Identification, sequencing and mutagenesis of the gene for 
a D-carbamoylase from Agrobacterium radiobacter

Alberto Buson, Alessandro Negro, Luigi Grassato, Massimo Tagliaro, 
Marina Basaglia, Claudio Grandi, Angelo Fontana, Marco P. Nuti * 

CRIBI Biotechnology Centre, University of Padua, Via Trieste 75, 35121 Padua, Italy

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Abstract

A clone positive for D-carbamoylase activity (7.7 kb HindIII-BamHI DNA fragment) was obtained by screening a genomic library of Agrobacterium radiobacter in Escherichia coli. This DNA fragment contains an open reading frame of 912 bp which is predicted to encode a peptide of 304 amino acids with a calculated molecular mass of 34,247 Da. The D-carbamoylase gene, named cauA, was placed under the control of T7 RNA-dependent promoter and expressed in E. coli BL21(DE3). After induction with isopropyl-thio-β-D-galactopyranoside, the synthesis of D-carbamoylase in E. coli reached about 40% of the total protein. The expressed protein was shown to possess a molecular mass, on SDS PAGE, of 36 kDa and showed an enhanced stability with respect to that of the wild-type enzyme derived from A. radiobacter. Site-directed mutagenesis experiments allowed us to establish that a Pro14-Leu14 exchange leads to an inactive enzyme species, while a Cys270→Ser270 exchange did not impair the functional properties of the enzyme.

Keywords: D-Carbamoylase; D-Amino acid; Agrobacterium radiobacter; Escherichia coli expression; cauA

1. Introduction

Optically active amino acids have a variety of useful applications as intermediates in the synthesis of pharmacologically active substances, such as for the preparation of semisynthetic penicillins and cephalosporins, pesticides or sweeteners [1]. At present, few microorganisms have been shown to be able to produce D-amino acids from 5-substituted hydantoins (2,4-imidazolidinedione). This bioconversion consists of spontaneous or enzymatic racemization of 5-substituted hydantoins to N-carbamoyl-D-amino acids by a D-hydantoinase and stereoselective hydrolysis of N-carbamoyl-D-amino acids to the corresponding D-amino acids by N-carbamoyl-D-amino acid amidohydrolase (D-carbamoylase). Hydantoin racemase [2] and D-hydantoinase [3-10] have been found in a number of microorganisms, while D-carbamoylase activity has been detected in Agrobacterium [4,11], Pseudomonas [12], Arthrobacter [8], Comamonas [13] and Blastobacter [14].

In this paper, we describe the cloning and sequenc-
ing of a β-carbamoylase gene from Agrobacterium radiobacter and its heterologous expression in Escherichia coli, along with structure-activity studies employing site-directed mutagenesis experiments. The properties of the β-carbamoylase synthesized in E. coli have been investigated and compared with those of the A. radiobacter enzyme. Some structure-function studies have also been conducted on enzyme mutants.

2. Materials and methods

2.1. Materials

A. radiobacter NRRL B 11291, kindly provided by Recordati SpA (Milan, Italy), was grown at 30°C in TY medium (per 1: 5.0 g tryptone, 3.0 g yeast extract, 0.9 g CaCl₂, pH 6.8). E. coli LE392 was used as host for the cosmid library and E. coli JM109 as host for recombinant plasmids. Plasmid pTZ19R, obtained from Pharmacia, was used for subcloning and sequencing. E. coli BL21(DE3) was used as the host strain for the Invitrogen expression vector pRSETA. Restriction endonucleases and T4 DNA ligase were purchased from Boehringer Mannheim. Polymerase chain reaction (PCR) was performed using Thermoprime Plus DNA Polymerase. Oligonucleotide primers were purchased from Promega, or custom-synthesized by Primm (Milan, Italy). DNA manipulation, transformation and plasmid purification were performed according to established procedures [15]. DNA fragments were isolated from agarose gels using a gel extraction kit (Qiaex, Qiagen). Dideoxy nucleotide sequencing was carried out from double-stranded DNA using the Applied Biosystems Taq DyeDeoxy Cycle Sequencing Kit in a thermal cycler utilizing the temperature program suggested by the manufacturer and an automatic DNA sequencer (model 370A, Applied Biosystems). Sequences were processed, compared and analyzed by the DNA STAR for Windows (DNA STAR Inc.) software. Homology searches were made with the FASTA and BLAST programs on EMBL and SwissProt databases. The hydrophilicity profile was constructed using the ANTIGEN program available from PCGENE software (Intelligenetics). Amino-terminal sequence analysis was performed using an Applied Biosystems sequencer model 477A with a model 120A on-line PTH-analyzer, according to the procedures recommended by the manufacturer.

Samples of N-carbamoyl-α-phenylglycine and N-carbamoyl-α-hydroxyphenylglycine were obtained from Recordati SpA.

2.2. Molecular cloning of β-carbamoylase

A cosmid DNA library from A. radiobacter was prepared as described by Hohn [16]. Total DNA was extracted and partially digested with SauIIIa. Phenol/chloroform-purified fragments were dephosphorylated using alkali phosphatase and subsequently ligated to BamHI digested cosmID pH79 at a ratio of 10:1. The ligated DNA was packaged with λ coat proteins by using the Boehringer Mannheim DNA packaging kit and transfected into E. coli LE392. This library was screened for β-carbamoylase activity (see below) and a positive clone (pECS37) was identified. Clone pECS37 was further digested with different endonucleases and a subclone bearing a 2.7 kb (HindIII-BamHI) fragment, still positive for the β-carbamoylase activity, was isolated. This fragment was cloned in pTZ19R (called pECA-HB) and partially sequenced.

2.3. Expression of β-carbamoylase in E. coli

Two oligonucleotides, (γ, forward) 5′-AGGCA-TATGACACGTCAGATGATACTTG-3′ and (δ, reverse) 5′-CGAAAGCTTTCTCGATCGGATAGGCGT-3′, derived from the 5′ and 3′ coding region of β-carbamoylase were used as primers for amplification, using pECA-HB as template. The forward primer was placed between bp -6 and bp 22 and contains the restriction site Ndel, which is absent in the β-carbamoylase gene. The reverse primer was placed between bp 993 and bp 967, corresponding with the HindIII site present downstream of the β-carbamoylase gene. The PCR amplified sequences were cut at Ndel and HindIII and cloned directly into the expression vector pRSETA between the same restriction sites to give pECA1. This plasmid contains the same sequence of pECA-HB (verified by complete dideoxynucleotide sequence).

A Cys²⁷⁰ → Ser²⁷⁰ mutant of β-carbamoylase was obtained by PCR using two oligonucleotides inver-
sely oriented and partially overlapping on the gene. The first primer (α) 5' ACAGATCTCGGGAA-CTCGGCAACACATCT 3' was placed between bp 830 and bp 859, the second (β) 5' GTAGATCTCGAGATCGGCGG 3' being placed between bp 839 and bp 812. Both oligonucleotides were designed in order to introduce the Cys→Ser mutation and a BglII site, which was utilized for subsequent screening of mutants. The clone obtained was named pECA-CS. The expression plasmids were transferred to *E. coli* cells BL21(DE3) in order to express recombinant proteins. Bacterial cells carrying the recombinant plasmid were grown at 30 or 37°C in Luria's broth with 100 μg/ml ampicillin until the absorbance at 590 nm of the culture suspension reached 0.6 OD units. Expression of protein was induced by adding 1 mM IPTG, which inactivates the lac repressor, thereby allowing synthesis of T7 RNA polymerase. The sample was then centrifuged, resuspended in 0.1 M phosphate buffer pH 7.3, sonicated and analyzed for the activity. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli [17].

2.4. Determination of α-carbamoylase activity

Samples were centrifuged at 4000 × g for 15 min at 4°C. The pellet was resuspended in 0.1 M phosphate buffer, pH 7.3, and the suspension was sonicated using a model 450 sonifier (Branson) in an ice-water bath for 40 s (maximum output) at 50% duty cycle. The extract was centrifuged at 10000 × g for 10 min at 4°C and the supernatant was kept on ice until analysis. The protein content was determined using the Coomassie Plus Protein Assay Reagent kit (Pierce) calibrated with bovine serum albumin. The standard assay reaction mixture contained 80 μmol N-carbamyl-α-phenylglycine, 400 μmol sodium phosphate buffer, pH 7.3, and the cell extract (total volume 4 ml). The reaction mixtures were incubated at 40°C and aliquots were taken at certain time intervals. The formation of NH₃ was determined by the Berthelot assay, as modified by Weatherburn [18]. One unit of enzyme activity was defined as the amount of enzyme which converts 1 μmol of N-carbamoyl-α-phenylglycine to α-phenylglycine in 1 min under the conditions of the assay. The specific activity was defined as the ratio of enzyme units per mg of protein.

3. Results

3.1. Cloning of α-carbamoylase

Clones of a cosmid genomic library (600 clones) containing inserts of approx. 40 kb of *A. radiobacter* in pHC79 were transformed into *E. coli*; transformants were screened using the Berthelot assay, revealing a α-carbamoylase positive clone (pECS37) showing a specific activity of 0.015 U. This clone was further digested using several endonuclease enzymes. The clone (pECA-HB), that carried a 2.7 kb HindIII-BamHI DNA fragment, showed an activity of 0.033 U in *E. coli* JM109. The DNA sequence analysis of this clone revealed one open reading frame (ORF) of 912 bases, which was predicted to encode a polypeptide chain of 304 amino acid residues with a calculated molecular mass of 34247 Da (Fig. 1). This ORF was renamed *cauA* (carbamoyl utilization). G+C content of this ORF was 61%, a value similar to that found in other *Agrobacterium* genes. A putative ribosome binding-site sequence (Shine-Dalgarno) is present in position -10 with respect to the initial Met codon. The NH₂-terminal amino acid sequence was also partially in agreement with that of the α-carbamoylase isolated from *Comamonas* [13] and *Blastobacter* [14]. Comparative analysis of this ORF with the protein sequences available in data banks established a weak homology with the aliphatic amidase of *Brevibacterium* [19] and *Pseudomonas aeruginosa* [20] (see Fig. 2). The lack of homology between *cauA* and the *hyuC* gene product from *Pseudomonas* that is involved in conversion of N-carbamoyl-L-amino acid to L-amino acid can be explained in view of the different type of final product [2].

3.2. Expression of α-carbamoylase

In order to demonstrate that the ORF sequence corresponds to α-carbamoylase activity and to increase the enzyme expression of pECA-HB (Fig. 3, lane 4), an expression vector was constructed placing this putative translated region under the control of a
T7 promoter. The E. coli strain BL21(DE3) transformed with the expression plasmid pECA1 (Fig. 3, lane 5) showed a new, abundantly expressed protein with a molecular mass of \( \sim 35 \) kDa on SDS-PAGE after induction with IPTG; no such protein was produced in BL21(DE3) transformed with pRSETA. The level of expression of this \( \sim 35 \) kDa protein reached about 40% of total protein and the pECA1 transformant expressed D-carbamoylase at a specific activity of 0.7 U, thus indicating that this gene is actively translated in E. coli.

Analysis of the N-terminal amino acid sequence of the recombinant protein revealed the presence of two overlapping sequences, the more abundant one being without the initial Met residue (Met-Thr-Arg-Gln-Met-Ile-Leu-Ala-Val-Gly).

The high expression of the D-carbamoylase in E. coli resulted in the formation of insoluble aggregates and a low D-carbamoylase activity when cells were grown at 37°C. A lower growth temperature (20–30°C) was routinely employed, since higher levels of expression and activity were obtained.

3.3. D-Carbamoylase mutants

The polypeptide chain of D-carbamoylase contains 5 cysteine residues, one of which (Cys\(^{279}\)) is predicted to be located in a highly hydrophilic region of the
protein due to the presence of a number of nearby charged residues and is thus particularly exposed on the surface of the protein, as indicated by the hydrophilicity profile plotted according to Hopp and Woods [21] (data not shown). Since the exposed location of Cys could likely cause sensitivity to oxidizing agents, a point mutation Cys\(^{279}\) → Ser\(^{279}\) was introduced into the \(cuaA\) gene (plasmid pECA-CS) and the mutant protein thus obtained was analyzed. The substitution was found not to impair the enzymatic activity, although an altered pattern of activity with respect to the wild-type was observed (see below).

Another \(\delta\)-carbamoylase mutant in which Pro\(^{14}\) was replaced by Leu\(^{14}\) was spontaneously obtained during cloning of the \(cuaA\) gene by PCR in plasmid pRSETA (plasmid pECA-PL). Although this mutant expressed the recombinant protein at the same level as the wild-type, it was inactive (Fig. 3, lane 7).

3.4. Functional characterization of \(\delta\)-carbamoylase

The functional properties of \(\delta\)-carbamoylase assayed in crude extracts of \(A.\ radiobacter\), the highly expressed enzyme from \(E.\ coli\) and the Cys\(^{279}\) → Ser\(^{279}\) mutant protein were compared. In previous studies it was shown that some cations exert an inhibitory action on the activity of \(\delta\)-carbamoylase from \(Comamonas\) [13]. The \(A.\ radiobacter\) enzyme and the \(\delta\)-carbamoylase expressed in \(E.\ coli\) were strongly inhibited in the presence of Cu\(^{2+}\),
Fig. 4. Enzymatic activity of wild-type and over-expressed d-carbamoylase. (A,B) Cell-free extracts were assayed for d-carbamoylase activity at the indicated pH and temperature, respectively. (C) Cell-free extract was kept at 4°C and assayed for residual enzymatic activity during incubation. Relative activity is expressed as percent of the maximum activity of each protein sample. Standard errors were within 5% of the respective means. Values are means of two independent experiment.
Zn^{2+}, Ag^{+} and Fe^{3+}, while Sn^{2+}, Pb^{2+}, Mg^{2+} and Mn^{2+} slightly enhanced the enzymatic activity. Similar effects of ions on the enzymatic activity were also established for the Cys^{270} → Ser^{270} γ-carbamoylase mutant protein. Specific sulfhydryl inhibitors, e.g., iodoacetamide, 5,5’-dithiobis(2-nitrobenzoic acid) and p-chloromercuribenzoic acid completely inhibited carbamoylase activity (data not shown).

The three enzyme species (from *A. radiobacter*, *E. coli* and the Cys^{270} → Ser^{270} mutant expressed in *E. coli*) show a similar pH dependence of activity (Fig. 4A). The Cys^{270} → Ser^{270} enzyme showed higher relative activity at lower temperatures (e.g., 40–50°C), while at high temperature (e.g., 65°C) the protein from *E. coli* and wild-type enzyme showed enhanced activity in respect to the mutant enzyme (Fig. 4B). γ-Carbamoylase from *A. radiobacter* is quite unstable, since after 14 days at 4°C the enzyme activity is reduced to ~20%. On the other hand, the over-expressed γ-carbamoylase from *E. coli* and the Cys^{270} → Ser^{270} mutant did not suffer essentially any inactivation when kept under the same experimental conditions (see Fig. 4C).

4. Discussion

Conversion of hydantoins into optically pure γ-amino acids is of great interest and has attracted the attention of several investigators over the years. While enzymes involved in this conversion (such as hydantoinase and racemase) have already been cloned and sequenced from *Pseudomonas*, the enzymes involved in the conversion of hydantoins to γ-amino acids have been partially characterized in *A. radiobacter* [4, 11], but no DNA sequence has been published until now. This paper describes the cloning of the γ-carbamoylase gene, cauA, from *A. radiobacter* and its heterologous expression in *E. coli*.

The identification and sequencing of the gene encoding γ-carbamoylase opens the possibility to produce this important enzyme on a large scale. Carbamoylase and hydantoinase derived from *A. radiobacter* are already employed for the production of γ-amino acids, but a major limitation for their practical use resides in the poor stability of γ-carbamoylase [14]. Since five cysteine residues are present in the polypeptide chain of γ-carbamoylase it is probable that one or more of them could be free and not involved in disulfide formation. In fact, γ-carbamoylase is more stable if kept in the presence of the reducing agent β-mercaptoethanol and thus likely inactivation of the enzyme could be caused by oxidation of cysteine residues. Replacement of the more exposed Cys^{270} into the Ser^{270} revealed that this amino acid is not involved in catalysis. The fact that some cationic ions (Cu^{2+}, Zn^{2+}, Ag^{+} and Fe^{3+}) and sulfhydryl group inhibitors affect the activity of over-expressed γ-carbamoylase indicates that SH-groups are involved in catalysis, but the Cys^{270} residue is probably not involved in this metal ion inhibition.

An interesting observation of this study is that the over-expressed γ-carbamoylase from *E. coli* is more stable than the enzyme from *A. radiobacter*. While an understanding of this observation requires the outcome of additional studies, it is likely that a different cytoplasmic environment between the two microorganisms could be responsible for the different behavior of the enzyme. Inactivation of γ-carbamoylase from *A. radiobacter* by proteolytic degradation does not seem to be involved, since both the molecular mass and the NH₂-terminal amino acid sequence remain unchanged even after inactivation (not shown). On the other hand, the loss of activity due to the Pro^{14} → Leu^{14} substitution suggests that the NH₂-terminal portion of the protein is critical for the enzyme activity. Since proline is a helix-breaking residue, a significant change of the conformation of the polypeptide chain of the protein is expected to occur, thus impairing critical structural determinants of the functional protein.

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References


