Topological Mapping of the Cysteine Residues of N-Carbamyl-D-amino-acid Amidohydrolase and Their Role in Enzymatic Activity

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The N-carbamyl-D-amino-acid amidohydrolase from Agrobacterium radiobacter NRRL B11291, the enzyme used for the industrial production of D-amino acids, was cloned, sequenced, and expressed in Escherichia coli. The protein, a dimer constituted by two identical subunits of 34,000 Da with five cysteines each, was susceptible to aggregation under oxidizing conditions and highly sensitive to hydrogen peroxide. To investigate the role of the cysteines in enzyme stability and activity, mutant proteins were constructed by site-directed mutagenesis in which the five residues were substituted by either Ala or Ser. Only the mutant carrying the Cys372 substitution was catalytically inactive, and the other mutants maintained the same specific activity as the wild type enzyme. The crucial role of Cys372 in enzymatic activity was also confirmed by chemical derivatization of the protein with iodoacetate. Furthermore, chemical derivatizations using both acrylamide and Ellman's reagent revealed that (i) none of the five cysteines is engaged in disulfide bridges, (ii) Cys372 is easily accessible to the solvent, (iii) Cys393 and Cys250 appear to be buried in the protein core, and (iv) Cys433 and Cys279 seem to be located within or in proximity of external loops and are derivatized under mild denaturing conditions. These data are discussed in light of the possible mechanisms of enzyme inactivation and catalytic reaction.

Optically active D-amino acids have attained a wide variety of commercial applications as intermediates for the production of fine chemicals, including β-lactam antibiotics, peptide hormones, and pesticides (1, 2). In particular, D-phenylglycine and D-p-hydroxyphenylglycine are among the most important chiral building blocks for the production of semisynthetic penicillins and cephalosporins such as ampicillin and amoxicillin.

Several optically active D-amino acids are currently produced in a two-step reaction process starting from D,L-5-monosubstituted hydantoins that are inexpensively synthesized from the corresponding aldehydes (3). In the first step, the substrate is hydrolyzed by a D-specific hydantoinase to give a D-carbamyl derivative. Subsequently, the carbamyl derivative is converted to the corresponding D-amino acid either by chemical methods (4) or by a second enzymatic step catalyzed by an N-carbamyl-D-amino-acid amidohydrolase (hereinafter carboxamidase) (5). Because chemical methods have high reaction temperatures, low yields, long reaction times, and generate large amounts of waste, the enzymatic hydrolysis of the N-carbamyl derivatives is highly preferred. Indeed, the use of the hydantoinase plus carboxamidase two-enzyme system is considered one of the most successful industrial applications of enzyme technology.

Several microorganisms expressing both enzymatic activities have been isolated, and the optimal reaction conditions and the biochemical properties of the two enzymes have been studied in some detail (6, 7). From what has been published so far, it appears that both the activity and stability of the carboxamidase are negatively affected by oxidizing conditions, suggesting that one or more cysteine residues are present in the enzyme. Indeed, the sensitivity of the enzyme to oxidizing conditions is one of the most serious drawbacks of the enzymatic D-amino acid production process, and a strict anaerobic regime is required to allow the completion of the substrate to product conversion (8).

To shed light on the role of the cysteines in the activity and stability of this important industrial enzyme, we decided to study in detail the N-carbamyl-D-amino-acid amidohydrolase from Agrobacterium radiobacter NRRL B11291, a strain that is used industrially for D-amino acid production. In this paper we describe the characterization of the recombinant A. radiobacter N-carbamyl-D-amino-acid amidohydrolase expressed in Escherichia coli. In particular, the role of the five cysteines in the activity of the enzyme has been thoroughly investigated using both chemical and genetic methods. In addition, a topological mapping has been undertaken using a chemical approach in which the availability of the cysteine residues to derivatizing agents has been assessed in native and denatured enzyme.

MATERIALS AND METHODS

Plasmids and Strains—E. coli 71/18(pSM214) was used for the expression of the recombinant N-carbamyl-D-amino-acid amidohydrolase. pSM214 is an E. coli/β-lactamase shuttle expression vector constructed in our laboratory (9). Phase M13mp8 and E. coli TG1 were used for site-directed mutagenesis. E. coli was transformed using either the CaCl₂ method (10) or electroporation (11).

Enzymes and Reagents—Restriction and modification enzymes were purchased from Boehringer Mannheim and from New England Biolabs and used according to the manufacturers’ specifications. Oligodeoxynucleotides were synthesized using a Beckman P1000 DNA synthesizer. Sequencing was performed manually on both strands using the Sequenase® sequencing kit (U.S. Biochemical Corp.) and [α-32P]dATP from DuPont NEN. Chromatographic material was from Pharmacia Biotech Inc. Guanidium hydrochloride, β-mercaptoethanol, dithiothreitol, acrylamide, iodoacetate, and 5,5'-dithiobis-2-nitrobenzoic acid (Ellman’s reagent) were from Fluka, and N-carbamyl-D-p-hydroxyphenylglycine was prepared from the corresponding amino acid and potassium cyanate according to the procedure described by Stark and Smyth (12).

Preparation of N-Carbamyl-D-amino-acid Amidohydrolase Mutants—Site-directed mutagenesis was performed using the Sculptor in vitro mutagenesis system from Amersham Corp. The mutagenesis primers used for the substitutions of cysteine with alanine or serine were: C172A, 5'-GGCGATCGTGGGCGATGAACATCC-3'; C193A, 5'-TGTAAGCCGCGGCGATGCCTGCG-3'; C243A, 5'-GAGCAGCATGGAGCAGCATGC-3'; C279A, 5'-GCAGTTCCGGCGCGCGGTGAGAT-3'. C172S, 5'-GGCGATCGTGGGCGATGAACATCC-3'; C193S, 5'-TGTAAGCCGCGGCGATGCCTGCG-3'; C243S, 5'-GAGCAGCATGGAGCAGCATGC-3'; C279S, 5'-GCAGTTCCGGCGCGCGGTGAGAT-3'.

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Role of Cysteines in N-Carbamyl-o-amino-acid Amidohydrolase

Hewlett Packard) using a Beckman System Gold equipped with a diode array detector. The separation was achieved at a flow rate of 1 ml/min, applying a linear gradient of 10–100% Buffer B in Buffer A (Buffer A: water containing 0.1% trifluoroacetic acid; Buffer B: 90% acetonitrile/10% water/0.075% trifluoroacetic acid).

N-terminal Protein Sequencing—Automated Edman degradation of peptides from CNBr cleavage was performed on a Beckman LF 3000 Protein Sequencer by means of the standard Porton sequencing routine 40 and on-line reverse phase HPLC analysis of the PTH amino acids. PTH S-carbamylcysteine derived from the iodoacetate reaction eluted just before PTH serine, whereas PTH Cys-S-β-propionamide (PTH Cys-Pam) obtained upon acrylamide treatment eluted between PTH Glu and PTH His. Finally, the diithreitol adduct of dehydroalnine that is obtained during the Edman degradation reaction from cysteine-Ellman’s reagent mixed disulfides (17) eluted between PTH Ala and PTH Tyr.

H$_2$O$_2$ Inactivation of N-Carbamyl-o-amino-acid Amidohydrolase and Catalase Protection Assay—N-Carbamyl-o-amino-acid amidohydrolase was exposed to increasing concentrations of H$_2$O$_2$ (0–0.2 mM) at room temperature. After a 15-min incubation, the residual activity was determined under the standard assay conditions. Catalase protection experiments were carried out by incubating the enzyme in the presence of increasing concentrations of catalase (0–5 units) at a H$_2$O$_2$ concentration of 0.1 mM. One catalase unit is defined as the amount of enzyme that decomposes 1 μmol of H$_2$O$_2$ in 1 min at 37°C.

RESULTS

Characterization of the N-Carbamyl-o-amino-acid Amidohydrolase Gene and Its Expression in E. coli—The sequence of the N-carbamyl-o-amino-acid amidohydrolase gene, whose isolation will be described elsewhere, is shown in Fig. 1. The gene encodes a protein of 304 amino acids with a calculated molecular weight of 34,000 and pl value of 5.99. From the sequence analysis, five cysteines are present at positions 172, 193, 243, 250, and 279.

The gene coding for the N-carbamyl-o-amino-acid amidohydrolase was inserted into plasmid pSM214 to give plasmid pSM637 (Fig. 2), which was used for its heterologous expression in E. coli. The enzyme was expressed under the control of a constitutive promoter at a level that on the basis of SDS-PAGE analysis was estimated to be more than 5% of the total soluble proteins (data not shown).

Properties of N-Carbamyl-o-amino-acid Amidohydrolase Purified from E. coli—Table I summarizes the results of the procedure developed to purify the recombinant N-carbamyl-o-amino-acid amidohydrolase. The procedure, which allowed a 20-fold purification with a final yield of 34%, gave an enzyme more than 95% pure as judged by SDS-PAGE and HPLC analysis. Under the standard assay conditions, the specific activity of the purified enzyme was 10 μmol min$^{-1}$ mg$^{-1}$.

On SDS-PAGE the molecular mass of the enzyme was estimated to be about 32,000 Da, in good agreement with the theoretical calculation from the sequence analysis. However, on Superose 12 HR 10/30 the protein eluted at a apparent molecular mass of about 67,000 Da, suggesting that the active form of the enzyme is a homodimer. The recombinant enzyme co-eluted with bovine serum albumine (67,000 Da) even in the presence of 10 mM β-mercaptoethanol, indicating that the two subunits of the homodimeric structure are not covalently associated (data not shown).

Under prolonged storage at 4°C in phosphate buffers, the enzyme progressively lost activity that could be recovered only partially by treatment with reducing agents such as β-mercaptoethanol (data not shown). The loss of activity paralleled the appearance of insoluble inactive aggregates in the enzyme preparation, suggesting that covalent intermolecular reactions took place under the storage conditions used. Nevertheless the

1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; PTH, phenylthiohydantoin; Cysβ-Pam, Cys-S-β-propionamide.

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The purified enzyme was more stable in the absence rather than in the presence of β-mercaptoethanol. This can be explained by the fact that in the presence of air thiols have a propensity to generate hydrogen peroxide and superoxide anions, which in turn can lead to irreversible enzyme damage (18, 19). To test the sensitivity of the enzyme to hydrogen peroxide, the activity was measured after incubation either in the presence or in the absence of H₂O₂. As shown in Fig. 3A, the enzyme was fully inactivated after 15 min of incubation with 0.1 mM H₂O₂. The enzyme was protected from H₂O₂ inactivation when a sufficient amount of catalase was included in the reaction mixture before H₂O₂ addition (Fig. 3B). The sensitivity of the enzyme to H₂O₂ suggested that at least one cysteine residue takes part in the catalytic reaction.

To experimentally test whether one of the five cysteines present in the carbamylase from A. radiobacter NRRL B11291 is involved in catalysis, we expressed in E. coli five mutants of the enzyme in which each of the cysteine residues was replaced with alanine. Substitution of Cys172 by alanine resulted in a completely inactive enzyme under the standard assay conditions, whereas substitution of any of the other cysteines had no effect on enzyme activity. Substitution of Cys172 with serine also resulted in complete inactivation of the enzyme. A number of double mutants were also generated (Cys172/Cys42, Cys172/Cys250). Taken together, these data clearly indicated that only Cys172 is strictly required for enzymatic activity.

Identification by Chemical Methods of the Cysteine Residue Involved in Catalysis—When the carbamylase from A. radiobacter NRRL B11291 is hydrolyzed by CNBr, a number of fragments are generated that can be resolved on reverse phase HPLC and identified by sequence analysis (Fig. 4). If this procedure is carried out after full denaturation of the enzyme in 6 M guanidinium hydrochloride and treatment with 1 M acrylamide, it is possible to establish whether any of the cysteines of the molecule is engaged in a disulfide bond. Free cysteines are alkylated by acrylamide, and on Edman degradation the compound PTH Cysβ-Pam is easily recognized in HPLC by standard PTH separation (16). Using the approach described above, we found that all five cysteine residues were alkylated upon acrylamide treatment (Table II), indicating that their thiol groups are in the reduced state.

Treatment of the enzyme with 0.2 M iodoacetate before the denaturation and the alkylation step resulted in modification of available cysteine thiols, and the Edman degradation product of the modified cysteines (PTH S-carboxymethylcysteine) can be distinguished from PTH Cysβ-Pam. Only Cys172 was identified as PTH S-carboxymethylcysteine (Table II), whereas all other cysteines were recovered as PTH Cysβ-Pam, indicating that only Cys172 is exposed to the aqueous solvent. Because the iodoacetate-treated enzyme was fully inactive (data not shown), we concluded that in agreement with the site-directed mutagenesis experiments, Cys172 must be involved in the catalytic reaction.

Topological Mapping of the Cysteines in the N-Carbamyl-D-amino-acid Amidohydrolase—From the data described above, it appears that Cys172 is exposed to the solvent and therefore easily accessible to chemical reagents able to react with thiol groups. In fact Cys172 was the only cysteine derivatized with iodoacetate before the denaturation and the alkylation step resulted in modification of available cysteine thiolis, and the Edman degradation product of the modified cysteines (PTH S-carboxymethylcysteine) can be distinguished from PTH Cysβ-Pam. Only Cys172 was identified as PTH S-carboxymethylcysteine (Table II), whereas all other cysteines were recovered as PTH Cysβ-Pam, indicating that only Cys172 is exposed to the aqueous solvent. Because the iodoacetate-treated enzyme was fully inactive (data not shown), we concluded that in agreement with the site-directed mutagenesis experiments, Cys172 must be involved in the catalytic reaction.

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tured with 5 M guanidinium hydrochloride, and finally derivatized with the thiol-reacting Ellman's reagent. When the chemical modification of the five cysteines was analyzed by peptide sequencing, we found that cysteines 172, 243, and 279 had been alkylated by acrylamide (identified as PTH Cys$_{b}$-Pam), whereas Cys$_{193}$ and Cys$_{250}$ were identified as the dithiothreitol adducts of dehydroalanine (Table II), indicating that they had been modified by Ellman's reagent after full denaturation of the enzyme. Considering that a 1 M solution of acrylamide is a weak protein denaturant, the experiments described above can be interpreted as indicating that Cys$_{243}$ and Cys$_{279}$ are exposed to the solvent by partial unfolding of the protein and are thus located within or in proximity of external loops. On the other hand, Cys$_{193}$ and Cys$_{250}$ are expected to be buried in the protein core and become accessible to the thiol-reacting compounds only after full denaturation.

**DISCUSSION**

The carbamyl-D-amino-acid amidohydrolases are important enzymes for the industrial production of D-amino acids, some of which are key intermediates for the synthesis of the β-lactam antibiotics. The relevant role of these enzymes in the pharmaceutical industry prompted us to study in more detail the biochemical properties of the carbamyl-D-amino-acid amidohydrolase from A. radiobacter, a bacterial strain that, due to its ability to synthesize large quantities of this enzyme together with hydantoinase, is currently used for the production of p-OH-phenylglycine and phenylglycine starting from the corresponding hydantoines (5, 20). In particular, we focused our attention on the role of cysteine residues on the activity of the enzyme. Sequence analysis of the carbamylase gene revealed that it encodes a protein of 32,000 Da containing five cysteines. On the basis of gel filtration and SDS-PAGE analyses, the enzyme was found to be organized in a nonsulfide bonded homodimeric structure.

The organization in more than one subunit appears to be a typical feature of amidohydrolases. For example, the N-carbamyl-D-amino-acid amidohydrolase from Comamonas sp. E222c, which has been recently characterized and whose N-terminal sequence is highly homologous to the corresponding region of the A. radiobacter enzyme, was proposed to have a trimeric structure with three identical subunits (7). Furthermore, the bacterial amidohydrolases that catalyze the hydrolysis of asparagine and glutamine are active as tetramers of identical subunits.
Among the amide bond-hydrolyzing enzymes, many utilize either the hydroxyl group of serine and threonine side chains or the thiol group of cysteine as a nucleophile to attack the scissile bond. Typical examples are the serine and cysteine proteases (25, 26), the amydohydrolases, which catalyze the hydrolysis of asparagine and glutamine to their acidic forms (22, 27, 28), and the E. coli penicillin acylase responsible for the conversion of penicillin G to 6-aminopenicillanic acid (23). Interestingly, the N-carbamyl-\(\beta\)-amino-acid amidohydrolase from A. radiobacter shares about 25% identity with the aliphatic amidases of both Pseudomonas aeruginosa and Rhodococcus erythropolis (29, 30) with the highest degree of homology being found in the region surrounding and including Cys\(^{172}\).

Grouping the A. radiobacter carboxylase in the “cysteine and serine proteases family” implies that (i) the reaction proceeds through the formation of an acyl-enzyme intermediate and (ii) a neighboring residue such as histidine or lysine should serve as a base to enhance the nucleophilicity of Cys\(^{172}\). Both properties can be experimentally tested. In particular, we recently developed a rapid screening procedure able to identify on agar plates carboxylase-deficient mutants. We are utilizing such screening protocol to shed light on the amino acid residues crucial for the enzymatic activity.

It has been shown that in some cysteine-dependent enzymes the active site cysteine can be substituted for serine without completely destroying the enzyme activity. For example, the substituted thymidylate synthases of both E. coli and bacteriophage T4 retain 0.02 and 0.07% activity of the wild type enzymes, respectively (31, 32).

Our Cys\(^{172}\) → Ser mutant was at least 4 orders of magnitude less active than the wild type, indicating that the carboxylase has more structural and catalytic constraints than other cysteine-dependent enzymes. The resolution of the three-dimensional structure of the enzyme will be of great help to shed light on the details of the catalytic reaction. In this context, the availability of large quantities of pure enzyme will facilitate future protein crystallization experiments.

An interesting aspect of the work presented here is that by using different protocols for the derivatization of the thiol groups, including treatment of the enzyme under native, mild denaturing, and strong denaturing conditions, it has been possible to have some hints as to the topological position of the five cysteines present in the molecule. The data clearly show that the active site Cys\(^{172}\) is readily accessible to the solvent, Cys\(^{193}\) and Cys\(^{250}\) are buried in the protein core, and Cys\(^{243}\) and Cys\(^{279}\) are probably located near external loops. These last two cysteines may be the ones involved in the formation of intermolecular disulfide bridges that we found to occur under oxidizing conditions. If this is the case, the replacement of Cys\(^{243}\) and Cys\(^{279}\) with other amino acids should be beneficial for the enzyme stability.

### Table II

**Analysis of the five cysteines of N-carbamyl-\(\beta\)-amino-acid amidohydrolase by N-terminal sequencing**

| Sequential protein treatment before CNBr | Retention time of cysteines after Edman degradation |
|------------------------------------------|----------------------------------------------------|
| Guanidinium hydrochloride denaturation   | Cys\(^{172}\) 8.7                                   |
| Acrylamide derivatization               | Cys\(^{172}\) 8.7                                   |
| Iodoacetate derivatization              | Cys\(^{172}\) 8.7                                   |
| Acrylamide derivatization               | Cys\(^{172}\) 8.7                                   |
| Acrylamide derivatization               |
| Derivatization with the Ellman’s reagent |                                                   |

Retention times of the standards: PTH Cys\(^{172}\)-Pam, 8.7; PTH carboxymethyl cysteine, 6.66; dehydroalanine dithiothreitol adduct, 11.5.

The experiments described in this work clearly demonstrated that this is indeed the case. The Cys\(^{172}\) → Ala substitution produced a fully inactive enzyme, at least as judged by our standard assay, which can detect enzymes with specific activity 10,000-fold lower than the wild type. Similar conclusions were obtained following a chemical approach. Cys\(^{172}\) was the only cysteine that under nondenaturing conditions could be chemically modified by either iodoacetate or Ellman’s reagent. After modification, the enzyme was totally inactive.

The involvement of a cysteine in the enzymatic activity fits with the type of chemical reaction catalyzed by carboxylase. Among the amide bond-hydrolyzing enzymes, many utilize
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