramicidin S synthetase, GrsA (41).

The residual labeling observed in fragment T8, which comprises the PCP domain but lacks the adenylation box, occurred regardless whether labeling was previous to the proteolysis or not, suggesting either that the PCP domain suffers direct loading of α-[3H]AA or more likely that there is a collaboration with the adenylation domain in trans. Such in trans collaboration between functional domains resembles the mode of action of multienzyme complexes and has also been described for individual domains of multifunctional enzymes (42). In summary this work has allowed us to gain insight into how this fascinating enzyme operates.
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