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Potential Application of *N*-Carbamoyl-β-Alanine Amidohydrolase from *Agrobacterium tumefaciens* C58 for β-Amino Acid Production▽

Ana Isabel Martínez-Gómez,[1] Sergio Martínez-Rodríguez,[1] Joaquín Pozo-Dengra,[1] Davide Tessaro,[2] Stefano Servi,[2] Josefa María Clemente-Jiménez,[1] Felipe Rodríguez-Vico,[1] and Francisco Javier Las Heras-Vázquez[1]*

Departamento de Química-Física, Bioquímica y Química Inorgánica, Edificio CITE I, Universidad de Almería, La Cañada de San Urbano, E-04120 Almería, Spain,[1] and Dipartimento di Chimica, Materiali, Ingegneria Chimica G. Natta, Politecnico di Milano, Milan, Italy[2]

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An *N*-carbamoyl-β-alanine amidohydrolase of industrial interest from *Agrobacterium tumefaciens* C58 (βcar_{At}) has been characterized. βcar_{At} is most active at 30°C and pH 8.0 with *N*-carbamoyl-β-alanine as a substrate. The purified enzyme is completely inactivated by the metal-chelating agent 8-hydroxyquinoline-5-sulfonic acid (HQSA), and activity is restored by the addition of divalent metal ions, such as Mn^{2+}, Ni^{2+}, and Co^{2+}. The native enzyme is a homodimer with a molecular mass of 90 kDa from pH 5.5 to 9.0. The enzyme has a broad substrate spectrum and hydrolyzes nonsubstituted *N*-carbamoyl-α-, -β-, -γ-, and -δ-amino acids, with the greatest catalytic efficiency for *N*-carbamoyl-β-alanine. βcar_{At} also recognizes substrate analogues substituted with sulfonic and phosphonic acid groups to produce the β-amino acids taurine and ciliatine, respectively. βcar_{At} is able to produce monosubstituted β^2- and β^3-amino acids, showing better catalytic efficiency (k_{cat}/K_m) for the production of the former. For both types of monosubstituted substrates, the enzyme hydrolyzes *N*-carbamoyl-β-amino acids with a short aliphatic side chain better than those with aromatic rings. These properties make βcar_{At} an outstanding candidate for application in the biotechnology industry.

N-Carbamoyl-β-alanine amidohydrolase (NCβAA) (EC 3.5.1.6), also known as β-alanine synthase or β-ureidopropionase, catalyzes the third and final step of reductive pyrimidine degradation. In this reaction, *N*-carbamoyl-β-alanine or *N*-carbamoyl-β-aminoisobutyric acid is irreversibly hydrolyzed to CO_2, NH_3, and β-alanine or β-aminoisobutyric acid, respectively (43). Eukaryotic NCβAAs have been purified from several sources (10, 25, 33, 39, 42, 44). Nevertheless, only two prokaryotic NCβAAs, belonging to the *Clostridium* and *Pseudomonas* genera (4, 29), have been purified to date, although this activity has been inferred for several microorganisms due to the appearance of the reductive pathway of pyrimidine degradation (38, 45). *Pseudomonas* NCβAA is also able to hydrolyze L-*N*-carbamoyl-α-amino acids, and indeed, this activity is widespread in the bacterial kingdom (3, 23, 26, 46).

β-Amino acids have unique pharmacological properties, and their utility as building blocks of β-peptides, pharmaceutical compounds, and natural products is of growing interest (14). β-Alanine, a natural β-amino acid, is a precursor of coenzyme A and pantothenic acid in bacteria and fungi (vitamin B_5) (7). β-Alanine is widely distributed in the central nervous systems of vertebrates and is a structural analogue of γ-amino-*n*-butyric acid and glycine, major inhibitory neurotransmitters, suggesting that it may be involved in synaptic transmissions (20). Another important natural β-amino acid is taurine (2-aminoethanesulfonic acid), which plays an important role in several essential processes, such as membrane stabilization, osmoregulation, glucose metabolism, antioxidation, and development of the central nervous system and the retina (9, 28, 33). 2-Aminoethylphosphonate, the most common naturally occurring phosphonate, also known as ciliatine, is an important precursor used in the biosynthesis of phosphonolipids, phosphonoproteins, and phosphonoglycans (5). β-Homoalanine (β-aminobutyric acid) has been used successfully for the design of nonnatural ligands for therapeutic application against autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, or autoimmune uveitis (30). Substituted β-amino acids can be denominated β^2, β^3, and $\beta^{2,3}$, depending on the position of the side chain(s) (R) on the amino acid skeleton (18). β^2-Amino acids are not yet as readily available as their β^3-counterparts, as they must be prepared using multistep procedures (17).

We decided to characterize NCβAA (β-carbamoylase) from *Agrobacterium tumefaciens* C58 (βcar_{At}) after showing that some dihydropyrimidinases belonging to the *Arthrobacter* and *Sinorhizobium* genera are able to hydrolyze different 5- or 6-substituted dihydrouracils to the corresponding *N*-carbamoyl-β-amino acids (18, 22). If βcar_{At} could decarbamoylate the reaction products of dihydrouracils, different β-amino acids would be obtained enzymatically in the same way that α-amino acids are produced via the hydantoinase process (6, 21). We therefore describe the physical, biochemical, kinetic, and substrate specificity properties of recombinant βcar_{At}.

MATERIALS AND METHODS

General protocols and reagents. Standard methods were used for cloning and expression (1, 31). Restriction enzymes, T4 DNA ligase, and thermostable *Pwo* polymerase for PCR were purchased from Roche Diagnostic S.L. (Barcelona, Spain). β-Leucine, β-homoalanine, taurine, ciliatine, α-amino-β-alanine, and glycine were purchased from Sigma-Aldrich (Madrid, Spain), 5-aminopentanoic

* Corresponding author. Mailing address: Departamento de Química-Física, Bioquímica y Química Inorgánica, Edificio CITE I, Universidad de Almería, La Cañada de San Urbano, E-04120 Almería, Spain. Phone: 34 950 015055. Fax: 34 950 015008. E-mail: fjheras@ual.es.

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acid and 6-aminohexanoic acid were purchased from Alfa Aesar (Labortecnic, Granada, Spain), β-phenylalanine, 3-aminoisobutyric acid, 4-aminobutyric acid, and β-alanine were purchased from Acros (Labortecnic, Granada, Spain), and 2-amino-3-ureidopropionic acid (Albizziin) was purchased from Bachem (Cymitquimica S.L., Barcelona, Spain). 2-Phenyl-3-ureidopropionic acid was synthesized as previously described elsewhere (35). The rest of the *N*-carbamoyl-amino acids used in this work were synthesized according to methods in the literature (2).

Microbes and culture conditions. *Agrobacterium tumefaciens* ATCC 33970 (strain C58) was used as a possible donor of the β-carbamoylase gene. It was cultivated at 30°C for 20 h in Luria-Bertani medium (LB medium; 1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.2). *Escherichia coli* BL21 (36) was used to clone and express the β-carbamoylase gene.

Cloning and sequence analysis of the βcar_{At} gene. A DNA region from the *Agrobacterium tumefaciens* C58 circular chromosome (NC_003062) which was highly similar to the NCβAA gene from *Pseudomonas aeruginosa* PAO1 (AE004091) was amplified by PCR. The primers used for PCR amplification were Atβcar5 (5′-AATCTAGAACGGCGGGTAAAAACTTGACGGT-3′) and Atβcar3 (5′-TTCTGCAGTCAATGATGATGATGATGATGTTGCACGATC TCCGCAGTC-3′). The latter included a polyhistidine tag (His_6 tag) before the stop codon. The XbaI- and PstI-digested fragment was purified from agarose gel by use of a Qiaquick gel extraction kit (Qiagen) and then ligated into the pBluescript II SK (+) plasmid (Stratagene Cloning Systems) to create plasmid pAMG4.

Once the fragment had been cloned, it was sequenced using the dye termination dideoxy nucleotide sequencing method in an ABI 377 DNA sequencer (Applied Biosystems). Sequencing was carried out twice from both strands, using standard T3 and T7 primers, edited, and assembled. The assembled sequences were aligned and compared with different NCβAAs of proven activity (10, 15, 27, 39).

Site-directed mutagenesis. Three different substitution mutants of arginine 291 were developed (R291E, R291K, and R291Q mutants). Mutagenesis of the pAMG4 plasmid was performed using a QuikChange II site-directed mutagenesis kit from Stratagene following the manufacturer's protocol. Mutations were confirmed by using the dye termination dideoxy nucleotide sequencing method as described above.

Overexpression and purification of wild-type and mutated βcar_{At}. The transformant BL21 strain was grown in LB medium supplemented with 100 μg ml^{-1} of ampicillin. A single colony was transferred into 10 ml of LB medium with ampicillin at the above-mentioned concentration in a 100-ml flask. This culture was incubated overnight at 37°C with shaking. Five hundred milliliters of LB medium with the appropriate concentration of ampicillin was inoculated with 5 ml of the overnight culture in a 2-liter flask. After 3 h of incubation at 37°C with vigorous shaking, the optical density at 600 nm (OD_{600}) of the resulting culture was 0.3 to 0.5. For induction of β-carbamoylase gene expression, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM, and incubation was continued at 34°C for an additional 6 h. The cells were collected by centrifugation (Beckman JA2-21 rotor; 7,000 × *g*, 4°C, 10 min), washed twice, and resuspended in 50 ml wash buffer (300 mM NaCl, 0.02% NaN_3, 50 mM sodium phosphate, pH 7.0). The cell walls were disrupted by sonication, using a UP 200 S ultrasonic processor (Hielscher GmbH, Germany), on ice for six periods of 60 s at pulse mode 0.5 and 60% sonic power. The cell debris was precipitated by centrifugation (Beckman JA2-21 rotor; 10,000 × *g*, 4°C, 20 min), and the supernatant was applied to a column with 3 ml of Talon metal-affinity resin (Clontech Laboratories, Inc.). After being washed four times with wash buffer, β-carbamoylase enzyme was eluted with elution buffer (100 mM NaCl, 0.02% NaN_3, 300 mM imidazole, 2 mM Tris, pH 8.0). The purified enzyme was dialyzed against 100 mM sodium phosphate buffer, pH 8.0, and stored at 4°C until use.

To ensure that only a single class of metal ions is present at the active site, an apoenzyme form of βcar_{At} was prepared by incubating 45 to 50 μM of purified enzyme with 10 mM of 8-hydroxyquinoline-5-sulfonic acid (HQSA) at 4°C overnight. HQSA was removed by dialysis in four stages at 12-hour intervals, all at 4°C, using 100 mM sodium phosphate buffer, pH 8.0.

Enzyme assays. The standard enzymatic reaction was carried out with purified βcar_{At} (at a final concentration of 0.03 μM) along with *N*-carbamoyl-β-alanine substrate (25 mM) dissolved in 100 mM sodium phosphate buffer (pH 8.0) in a 500-μl reaction volume. The reaction mixture was incubated at 30°C for 20 min, and 25-μl aliquots were stopped by the addition of 475 μl of 1% H_3PO_4. After centrifugation, the resulting supernatants were analyzed by high-performance liquid chromatography (HPLC). *N*-Carbamoyl-β-alanine and β-alanine were detected by an HPLC instrument (Finnigan SpectraSystem HPLC system; Thermo, Madrid, Spain) equipped with a Hypersil-amino acid C_{18} column (4.6 × 250 mm; Thermo). The mobile phase was NaH_2PO_4 (20 mM; pH 4.5), pumped at a flow rate of 0.75 ml min^{-1} and measured at 200 nm. The specific activity of the enzyme was defined as the amount of enzyme that catalyzed the formation of 1 μmol of β-amino acid at 30°C min^{-1} and mg^{-1} of protein.

Substrate specificity studies were performed with each different *N*-carbamoyl-β-amino acid dissolved in 100 mM sodium phosphate buffer (pH 8.0) along with the purified enzyme at different concentrations (0.03 to 5 μM). Reactions were carried out at 30°C, with the apoenzyme preincubated (1 h) at 4°C with $NiCl_2$, and stopped by the addition of 1% H_3PO_4. The mobile phase of the different substrates and their corresponding β-amino acids was methanol-phosphoric acid (20 mM) (5:95 to 30:70 [vol/vol], depending on the compound) at pH 3.2, pumped at a flow rate of 0.75 ml min^{-1}. Compounds were detected with a UV detector at a wavelength of 200 nm.

Molecular mass analysis. Size-exclusion chromatography–HPLC analysis was performed to estimate the molecular mass of the native enzyme, using a nondenatured protein molecular weight marker kit (Bio-Rad, Madrid, Spain). The enzyme was eluted in 100 mM citrate, sodium phosphate, Tris, and borate buffers (pH 5.5 and 6.0, pH 6.0 to 8.0, pH 7.0 to 8.5, and pH 8.0 to 9.0, respectively) at a flow rate of 0.4 ml/min and measured at 280 nm in an HPLC system with a Biosep-SEC-S2000 column (Phenomenex, Madrid, Spain). The molecular mass of the monomeric form was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using a low-molecular-weight marker kit (GE Healthcare, Barcelona, Spain).

Protein characterization. The thermal stability of the β-carbamoylase enzyme was measured after 60 min of preincubation at temperatures from 25 to 50°C in 100 mM sodium phosphate buffer at pH 8.0. The enzyme assay was then carried out at 30°C for 20 min with the purified β-carbamoylase enzyme and *N*-carbamoyl-β-alanine substrate. The effects of different compounds on enzyme activity were evaluated. A 2 mM concentration of each metal ($HgCl_2$, $MgCl_2$, $NiCl_2$, $MnCl_2$, $CoCl_2$, $CuCl_2$, $ZnCl_2$, $CaCl_2$, $PbCl_2$, $FeCl_2$, $FeCl_3$, RbCl, CsCl, LiCl, NaCl, and KCl) and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and a 10 mM concentration of β-mercaptoethanol, dithiothreitol (DTT), EDTA, HQSA, 2-iodoacetamide, bicine, borate, and glycine buffers were incubated with the β-carbamoylase (0.03 μM) in 100 mM sodium phosphate buffer, pH 8.0 (final volume, 500 μl), at 4°C for 60 min. The effect of each compound on specific activity was determined by standard enzyme assay.

Modeling studies. The βcar_{At} model was obtained by the Swiss-Model server (34), using the substrate-bound structure of *Saccharomyces kluyveri* NCβAA (Skβas) (PDB accession no. 2V8H_A), which has been solved at 2.0 Å, as a template (19). The stereochemical geometry of the final model was validated by PROCHECK (16) and WHATCHECK (13), included in the model assessment tools of the Swiss-Model server. Manual model building of the structures was performed with the Swiss PDB viewer (12) and pymol (8).

Nucleotide sequence accession number. The nucleotide sequence of the βcar_{At} gene has been deposited in the GenBank database under accession number EF507843.

RESULTS AND DISCUSSION

Sequence analysis and structure prediction of βcar_{At}. The βcar_{At} amino acid sequence was compared to those of other NCβAAs of proven activity (*Saccharomyces kluyveri*, GenBank accession no. AAK60518; *Dictyostelium discoideum*, GenBank accession no. AAK60519; *Drosophila melanogaster*, GenBank accession no. AAK60520; *Homo sapiens*, GenBank accession no. NP_057411; *Rattus norvegicus*, GenBank accession no. Q03248; *Arabidopsis thaliana*, GenBank accession no. BAB09868) (10, 15, 27, 39). The highest sequence identity was found with that from *Saccharomyces kluyveri* (Skβas; 36.70%); the amino acid sequence identity with all other enzymes was <10%. Surprisingly, a BLAST analysis showed that βcar_{At} presented higher sequence similarity to several L-*N*-α-carbamoylases, among them the previously described *Sinorhizobium meliloti* L-*N*-α-carbamoylase (SmLcar; 79.89%) (23). The same results were previously reported for Skβas (10), which shows a higher sequence identity with bacterial L-*N*-α-carbamoylases than with mammalian NCβAAs.

Divergent evolution from a common ancestral gene was proposed for different *N*-carbamoyl amidohydrolases acting on *N*-carbamoyl-substituted compounds (10). This hypothesis has

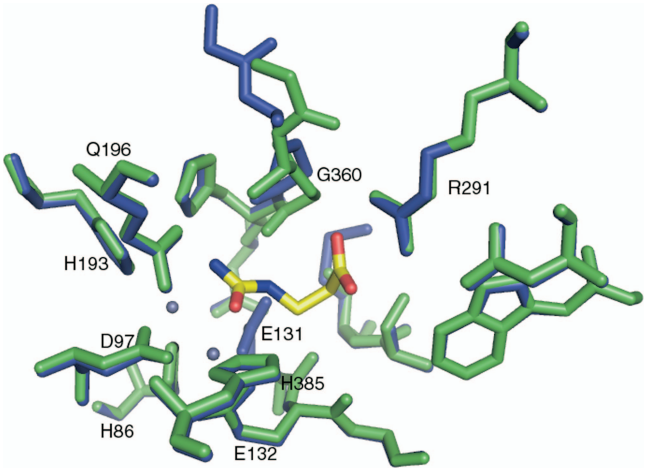


FIG. 1. Superposition of active sites of closed Skβas (green) bound to its natural substrate (PDB accession no. 2V8H_A) and of a βcar_{At} model (blue). *N*-Carbamoyl-β-alanine atoms are shown as follows: red, oxygen; yellow, carbon; and blue, nitrogen.

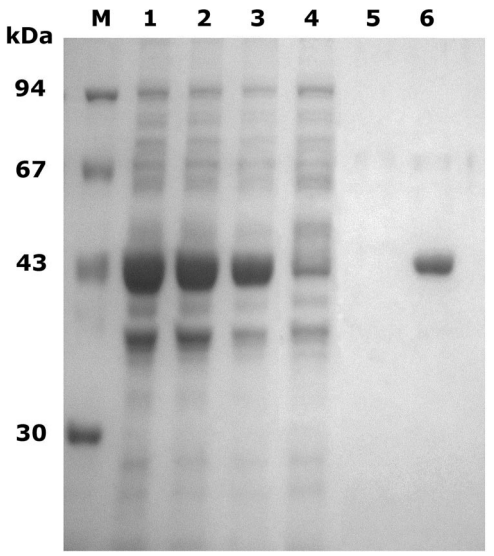


FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of each purification step of βcar_{At} from *E. coli* BL21 harboring the pAMG4 plasmid. Lane M, low-molecular-weight marker; lane 1, whole recombinant cells before rupture; lanes 2 and 3, supernatant and pellet of the resuspended crude extract after cell sonication; lane 4, eluate after adding the sonicated supernatant to the metal affinity column; lane 5, flowthrough after washing the metal affinity column with buffer; lane 6, purified enzyme.

been supported further by computational methods which have shown that several enzymes that have distantly related primary structures share the same structural scaffold (20, 24). βcar_{At} would be included in the subfamily composed of bacterial L-*N*-carbamoylases and Skβas. The structural model of βcar_{At}, determined using the known 2.0-Å resolution structure of substrate-bound Skβas (2V8H), clearly shows that the catalytic center is completely conserved (Fig. 1). An overall average G factor of −0.07 was recorded (G factor scores should be above −0.5). Ramachandran plot statistics indicated that 97.8% of the main-chain dihedral angles are found in the most favorable regions.

Overexpression, purification, and subunit structure of βcar_{At}. The His_6-tagged enzyme was purified in a one-step procedure using immobilized cobalt affinity chromatography, with an apparent molecular mass of approximately 45 kDa under denaturing conditions (estimated purity, >90%) (Fig. 2). Size-exclusion chromatography showed that the relative molecular mass of the native enzyme from pH 5.5 to 9.0 varied from 88 to 100 kDa (data not shown), proving that βcar_{At} is a homodimer, as reported for other NCβAAs and L-*N*-carbamoylases (Skβas [20], NCβAA from *Pseudomonas putida* [29] and SmLcar [23]). However, it varied considerably from those from rat (25) and calf liver (41), which have a hexameric structure (about 240 kDa), and those from *Arabidopsis thaliana* and *Zea mays*, which are decameric (about 440 kDa) (42).

Influence of pH and temperature on enzyme activity. βcar_{At} activity was assayed in several buffers from pH 6.0 to 10.5 (bicine, sodium phosphate, Tris, borate-HCl, borate-NaOH, and glycine-NaOH) at a concentration of 100 mM. The enzyme was active in sodium phosphate buffer (pH 6.5 to 8.0) and Tris buffer (pH 8.0 to 9.0), with an optimum pH for *N*-carbamoyl-β-alanine hydrolysis of 8.0. This is similar to those described for *P. putida* (29) and human liver (34) but slightly higher than those for other NCβAAs, which have an optimum pH of 7.5 (10) or 7.0 (25, 41, 42). Thermal stability studies performed on βcar_{At} have shown that residual activity gradually decreases at temperatures over 30°C for 60 min and that after incubation at 45°C for 60 min, only 20% of the activity remains (Fig. 3). Similar assays have been done only with the *P. putida* enzyme, which showed high thermostability, retaining 80% of the initial activity after incubation at 65°C for 30 min. The optimal temperature of βcar_{At} was evaluated at temperatures from 25 to 60°C, with maximum activity at 30°C (Fig. 3). In view of the above data, it should be borne in mind that the maximum activity may be achieved at higher temperatures than 30°C, but exact determination could be hampered by the duration of the activity assays. With the exception of NCβAA

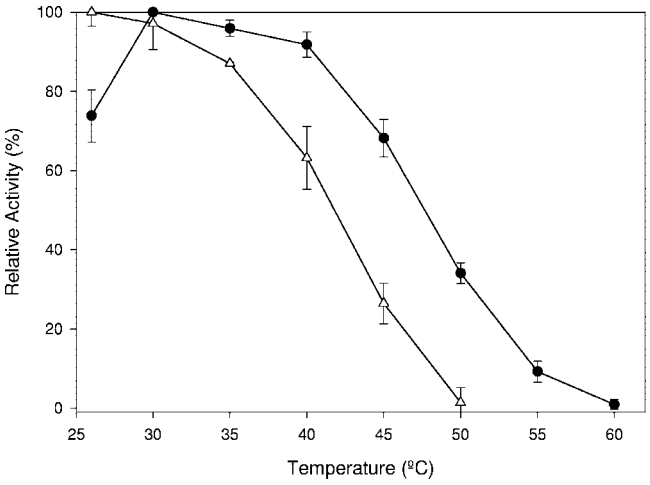


FIG. 3. Effect of temperature on βcar_{At} activity. βcar_{At} activity was measured at different temperatures by a standard enzyme assay (black circles). βcar_{At} activities after incubation for 60 min at the indicated temperatures are shown (white triangles). The remaining βcar_{At} activity was measured at 30°C for 20 min, using *N*-carbamoyl-β-alanine substrate.

TABLE 1. Effects of metal ions and chemical agents on the activity of βcar_{At}[a]

Compound	Non-HQSA-treated enzyme		HQSA-pretreated enzyme	
	Sp act (U mg^{-1})	Relative activity (%)	Sp act (U mg^{-1})	Relative activity (%)
None	1.49 ± 0.07	100 ± 5	0 ± 0	0 ± 0
Zn^{2+}	0.93 ± 0.10	63 ± 7	0 ± 0	0 ± 0
Cu^{2+}	0.53 ± 0.06	36 ± 4	0.01 ± 0.01	0 ± 0
K^{+}	1.81 ± 0.14	122 ± 10	0.32 ± 0.08	22 ± 5
Ca^{2+}	1.73 ± 0.16	116 ± 11	0.55 ± 0.04	37 ± 3
Hg^{2+}	1.15 ± 0.12	77 ± 8	0 ± 0	0 ± 0
Rb^{+}	1.69 ± 0.14	113 ± 10	0.24 ± 0.06	16 ± 4
Li^{+}	1.52 ± 0.12	102 ± 8	0.48 ± 0.04	32 ± 3
Co^{+}	3.17 ± 0.15	213 ± 10	7.02 ± 0.22	471 ± 15
Cs^{+}	1.62 ± 0.15	108 ± 10	0.80 ± 0.15	54 ± 10
Fe^{2+}	1.90 ± 0.15	127 ± 10	0.95 ± 0.06	64 ± 4
Fe^{3+}	1.56 ± 0.13	105 ± 9	0.46 ± 0.04	31 ± 2
Mg^{2+}	1.56 ± 0.10	105 ± 7	0.60 ± 0.05	40 ± 3
Mn^{2+}	2.93 ± 0.24	197 ± 16	7.71 ± 0.25	517 ± 17
Ni^{2+}	2.77 ± 0.24	186 ± 16	7.79 ± 0.51	523 ± 34
Pb^{2+}	1.37 ± 0.09	92 ± 6	0.25 ± 0.02	17 ± 1
Na^{+}	1.47 ± 0.13	98 ± 8	0.08 ± 0.01	5 ± 1
DTNB	1.18 ± 0.10	79 ± 7		
EDTA	0.22 ± 0.01	14 ± 1		
β-Mercaptoethanol	1.44 ± 0.13	96 ± 8		
DTT	1.41 ± 0.11	94 ± 7		
Iodoacetamide	1.44 ± 0.12	97 ± 8		

[a] The metal ion chloride salts and reducing, sulfhydryl, and chelating reagents were incubated with the enzyme at 4°C for 60 min. The restoration of βcar_{At} activity by metal ions after treatment with the HQSA chelating agent was also studied. The enzyme was incubated overnight with 10 mM HQSA at 4°C. The chelating agent was removed by dialysis in four stages at 4-hour intervals, all at 4°C. Divalent metal salt solutions were incubated with the enzyme at 4°C for 60 min. Activity assays were carried out in triplicate as described in Materials and Methods, with *N*-carbamoyl-β-alanine as the substrate.

from *P. putida* (29), which has an optimal temperature of 60°C, the optimal temperature range for hydrolysis of all previously studied NCβAA enzymes is 25 to 37°C.

Effects of chemical agents and metal ions. Buffers in which the enzyme had no detectable activity were studied as possible inhibitors. After preincubation in 10 mM borate, bicine, and glycine, remaining βcar_{At} activity was 6, 13, and 22%, respectively, compared to that of the untreated enzyme. The enzymatic function was not affected by reducing compounds such as DTT and β-mercaptoethanol or by the sulfhydryl reagents DTNB and iodoacetamide, all at 10 mM, thus showing that no cysteine residue is crucial for enzyme activity or protein stability. NCβAAs are described as metalloenzymes (15, 20, 42). This also proved true for βcar_{At}, since the chelating agents EDTA and HQSA, both at 10 mM, decreased its activity drastically, to 16% and 0%, respectively. To evaluate the activation or inhibition of βcar_{At} by different metal ions, its activity was assayed in the presence of a 2 mM concentration of these compounds, using the non-HQSA-treated and apoenzyme forms (Table 1). Incubation with Cu^{2+} resulted in strong inhibition, while Zn^{2+} and Hg^{2+} caused only slight inhibition. However, metal ions such as Ni^{2+}, Mn^{2+}, and Co^{2+} greatly enhanced activity. These metals may bind to His86, His193, Asp97, Glu132, and His385, as inferred from sequence comparison and modeling studies. Enzyme inactivation by HQSA was reversible after removal of the chelating agent by dialysis and addition of Ni^{2+}, Mn^{2+}, and Co^{2+} at 2 mM, but not Zn^{2+} (Table 1). Whether one of these three cations is the natural cofactor of the enzyme remains unclear. It is worth noting that rat, maize, and *S. kluyveri* NCβAAs have been described as Zn^{2+} dependent (although no other metals have been checked) (15, 20, 39). On the other hand, prokaryotic NCβAA (βcar_{At} and *P. putida* NCβAA) and L-*N*-carbamoylase activities are optimum with cofactors other than Zn^{2+} (23, 29). The question remains as to whether eukaryotic NCβAAs would be more active with Mn^{2+}, Co^{2+}, or Ni^{2+} catalytic cofactors than with Zn^{2+}. Of the three best cofactors detected for βcar_{At} under experimental conditions, Co^{2+}, Mn^{2+}, and Ni^{2+}, we continued our studies using Ni^{2+}. The best metal ion/protein ratio was found to be 25:1 when Ni^{2+} was present for at least 20 min at 4°C. Under these conditions, maximum activity was maintained and no inhibition was detected (Fig. 4).

Substrate specificity and kinetic characterization. The ability of purified βcar_{At} to hydrolyze different substrates was examined. To this end, the kinetic parameters K_m, k_{cat}, and k_{cat}/K_m were obtained from hyperbolic saturation curves by least-squares fitting of the data to the Michaelis-Menten equation. Reactions were carried out with different concentrations of substrates at 30°C after preincubation of the enzyme with $NiCl_2$.

The involvement and roles of several residues comprising the catalytic center of L-*N*-carbamoylases and Skβas have been proved (19, 24), and they are completely conserved in βcar_{At} (Fig. 1), in which Arg291 would be the key residue for recognition of the substrate carboxyl group. To reaffirm this hypothesis, three mutants were developed (R291E, R291K, and R291Q mutants). None of the mutants showed activity using the standard assay, although only the lysine mutant enzyme secondary structure elements were unaltered when analyzed by far-UV circular dichroism (data not shown). The R291K mutation suggests how essential this residue is in substrate binding, supporting previous results found for other enzymes (19, 24).

βcar_{At} is also able to hydrolyze substrate analogues in which the carboxyl group is replaced by a sulfonic or phosphonic group, although its affinity is somewhat lower for these compounds (Table 2). This is probably due to the different polarizability of the carbonyl, phosphonyl, and sulfonyl groups, which would affect their interaction with the Arg291 guanidinium group.

By analogy with Skβas, βcar_{At} catalytic activity comes from large-scale movement of the catalytic domain (19, 20). We chose this feature to find out whether βcar_{At} might accommodate longer or shorter *N*-carbamoyl-amino acids between the lid and the catalytic domains. βcar_{At} hydrolyzed compounds from *N*-carbamoyl-glycine (ureidoethanoic acid) to *N*-carbamoyl-δ-aminopentanoic acid (5-ureidopentanoic acid), but not longer substrates, showing the highest affinity for *N*-carbamoyl-β-alanine (3-ureidopropanoic acid) (Fig. 5). These results confirm that βcar_{At} is a ureidohydrolase and mainly a β-ureidopropionase or NCβAA. *P. putida* NCβAA was the first member shown to hydrolyze *N*-carbamoyl-α-, -β-, and -γ-amino acids (29), but to the best of our knowledge, βcar_{At} is the first enzyme to hydrolyze *N*-carbamoyl-δ-amino acids as well.

βcar_{At} was able to produce several monosubstituted β^2- and

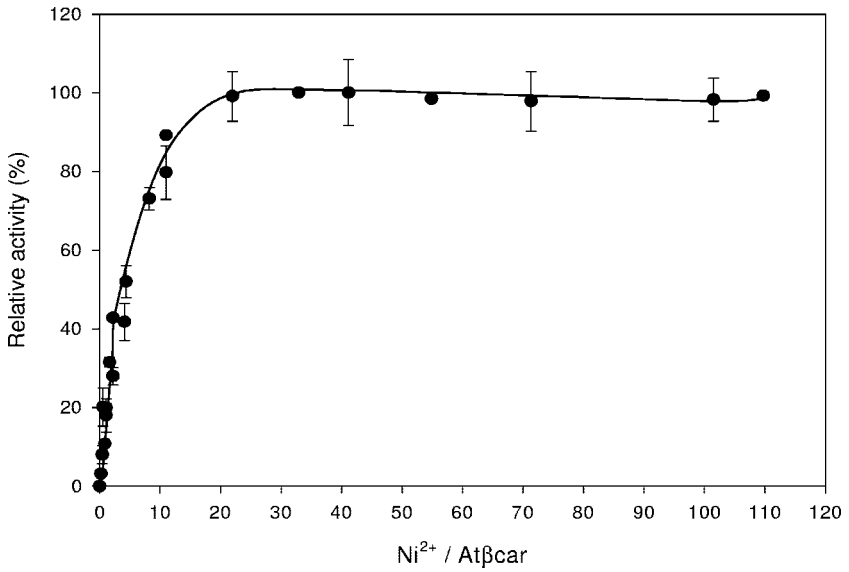


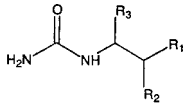
FIG. 4. Effect of Ni^{2+} metal ion on βcar_{At} activity. The reaction mixture contained 100 mM sodium phosphate buffer (pH 8.0), 25 mM *N*-carbamoyl-β-alanine substrate, 0.03 μM of βcar_{At}, and several different Ni^{2+} concentrations. Data are the means of three independent experiments and are shown as percentages of the enzyme activity at different $Ni^{2+}/\beta car_{At}$ ratios.

β^3-amino acids, showing better catalytic efficiency (k_{cat}/K_m) for the production of the first class (Table 2). For example, the k_{cat}/K_m ratio for 2-methyl-3-ureidopropionic acid was 60 times better than that for 3-methyl-3-ureidopropionic acid, decreased drastically with a larger substituent (phenyl group) for β^2-carbamoyl, and was not detected at all for the β^3-counterpart (Table 2). Up to now, β^2-amino acids have not been as readily available as their β^3-counterparts and must be prepared using multistep procedures (17). However, the βcar_{At} enzyme will simplify their synthesis.

Bearing in mind the flaws that any model has, the relative position of the substrate in the model provides an idea of the specificity of βcar_{At} for *N*-carbamoyl-β^2- and -β^3-amino acids. The bulkiness of the *N*-carbamoyl-β^2-amino acid side chains causes a lower affinity for larger groups, and therefore steric clashes might make the largest contribution to affinity loss. A closer inspection of the homology model identifies the side chains of Trp218 (Trp251 in Skβas) and Ala139 (Ser167 in Skβas) located where the substrate substituent would fit, thus explaining the loss of affinity due to the steric effect (Fig. 6). It is worth noting that the presence of an amino group instead of a methyl group in the substrate increased the affinity approximately 100 times, thus showing that some interactions caused by the presence of the amino group favor binding. However, this fact cannot be explained by the homology model, as no hydrogen bonding or van der Waals contacts can be inferred.

Hydrolysis of *N*-carbamoyl-β^3-amino acids was achieved with a methyl group in this position, but not with phenyl or isopropyl moieties. Visual inspection of the βcar_{At} model suggests that it would be impossible to allocate a substituent larger than a single atom group because of steric clashes, probably with the Glu132 carbonyl group and/or the catalytic Glu131 side chain (Fig. 6). Interestingly, although the affinity for the 3-methyl-substituted substrate was almost the same as that for *N*-carbamoyl-β-alanine (3.44 and 2.14 mM, respectively), the k_{cat} of the enzyme showed a 100-fold decrease for the substituted compound. The presence of the methyl substituent might perturb the catalytic Glu131 carboxyl group environment, thus hindering its proton-shuttling task for activation of the polar-

TABLE 2. Kinetic parameters of βcar_{At} enzyme

Substrate model 

Substrate	R_1	R_2	R_3	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	Product
3-Ureidopropionic acid	-COOH	-H	-H	2.14 ± 0.20	25.71 ± 1.00	12,030.88 ± 1,571.85	β-Alanine
2-Ureidoethanesulfonic acid	$-SO_3H$	-H	-H	10.58 ± 0.82	5.84 ± 0.21	551.86 ± 62.89	Taurine
2-Ureidoethanephosphonic acid	$-PO_3H_2$	-H	-H	20.15 ± 2.37	0.23 ± 0.01	11.56 ± 1.75	Ciliatine
2-Methyl-3-ureidopropionic acid	-COOH	$-CH_3$	-H	6.59 ± 0.55	24.39 ± 1.06	3,695.86 ± 467.83	α-Methyl-β-alanine
2-Amino-3-ureidopropionic acid	-COOH	$-NH_2$	-H	0.07 ± 0.02	0.15 ± 0.01	2,191.58 ± 799.20	α-Amino-β-alanine
2-Phenyl-3-ureidopropionic acid	-COOH	-Phenyl	-H	84.37 ± 23.07	0.13 ± 0.02	1.51 ± 0.66	α-Phenyl-β-alanine
3-Methyl-3-ureidopropionic acid	-COOH	-H	$-CH_3$	3.44 ± 0.76	0.20 ± 0.01	59.66 ± 16.91	β-Homoalanine
3-Phenyl-3-ureidopropionic acid	-COOH	-H	-Phenyl	Not detected	Not detected	Not detected	β-Phenylalanine
3-Isopropyl-3-ureidopropionic acid	-COOH	-H	$-CH(CH_3)_2$	Not detected	Not detected	Not detected	β-Leucine

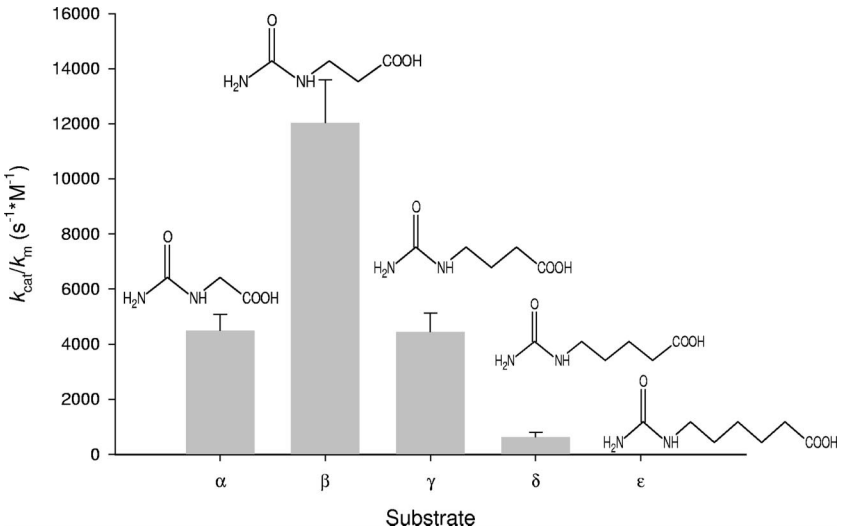


FIG. 5. Comparison of nonsubstituted carbamoyl compounds, precursors of α-, β-, γ-, δ-, and ε-amino acids, as substrates. Bar α, *N*-carbamoyl-α-glycine (ureidoethanoic acid); bar β, *N*-carbamoyl-β-alanine (3-ureidopropanoic acid); bar γ, *N*-carbamoyl-γ-aminobutyric acid (4-ureidobutanoic acid); bar δ, *N*-carbamoyl-δ-aminopentanoic acid (5-ureidopentanoic acid); and bar ε, *N*-carbamoyl-ε-aminohexanoic acid (6-ureidohexanoic acid). Data, shown as k_{cat}/K_m, are the means of three independent experiments.

ized water molecule which bridges the enzyme's binuclear metal center.

Our laboratory is currently evaluating the application of βcar_{At} in the same context as the "hydantoinase process," along with different dihydropyrimidinases. The broad substrate specificity of this enzyme makes it an attractive tool for the production of substituted and nonsubstituted β-amino acids.

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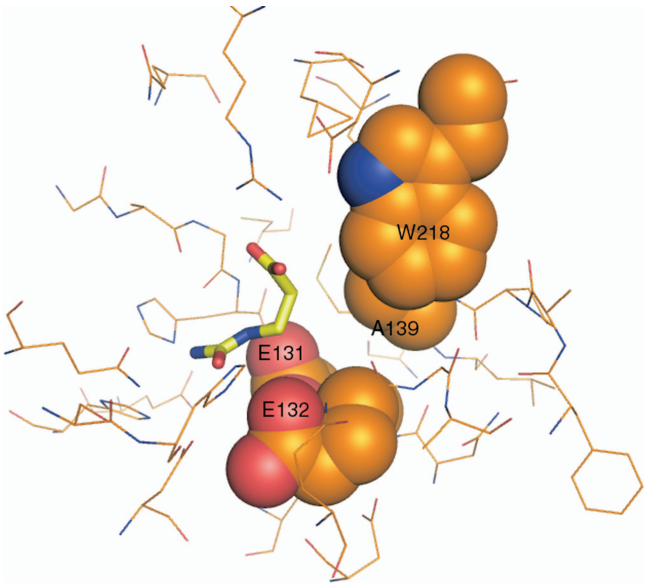

FIG. 6. Modeled βcar_{At} showing the most probable residues involved in steric impediments of 2- and 3-substituted *N*-carbamoyl-β-amino acids.

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