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Alanine racemase from *Tolypocladium inflatum*: A key PLP-dependent enzyme in cyclosporin biosynthesis and a model of catalytic promiscuity

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ABSTRACT

Cyclosporin A, a cyclic peptide produced by the fungus *Tolypocladium inflatum*, is a widely employed immunosuppressant drug. Its biosynthesis is strictly dependent on the action of the pyridoxal 5′-phosphate-dependent enzyme alanine racemase, which produces the D-alanine incorporated in the cyclic peptide. This enzyme has a different fold with respect to bacterial alanine racemases. The interest elicited by *T. inflatum* alanine racemase not only relies on its biotechnological relevance, but also on its evolutionary and structural similarity to the promiscuous enzymes serine hydroxymethyltransferase and threonine aldolase. The three enzymes represent a model of divergent evolution from an ancestral enzyme that was able to catalyse all the reactions of the modern enzymes.

A protocol to express and purify with high yield recombinant *T. inflatum* alanine racemase was developed. The catalytic properties of the enzyme were characterized. Similarly to serine hydroxymethyltransferase and threonine aldolase, *T. inflatum* alanine racemase was able to catalyse retroaldol cleavage and transamination reactions. This observation corroborates the hypothesis of the common evolutionary origin of these enzymes. A three-dimensional model of *T. inflatum* alanine racemase was constructed on the basis of threonine aldolase crystal structure. The model helped rationalise the experimental data and explain the catalytic properties of the enzymes.

Introduction

Cyclosporin A is a potent immunosuppressant drug, mainly used to prevent rejection after bone marrow and organ transplantation [1–3]. In addition, it has anti-inflammatory, antiparasitic and antifungal properties [4] and has been recommended for a number of clinical applications, such as the reversal of multidrug resistance, treatment of rheumatoid arthritis, psoriasis, atopic dermatitis, type I diabetes and also as an anti-human immunodeficiency virus 1 (HIV-1) agent [5,6]. Cyclosporin A is a cyclic undecapeptide initially isolated from the fungus *Tolypocladium inflatum*, in which it is synthesised in a non-ribosomal manner by a large multifunctional enzyme complex named cyclosporin synthetase [7–9]. Among the substrates used by this multifunctional enzyme, D-alanine is the only D-amino amino acid and is the first one to be incorporated during the synthesis of the undecapeptide [10]. In *T. inflatum*, D-alanine is synthesised exclusively for the biosynthesis of cyclosporine A from L-alanine by the action of a pyridoxal 5′-phosphate (PLP[4])-dependent enzyme, alanine racemase (*t*AlaRac). This enzyme obviously plays a very important role in cyclosporin biosynthesis, as it is demonstrated by the fact that its expression levels affect the amounts of cyclosporine produced in the fungus [11]. *t*AlaRac was the first alanine racemase to be purified from a eukaryotic organism (alanine racemases are in fact well known as a typical prokaryotic enzyme involved cell wall biosynthesis [12,13]), and only partially characterised, almost 20 years ago [11]. Ever since, in spite of its pivotal role in cyclosporin A biosynthe-

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[4] *Abbreviations used:* *t*AlaRac, *Tolypocladium inflatum* alanine racemase; BES, NN-bis(2-hydroxyethyl)-2-aminoethanesulfonate; PLP, pyridoxal 5′-phosphate; SHMT, serine hydroxymethyltransferase (**EC 2.1.2.1**); *e*SHMT, serine hydroxymethyltransferase from *Escherichia coli*; TA, threonine aldolase (**EC 4.1.2.48**); *e*TA, *Escherichia coli* threonine aldolase; *tm*TA, *Thermotoga maritima* threonine aldolase.

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sis (the nucleotide sequence of the gene encoding *T. inflatum* alanine racemase is part of a European patent EP0696323), *t*AlaRac has not been characterised further and to our knowledge its purification yield (quite poor when the enzyme is directly extracted from *T. inflatum*) has not been improved by recombinant expression techniques. The difficulty to obtain a soluble, recombinantly expressed *t*AlaRAc, which we experimented in the early stage of our work, may be one reason of this apparent lack of interest in *t*AlaRAc. We also tried to recombinantly express and purify, without any success, another fungal AlaRac, which has been identified in the pathogenic fungus *Cochliobolus carbonum* but never isolated or characterized. Also in this organism, the D-alanine produced is vital for the synthesis of a cyclic tetrapeptide, the HC-toxin, an inhibitor of histone deacetylase which is an essential virulence determinant [14].

Our main interest in fungal AlaRac resides in its structural similarity and close evolutionary relationship with serine hydroxymethyltransferase (SHMT; **EC 2.1.2.1**) and threonine aldolase (TA; **EC 4.1.2.48**), two other PLP-dependent enzymes, which are endowed with catalytic promiscuity and show overlapping catalytic properties [15–17]. Although TA and SHMT possess quite a pronounced substrate and reaction specificity, when presented with appropriate substrate analogues, both enzymes are able to catalyse the same set of "anomalous" reactions, including racemization and transamination of both alanine enantiomers. The observed similarities in the catalytic properties of the two enzymes are likely to derive from the particular catalytic apparatus of a common ancestor, from which they evolved to catalyse different but related reactions. In a recent study [18], it has been proposed that TA and SHMT represent an example of divergent evolution from an ancestral generalist enzyme (oppositely to specialist enzymes, generalist enzymes act on a variety of substrates and catalyse more than one reaction). The acquisition of more specialised reactions occurred in order to increase or regulate the metabolic flux through different enzyme-catalysed reactions. Together with SHMT and TA, fungal AlaRac represents a model to investigate how homologous enzymes evolved from a common ancestor to catalyse different, but chemically related, reactions [19].

Our attempts to express and purify *t*AlaRac and to model its three-dimensional structure were carried out mainly in view of a characterization of its catalytic properties and promiscuity features. However, the comparative study of fungal alanine racemase, threonine aldolase and serine hydroxymethyltransferase is also justified by the potential importance of these enzymes as drug targets and biotechnological tools [20].

Experimental procedures

Materials

Ingredients for bacterial growth were from Fluka. Ni-NTA Agarose for purification of 6xHis-tagged proteins was from Qiagen. Oligonucleotides and DNA sequencing were from Eurofins MWG Operon. All other chemicals were from Sigma–Aldrich and Carlo Erba. *E. coli* SHMT and L-TA were purified as previously described [15,21]. The cDNA of the gene encoding *Tolypocladium inflatum* alanine racemase (*cssB* gene; GeneBank entry #A40406) was kindly provided by Sandoz GmbH (Biochemiestrasse 10, A-6250 Kundl, Tirol, Austria).

Cloning of the gene encoding T. inflatum alanine racemase

The coding sequence of the *cssB* gene (GeneBank entry #A40406) was amplified by PCR using the following primers: upstream primer, 5′–CGGCATATGAAGATTTGCCCGATACTG–3′; downstream primer, T7 Rev: 5′–GCGGATCCCTAAAGGCGTTCTAGAC CTGC–3′. The amplified DNA segment was inserted into a pET22b vector, between *Nde*I and *BamH*I restriction sites (underlined in the above primer sequences). The nucleotide sequence of the insert was determined to confirm that no mismatching had occurred during the PCR amplification. Following the initial unsuccessful expression trials, the same insert was cloned into a pET43.1a vector, between *Sma*I (blunt end cloning) and *Bam*HI (present right after the *cssB* stop codon) restriction sites. The primers used to PCR amplify and cloning *cssB* into pET43.1a were the following: upstream primer, 5′–GGGGCAGCATGAAGATTTGCCCGATACTG–3′; downstream primer, T7 Universal Terminator Primer: 5′– GCTAGTT ATTGCTCAGCGG–3′. This new construct, named pET43-cssB, was used to transform *E. coli* HMS174(DE3) competent cells, which expressed the soluble fusion protein.

Purification of T. inflatum alanine racemase

An overnight culture (90 mL) of *E. coli* HMS174(λDE3) cells transformed with plasmid pET43-cssB was used to inoculate 4.5 L of Luria–Bertani broth containing ampicillin (100 mg L^{-1}). Bacteria were allowed to grow for approximately 3 h at 37 °C (until their OD_{600} reached to ~0.4), then the growing temperature was lowered to 25 °C and the expression of *t*AlaRac induced with 0.2 mM isopropyl thio-β-D-galactoside (IPTG). Bacteria were harvested after 18 h and suspended in 50 mL of 50 mM potassium phosphate buffer at pH 7.2 containing 150 mM NaCl, 100 μM EDTA, 2 mM 2-mercaptoethanol and SIGMAFAST™ protease inhibitor (half cocktail tablet). Cell lysis was carried out by the addition of 40 mg lysozyme (followed by incubation for 30 min at room temperature), followed by freezing, thawing and sonication on ice (2-min in short 5-s pulses with 20-s intervals). Lysate was centrifuged at 12,000 g for 30 min and the pellet was discarded. The supernatant was added to a Ni-NTA (SIGMA–Aldrich) column (2 cm ∅ × 6 cm), previously equilibrated with 50 mM potassium phosphate buffer at pH 7.5, containing 150 mM NaCl and 0.2 mM DTT. The column was washed with 300 mL of the same buffer and eluted with a linear 0 to 300 mM imidazole gradient (the mixing chamber contained 250 mL of the equilibration buffer and the reservoir 250 mL of the same buffer including 300 mM imidazole; in the buffer containing imidazole, pH was adjusted to 7.5 with HCl). Collected fractions (3.5 mL) were analysed by SDS–PAGE and those containing the fusion protein (M.W. 103 KDa; fractions 10–30) were pooled, concentrated to 10 mL volume using Amicon Ultra-15 centrifugal filters (30 kDa cut-off; Millipore) and dialysed overnight against 2 L of 20 mM potassium phosphate buffer at pH 7.2, containing 0.2 mM DTT, glycerol (5% v/v), 100 μM PLP, after the addition of 40 μg of thrombin. SDS–PAGE analysis of the dialysed sample showed that the fusion protein was entirely cut by thrombin into two proteins with molecular masses corresponding to those of NusA (66 KDa) and *t*AlaRac (41 KDa). This sample was loaded on a DEAE-Sepharose column (1.5 cm ∅ × 8 cm), which had been equilibrated with 20 mM potassium phosphate buffer at pH 7.2 containing 0.2 mM DTT and glycerol (5% v/v). The column was washed with 100 mL of the same buffer and eluted with a linear 0 to 300 mM NaCl gradient (the mixing chamber contained 100 mL of the equilibration buffer and the reservoir 100 mL of the same buffer including 300 mM NaCl). Collected fractions (5 mL) were analysed by SDS–PAGE and those containing pure *t*AlaRac (fractions 14–34) were pooled, concentrated to 5 mL volume and dialysed against 20 mM potassium phosphate buffer at pH 7.2, containing 0.2 mM DTT and glycerol (5% v/v). The purification yield was about 5 mg *t*AlaRac per litre of bacterial culture. The enzyme was stored on ice. Enzyme subunit concentration was calculated on the basis of PLP content [22,23] and using a theoretical extinction coefficient at 280 nm of 36,000 M^{-1} cm^{-1} (calculated with the Expasy ProtParam tool).