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N-Carbamoyl-β-alanine amidohydrolase from *Agrobacterium tumefaciens* C58: A promiscuous enzyme for the production of amino acids☆

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ABSTRACT

The availability of enzymes with a high promiscuity/specificity relationship permits the hydrolysis of several substrates with a view to obtaining a certain product or using one enzyme for several productive lines. *N*-Carbamoyl-β-alanine amidohydrolase from *Agrobacterium tumefaciens* (Atβcar) has shown high versatility to hydrolyze different *N*-carbamoyl-, *N*-acetyl- and *N*-formyl-amino acids to produce different α, β, γ and δ amino acids. We have calculated the promiscuity index for the enzyme, obtaining a value of 0.54, which indicates that it is a modestly promiscuous enzyme. Atβcar presented the highest probability of hydrolysis for *N*-carbamoyl-amino acids, being the enzyme more efficient for the production of α-amino acids. We have also demonstrated by mutagenesis, modelling, kinetic and binding experiments that W218 and A359 indirectly influence the plasticity of the enzyme due to interaction with the environment of R291, the key residue for catalytic activity.

1. Introduction

N-Carbamoyl-β-alanine amidohydrolase (βcar, E.C. 3.5.1.6) catalyzes the last reaction of pyrimidine reductive pathway, which is composed of three enzymatic steps (Fig. 1). Firstly, dihydropyrimidine dehydrogenase (DPD, EC 1.3.1.1/EC 1.3.1.2) reduces the pyrimidine bases uracil and thymine, yielding dihydrouracil and dihydrothymine, respectively. In a second step, dihydropyrimidinase (DHPase, EC 3.5.2.2) converts the dihydropyrimidines to β-ureidopropionic and β-ureidoisobutyric acid. The third reaction is the irreversible hydrolization of the *N*-carbamoylated-β-amino acids by β-ureidopropionase (EC 3.5.1.6) to β-alanine or β-aminoisobutyric acids, CO_2 and NH_3.

The βcar enzyme from *Agrobacterium tumefaciens* C58 (Atβcar) has previously shown its capacity to hydrolyze *N*-carbamoyl compounds to α-, β-, γ-, and δ-amino acids, making it an attractive tool for the production of several interesting compounds [1]. The enzyme's capacity to metabolize structurally distinct substrates or convert a single substrate to multiple products has been of increasing interest to researchers due to its many possible applications. This condition has been defined as promiscuity, and it has been exploited in numerous synthetic applications, from the laboratory to industrial scale [2,3]. Promiscuity is a very broad term which can be broken down into conditions promiscuity, substrate promiscuity and catalytic promiscuity. Conditions promiscuity is shown by enzymes whose catalytic activity depends on the reaction conditions; substrate promiscuity refers to enzymes with relaxed or broad substrate specificity; and catalytic promiscuity refers to enzymes that can catalyze different chemical transformations, and it can be either accidental (a side reaction by the wild-type enzyme) or induced (mutated enzyme) [3]. Substrate promiscuity is a feature of ancestral enzymes [4], because it was necessary to perform multiple functions to maintain primary organisms; the vestiges of these processes are maintained until the present day.

Thus, the promiscuity of Atβcar is interesting in the context of the "Hydantoinase Process", because Atβcar can decarbamoylate the products of the hydrolysis of 5- or 6-substituted dihydrouracils carried out by dihydropyrimidinases [5]. This enzymatic tandem opens the versatility of the "Hydantoinase Process" to the production of more pharmacological compounds than those described to date [6]. It is then necessary to study the catalytic versatility and limitations of Atβcar in order to open up new possibilities. In this work we have continued the previous studies on the promiscuity of the enzyme [1] by assaying with other different substrates, and we have calculated its promiscuity index. This index was developed by Nath and Atkins [2] based on the rate of product formation and the K_m. According to their definition, an enzyme is more promiscuous when it is able to metabolize two chemically dissimilar substrates

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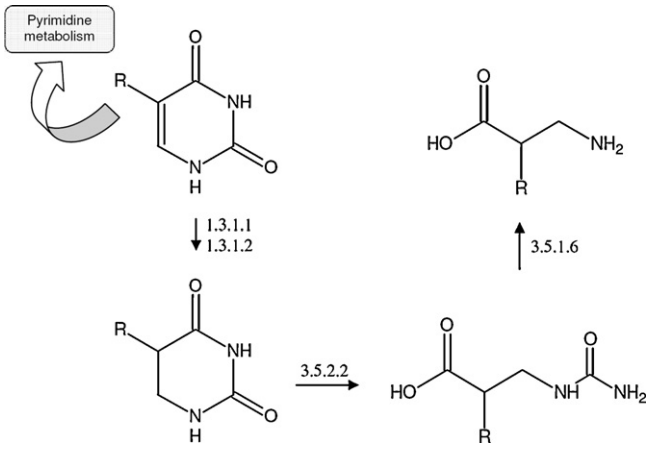

Fig. 1. Pyrimidine reductive pathway where the *N*-carbamoyl-β-alanine amidohydrolase is the enzyme responsible of the hydrolysis from *N*-carbamoyl-β-alanine to β-alanine.

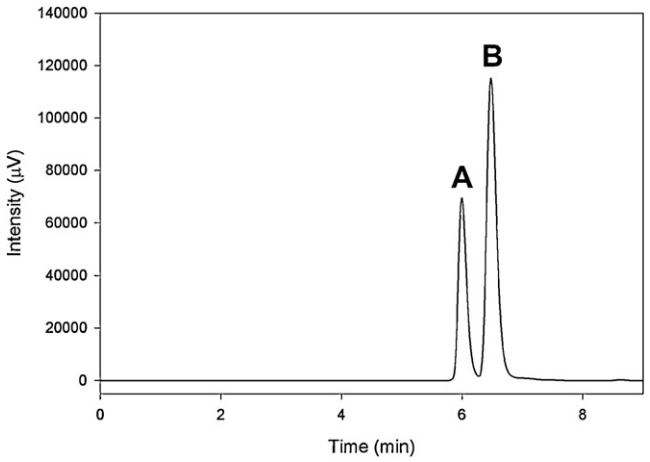

Fig. 2. HPLC separation of ornithine (A) and *N*-carbamoyl-ornithine (B). Conditions: HPLC Finnigan Spectra System equipped with a Hypersil-amino acid C18 column (4.6 mm × 250 mm; Thermo); solvent system NaH_2PO_4 (20 mM, pH 4.5); flow-rate from 0.2 mL min^{-1} and measured at 200 nm.

at the same rates of product formation as it metabolizes two similar ones. Simultaneously, based on the model of the enzyme [1,7], we have mutated some amino acids in the environment of the catalytic centre, to evaluate their implication in the enzymatic activity and their effect on different substrates. We have also, for the first time, checked the enantioselectivity of a *N*-carbamoyl-β-alanine amidohydrolase toward a substrate different than a *N*-carbamoyl-β-amino acid.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were of analytical grade, and were used without further purification. TALON™ metal affinity resin was purchased from Clontech Laboratories, Inc. *N*-carbamoyl-amino acids were synthesized according to the methods described in the literature [8]. *N*-carbamoyl-D-ornithine (also called D-citrulline) was from Bachem, *N*-carbamoyl-L-ornithine (also called L-citrulline), *N*-acetyl-L-methionine and *N*-formyl-L-methionine were from Sigma, *N*-acetyl-L-alanine from Avocado and *N*-acetyl-β-alanine form Panreac.

2.2. Site-directed mutagenesis

Mutagenesis (A139G, W218F, W218H, W218A and A359G) was performed using QuikChange II Site-directed mutagenesis kit from Stratagene following the manufacturer's protocol. For the mutations, the C-terminal His-tagged expression plasmid pAMG4 harbouring the wild type Atβcar gene was used as template [1]. Mutations were confirmed by using the dye dideoxy nucleotide sequencing method in an ABI 377 DNA Sequencer (Applied Biosytems).

2.3. Purification of Atβcar and mutants

Procedures for the purification of Atβcar are described in detail elsewhere [1,7]. Briefly, the recombinant enzyme was overproduced in *Escherichia coli*, including a His6-tag and purified using TALON™ metal affinity resin (CLONTECH Laboratories, Inc.). The apoenzyme form of the enzyme was prepared by incubating 25–50 μM of purified Atβcar with 10 mM of 8-hydroxyquinoline-5-sulphonic acid (HQSA) at 4 °C overnight. At this step, the protein was concentrated with an Amicon ultrafiltration YM-30 device, and HQSA was removed by Gel Filtration on a Superdex-200 column (GE Healthcare), using 100 mM sodium phosphate buffer (pH 8.0), on a Bio-Rad BioLogic Duo Flow FPLC system. Additional dialysis was carried out at 12-h intervals, all at 4 °C, with 100 mM sodium phosphate buffer (pH 8.0), and the enzyme was stored at 4 °C, until use. As Atβcar activity required the presence of divalent metal ions such as Ni^{2+} [1], holoenzyme preparation was achieved adding 0.5 mM of $NiCl_2$ to 10–25 μM apoenzyme, and incubating it for 1 h, after which activity measurement was carried out. This holoenzyme form of Atβcar was used in all the experiments, except where otherwise indicated. W218F, W218H, W218A and A359G Atβcar mutants were purified following the same protocol.

2.4. Enzyme assay

Enzymatic reactions were carried out with purified Atβcar at final concentrations from 0.03 μM to 5 μM, with each substrate dissolved in 100 mM sodium phosphate buffer (pH 8.0) at a final volume of 500 μl. 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 supernatants were analyzed by high-performance liquid chromatography (HPLC) (Finnigan Spectra System HPLC system; Thermo, Madrid, Spain) with a Hypersil-amino acid C18 column (4.6 mm × 250 mm; Thermo). The mobile phase for the detection of β-alanine, glycine, alanine, γ-amino butyric acid (GABA), γ-amino-β-hydroxy butyric acid (GABOB), homoisoserine and ornithine was NaH_2PO_4 (20 mM, pH 4.5), pumped at a flow rate from 0.2 to 0.75 ml min^{-1} depending of the compound and measured at 200 nm (Fig. 2). For the remaining amino acids, the mobile phase 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} and measured at 200 nm.

The separation of D- and L-forms of α-methyl-β-alanine and GABOB was made in the HPLC system (LC2000Plus HPLC System, Jasco, Madrid, Spain) equipped with a Chirobiotic T column (4.6 mm × 250 mm, Sigma–Aldrich, Madrid, Spain). The mobile phase for separation of D- and L-α-methyl-β-alanine was 20% triethylammonium acetate (TEAA) 0.1% pH 4.1 and 80% methanol pumped at a flow rate of 0.4 ml min^{-1} [9], and for separation of D- and L-GABOB was methanol 100% at 1 ml min^{-1}. All compounds were measured at 200 nm.

2.5. Circular dichroism experiments

2.5.1. Secondary structure determination

The secondary structures of mutants W218F, W218H, W218A and A359G of Atβcar were compared with the native protein using Far-UV and Near-UV circular dichroism (CD) spectra, recorded with a Jasco J850 CD spectrometer in the Laboratory of Dr. J.L. Neira. The protein concentrations, with and without cation, were adjusted to give a final concentration of 2 μM at 280 nm in 100 mM phosphate sodium buffer pH 8. CD measurements were taken at room temperature using a 0.1 and 0.5 path length cuvette for Far-UV or Near-UV, respectively. Spectra were acquired from 340 to 250 nm (Near-UV) and 250 to 190 (Far-UV) at a rate of 50 nm/min. For each protein, a baseline scan (buffer) was subtracted from the average of six scans to give the final averaged scan.

2.5.2. Thermal denaturation

Circular dichroism spectra were measured in 100 mM sodium phosphate, with and without 0.5 mM $NiCl_2$ at pH 8 using a Jasco J850 CD spectrometer equipped with a JASCO PTC-423S/15 Peltier accessory. All measurements were carried out at a protein concentration of 2 μM in a 1 mm cuvette. Thermal denaturation measurements were monitored by measuring the changes in helix α at 222 nm. Denaturation data were collected at a scan rate of 0.2 °C/min and the temperature was increased from 25 °C to 95 °C.

The thermal transition of native β-carbamoylase and of mutants W218F and A359G was analysed using a two-state model. The spectral parameters were fitted directly in the following equation by non-linear least square analysis:

$$S_{obs} = \frac{S_N + S_U \exp(-\Delta H_{VH}/R(1/T - 1/T_m))}{1 + \exp(-\Delta H_{VH}/R(1/T - 1/T_m))}$$

where S_{obs} is the observed spectral parameter, and $S_N = A_N + B_{NT}$ and $S_U = A_U + B_{UT}$ refer to the linear dependence of the native (N) and unfolded (U) states, which have the slopes B_N and B_U, respectively. ΔH_{VH} is the apparent change in van't Hoff enthalpy, R is the universal gas constant, T is the temperature in Kelvin and T_m is the melting temperature or the transition midpoint at which 50% of the protein is unfolded.

2.6. Index of substrate promiscuity

The catalytic efficiency toward various substrates was defined as

$$e = \frac{k_{cat}}{K_m} \quad (1)$$

where k_{cat} is the rate at which product is generated by an enzyme under saturating substrate concentrations. K_m is the Michaelis constant and represents the concentration of substrate that yields a half-maximal rate.

When N substrates are assayed, each with an associated catalytic efficiency e_i, p_i is defined as:

$$p_i = \frac{e_i}{\sum_{i=1}^{N} e_i} \quad (2)$$

where p_i is the probability that the ith substrate will be the first to metabolize when an enzyme is simultaneously exposed to equal, low concentrations of all N substrates. The promiscuity index I is defined as:

$$I = -\frac{1}{\log N} \sum_{i=1}^{N} \frac{e_i}{\sum_{j=1}^{N} e_j} \log \frac{e_i}{\sum_{j=1}^{N} e_j} \quad (3)$$

According to Nath and Atkins [2], a value of $I = 1$ means that the enzyme is perfectly promiscuous and all the substrates in the set are equally well-metabolized. A value of $I = 0$ denotes that the enzyme only turns over a single substrate, and the enzyme can be described as perfectly specific.

2.7. Isothermal titration calorimetry

Titrations were performed using the MCS high-sensitive microcalorimeter manufactured by Microcal Inc. (Microcal, Northampton, MA, USA), which has been described elsewhere [10,11]. A circulating water bath (Neslab RTE-111) was used to stabilize the temperature. The instrument was allowed to equilibrate overnight. The enzyme was dialyzed extensively against 100 mM sodium phosphate, 0.5 mM $NiCl_2$ at pH 7 prior to all titrations. The ligands were prepared in the final dialysis buffer. The enzyme was loaded into the sample cell of the calorimeter (V = 1.38 ml) using enzyme concentrations from 24.9 to 30.7 μM, while concentrations of inhibitors ranged from 129.6 to 153.4 mM for acetate, from 75.9 to 190.1 mM for propionate, from 161.0 to 214.7 mM for isobutyrate and from 89.3 to 158.7 mM for 2-phenylpropionate.

The system was allowed to equilibrate and a stable baseline was recorded before initiating an automated titration. The titration experiment consisted of 25 injections of 10 μl each into the sample cell, carried out at 4-min intervals and at different temperatures. The sample cell was stirred at 400 rpm. Dilution experiments were performed by identical injections of different substrates into the cell containing only buffer. The thermal effect of protein dilution was negligible in all cases. The peaks of the obtained thermograms were integrated using the ORIGIN software (Microcal, Inc.) supplied with the instrument.

3. Results and discussion

3.1. Substrate promiscuity of Atβcar

The starting point of a biocatalytic process is the availability of enzymes with high activity which permit conversion form suitable and cheap substrates. It is interesting to exploit the relationship between promiscuity and specificity to develop new industrial applications for old enzymes. We have continued our studies on the enzyme Atβcar, whose function in nature is involved in the pyrimidine metabolism pathway (Fig. 1). This enzyme is able to produce α, β, γ and δ amino acids from non-ramified *N*-carbamoyl-amino acids [1], showing greatest catalytic efficiency for *N*-carbamoyl-β-alanine (3-ureidopropanoic acid). To explore the versatility of the enzyme we have broadened the type of substrate to obtain different amino acids (Table 1). The *in vitro* assays sometimes reveal activity other than the physiological function [12], and substrate promiscuity has drawn considerable attention in the recent years due to the renovation of industrial processes.

Different possible precursors for the production of amino acids have been tested; seven precursors for α-amino acids (carbamoyls, acetyls and formyl), all of them showing activity; eight for β-amino acids (carbamoyls and acetyls); three for γ-amino acids (carbamoyls) and three for δ-amino acids (carbamoyl). Using the standard conditions, we determined the kinetic parameters K_m, k_{cat} and k_{cat}/K_m for the above-mentioned precursors (Table 1). The enzyme showed better affinity constant and catalytic efficiency when *N*-carbamoyl-L-methionine was used as substrate. This is in concordance with the previous report on the β-ureidopropionase from *Pseudomonas putida* (Ppuβcar) [13], where the best catalytic efficiency was found for a substrate other than the natural ones. For methionine production, the best affinity constant was obtained when the precursor was *N*-carbamoylated, followed by the *N*-acetyl and *N*-formyl derivatives, the best catalytic efficiency also being obtained for the *N*-carbamoyl derivative. For α- and β-alanine

Table 1
Kinetic parameters of Atβcar for several precursors of α, β, γ and δ amino acids. Racemic mixture of some compounds where used as they are not commercially available nor could be obtained by synthesis. Atβcar showed activity towards *N*-c-Trp, but the kinetic parameters could not be calculated due to the low solubility of the substrate. Activity was not detected for the following compounds: *N*-carbamoyl-β-phenylalanine*, *N*-carbamoyl-β-leucine, *N*-carbamoyl-2-amino-1-ciclohexanocarboxilate, *N*-carbamoyl-β-homomethionine, *N*-4-acetamidobutirate, 4-guanidinobutirate, *N*-carbamoyl-6-aminohexanoate. Data for substrates marked with an asterisk (*) were obtained in a previous work [1]. N.D., non-detected under standard assay conditions.

	Substrate	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m	p_i (%)
α	*N*-Carbamoyl-glycine*	4.75 ± 0.43	21.88 ± 0.76	4.61 ± 0.58	3.21
	N-Acetyl-L-alanine	2.96 ± 0.33	0.89 ± 0.04	0.30 ± 0.05	$2.09e^{-1}$
	N-Acetyl-D-alanine		N.D.		
	N-Carbamoyl-L-alanine	0.26 ± 0.04	6.27 ± 0.28	24.12 ± 4.59	16.79
	N-Carbamoyl-L-phenylalanine	0.89 ± 0.05	5.81 ± 0.11	6.53 ± 0.48	4.55
	N-Carbamoyl-D-phenylalanine		N.D.		
	N-Acetyl-L-methionine	0.84 ± 0.09	0.15 ± 0.00	0.18 ± 0.03	$1.24e^{-1}$
	N-Acetyl-D-methionine		N.D		
	N-Carbamoyl-L-methionine	0.11 ± 0.01	8.64 ± 0.13	78.55 ± 7.81	54.71
	N-Carbamoyl-D-methionine		N.D.		
	N-Formyl-L-methionine	6.01 ± 0.27	23.03 ± 0.35	3.83 ± 0.23	2.67
β	*N*-Acetyl-β-alanine	169.19 ± 7.95	3.93 ± 0.10	0.02 ± 0.00	$1.52e^{-2}$
	N-Carbamoyl-β-alanine*	2.14 ± 0.19	25.71 ± 1.01	12.01 ± 1.22	8.37
	N-Carbamoyl-β-homoalanine*	3.44 ± 0.76	0.21 ± 0.01	0.06 ± 0.02	$4.25e^{-2}$
	N-Carbamoyl-α-amino-β-alanine*	0.07 ± 0.02	0.15 ± 0.01	2.14 ± 0.82	1.49
	N-Carbamoyl-α-methyl-β-alanine*	6.59 ± 0.55	24.39 ± 1.07	3.70 ± 0.47	2.58
	N-Carbamoyl-α-phenyl-β-alanine*	84.38 ± 23.07	0.13 ± 0.00	$1.54e^{-3}$ ± 0.00	$1.07e^{-3}$
	N-Carbamoyl-taurine*	10.58 ± 0.82	5.84 ± 0.22	0.55 ± 0.06	$3.85e^{-1}$
	N-Carbamoyl-ciliatine*	20.15 ± 2.37	0.23 ± 0.01	0.01 ± 0.00	$7.95e^{-3}$
γ	*N*-Carbamoyl-GABA*	5.17 ± 0.65	24.03 ± 1.02	4.65 ± 0.78	3.24
	N-Carbamoyl-GABOB	6.94 ± 0.86	1.95 ± 0.14	0.28 ± 0.06	$1.96e^{-1}$
	N-Carbamoyl-homoisoserine	6.55 ± 0.66	4.27 ± 0.26	0.65 ± 0.11	$4.54e^{-1}$
δ	*N*-Carbamoyl-5-aminopentanoate*	1.69 ± 0.22	2.28 ± 0.12	1.35 ± 0.25	$9.39e^{-1}$
	N-Carbamoyl-D-ornithine	9.09 ± 2.98	0.01 ± 0.00	$8.82e^{-3}$ ± 0.00	$6.15e^{-3}$
	N-Carbamoyl-L-ornithine	9.47 ± 1.79	0.08 ± 0.01	$1.70e^{-3}$ ± 0.00	$1.19e^{-3}$

the best affinity and catalytic efficiency was also obtained for the *N*-carbamoyl derivative. This capacity to hydrolyze different precursors is an advantage for industrial applications, because it allows choice between the most available or cheapest precursors.

The quantification of substrate promiscuity of Atβcar was evaluated by means of the index *I*, applying Eq. (3). Table 1 shows that the probability "p_i" of using *N*-carbamoyl-L-methionine as substrate is 54.71% (which indicates that the enzyme is modestly promiscuous), followed by *N*-carbamoyl-L-alanine (16.79%) and *N*-carbamoyl-β-alanine (8.37%), the first β precursor. These findings confirm this enzyme as the second β-ureidopropionase with broad activity towards *N*-carbamoyl-α-amino acids [1,13,14], and corroborate the relationships between β-ureidopropionases and L-carbamoylases. Some authors have related promiscuity with conformational diversity, in particular with plasticity, as conformational changes enable it to accommodate different substrates [15]. The mobility of active site loops appears to play a key role in mediating promiscuity. Based on the amino acid sequence comparison, Atβcar belongs to the peptidase family M20/M25/M40, which shows a common architecture formed by two domains: catalytic and lid or dimerization [7,16,17]. The catalytic domain has a large-scale movement after binding, allowing the substrate to reach the bimetallic centre. The plasticity generated by this movement could be one of the reasons for the promiscuity of the enzyme [18].

3.2. Stereospecificity and enantioselectivity of Atβcar

Atβcar has proved to be enantiospecific toward the L-enantiomer of *N*-carbamoyl-α-amino acids, whereas it was not able to hydrolyze the D-enantiomer of different *N*-carbamoyl amino acids (concretely those derived from D-Ala, D-Phe, D-Trp and D-Met). The same enantiospecificity was detected with the homolog Ppuβcar [13]. When using a racemic mixture of *N*-carbamoyl-α-amino acids, only a 50% is converted toward the L-amino acid. However, when the reaction was carried out with racemates of *N*-carbamoyl-β-, γ- and δ-amino acids, we observed a total consumption of the substrates. To evaluate whether Atβcar would be enantioselective towards one of the enantiomers, for β- and γ-amino acids the compounds were measured with a chiral quirobiotic T due to the lack of commercially available enantiomerically pure substrates. The consumption curves provide evidence suggesting that the L-enantiomers were being hydrolyzed faster than the D (Fig. 3). For δ-amino acids we used D- and *N*-carbamoyl-L-ornithine as substrates (Table 1). Interestingly, although the K_m for both enantiomers were almost identical (8.09 and 9.47 mM, respectively), Atβcar hydrolyzed 6–7 times faster the L-enantiomer, proving that Atβcar is also enantioselective, for δ-amino acids precursors.

3.3. Mutant studies

Based on the three-dimensional structures of β-alanine synthase from *Saccharomyces kluyveri* (Skβas, PDB accession no. 2V8H) [16,17], homology models of Atβcar were generated in previous works, showing A139 and W218 residues putatively involved in the activity of the enzyme [1,7]. Another residue, identified as A359, has since been located in the proximity of the catalytic centre. The three residues were mutated to asses their implications in the catalytic activity (A139G, W218F, W218H, W218A and A359G). We were not successful in the purification of A139G, as this mutation caused the absence of overexpression of the enzyme (data not shown). To evaluate the correct folding of W218H, W218A, W218F and A359G, far-UV circular dichroism (CD) spectra were collected. W218F and A359G did not show significant differences in their heir secondary structure compared to the wild type (data not shown). However, W218H and W218A showed a totally different secondary structure, and thus we decided not to continue the experiments with them, as the different secondary structure precluded any hypothesis on the changes arisen from the mutations. Thermal denaturations were followed by CD for the wild-type enzyme and W218F and

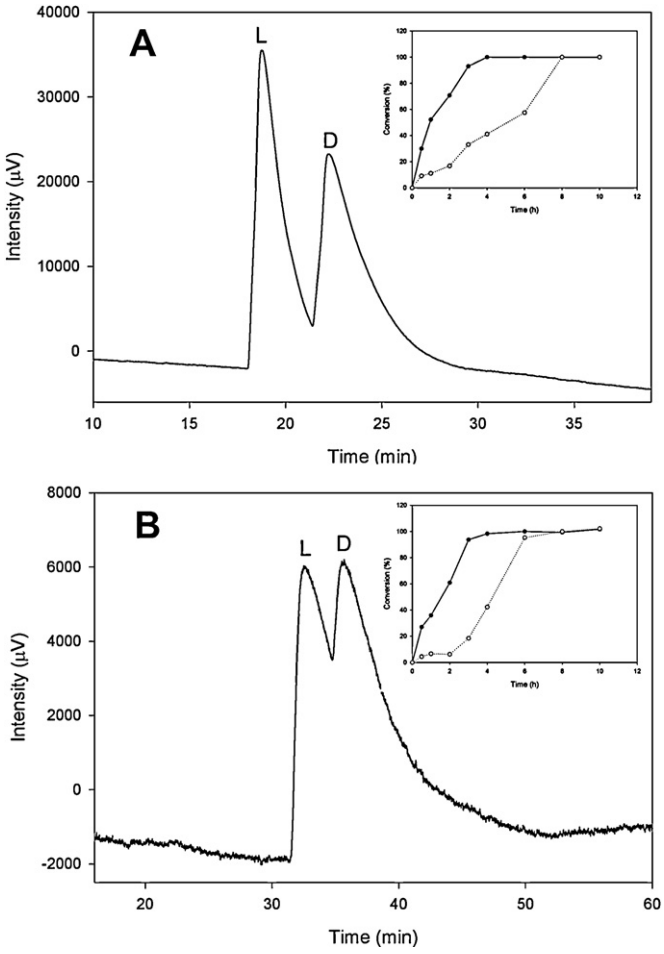

Fig. 3. Chiral-HPLC separation of α-methyl-β-alanine (A) and GABOB (B). The insets show the enzymatic conversion by Atβcar to produce the D-isomer (○) and L-isomer (●). The reactions were performed as described in Section 2. Conditions: HPLC separation of D- and L-forms of α-methyl-β-alanine and GABOB were made in LC2000Plus HPLC System (Jasco, Madrid, Spain), equipped with a Chirobiotic T column (4.6 mm × 250 mm, Sigma–Aldrich, Madrid, Spain). Mobile phase for separation of D- and L-α-methyl-β-alanine: 20% triethylammonium acetate (TEAA) 0.1% pH 4.1 and 80% methanol pumped at a flow rate of 0.4 ml min^{-1}. Mobile phase for separation of D- and L-GABOB: methanol 100% at 1 ml min^{-1}. All compounds were measured at 200 nm.

A359G mutants, whether or not the ion was present. In all cases the thermal denaturations were irreversible, and therefore we did not try to estimate the thermodynamic parameters governing thermal unfolding, but we were able to determine the T_m, as has been described for other proteins showing irreversible thermal denaturations [19–21]. The presence of the Ni^{2+} cation increases the thermal stability of WT and mutated Atβcar by 7–10 K (Table 2). Stabilization of the folding of different metalloenzymes by different cations have been described elsewhere [22, and references therein). On the other hand, the slight differences in the T_m of wild-type and mutated Atβcar (lower than 3 K in all cases, see Table 2), suggest that the mutations carried out do not significantly alter Atβcar folding, allowing us to hypothesize on the role of W218 and A359.

Table 2
Unfolding temperature following Circular Dichroism of the wild-type protein and mutants with and without cation.

PROTEIN	Tm (K)
WT holoenzyme	326.23
WT apoenzyme	319.10
W218F holoenzyme	327.28
W218F apoenzyme	316.90
A359G holoenzyme	329.68
A359G apoenzyme	320.60

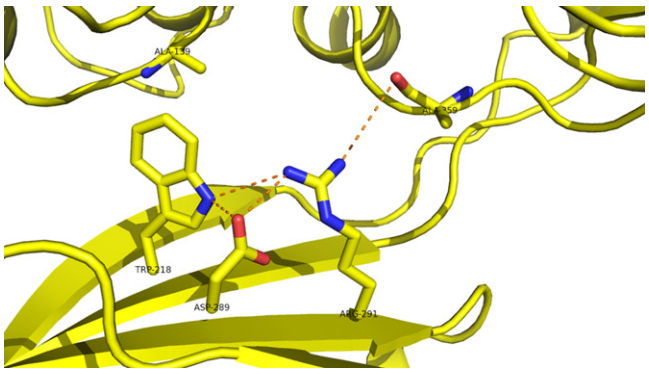

Fig. 4. Modelled structure of Atβcar showing the environment of the mutated residues and their probable interactions.

To evaluate the involvement of these residues in the activity of the enzyme, we used two different strategies: estimation of the kinetic parameters toward two substrates (*N*-carbamoyl-glycine and *N*-carbamoyl-β-alanine), and measurement of the thermodynamic parameters of binding for several inhibitors. While the K_m values of A359G were similar to the ones obtained for the wild-type enzyme (Table 3), W218F showed slightly higher values, although in the same order of magnitude. The k_{cat}, on the other hand, decreased in all cases except for A359G mutant when *N*-carbamoyl-glycine was used as substrate. Taking all together, values for the catalytic efficiency of the enzyme were one order of magnitude lower than for the wild-type enzyme, except for A359G mutant in the presence of *N*-carbamoyl-glycine. These results suggest that W218 is indirectly involved in the enzymatic activity for both kinds of substrates, whereas A359 is only related to the activity towards *N*-carbamoyl-β-amino acid, but not for the shorter *N*-c-α-amino acid.

Studies of protein-inhibitor binding by ITC were made to evaluate the implication of the mutations. Propionate, acetate, isobutyrate and 2-phenylpropionate, inhibitors of Atβcar [7], were selected. For the mutant A359G a decrease of one order of magnitude of the binding constant value was observed for propionate, acetate and isobutyrate, and no binding could be detected for 2-phenylpropionate (Table 4). Based on the modelled structure of Atβcar, the carbonyl group of the previous residue (G358) forming part of the peptidic bond with A359 is at binding distance from the binding residue R291 (Fig. 4). The A359G mutant might increase the flexibility of this zone, destabilizing the hydrogen bond with the key role arginine, and decreasing the affinity of A359G for the inhibitors. This change in the binding constants is caused by a less favourable entropic contribution, accompanied by a more favourable enthalpic contribution. Both enthalpic and entropic changes support the hypothesis of a disruption in the hydrogen bond network of the peptidic carbonyl group. No heat of binding was detected with any of the inhibitors for W218F. Triptophan 218 was already hypothesized to be in the proximity of the carboxylate moiety of D289 of the enzyme [7]. This aspartate residue is also at hydrogen-bond distance from the key binding residue R291. This leads us to postulate that non-detection of binding of W218F towards the inhibitors used might be due to alterations in the interactions of W218-D289-R291 (Fig. 4). This is further supported by the lower K_m calculated for the two different substrates, which are higher than those calculated for the wild-type enzyme (Table 3).

Table 3
Kinetic values obtained for W218F and A359H to evaluate their involvement in the activity of the enzyme. The substrates used were *N*-carbamoyl-glycine and *N*-carbamoyl-β-alanine.

	C-β-alanine			C-glycine		
	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} mM^{-1})	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} mM^{-1})
WT	5.14 ± 0.68	23.40 ± 1.05	4.56 ± 0.81	2.61 ± 0.29	7.49 ± 0.30	2.87 ± 0.433
W218F	9.41 ± 1.00	5.06 ± 0.26	0.54 ± 0.08	5.39 ± 0.48	3.33 ± 0.12	0.62 ± 0.08
A359G	5.02 ± 0.50	4.97 ± 0.17	0.99 ± 0.13	1.99 ± 0.18	6.17 ± 0.17	3.10 ± 0.36

Table 4
Thermodynamic parameters for wild-type protein and mutants calculated by ITC for several inhibitors. N.D. not detected.

Ligand	Protein	K (M^{-1})	ΔH (J/mol)	ΔG (kJ/mol)	ΔS (J/kmol)
Propionate	Wt	5408.0	−3745.7 ± 14.6	−21.3 ± 0.1	58.9 ± 0.4
Acetate		4000.0	−3384.9 ± 24.7	−20.6 ± 0.1	57.8 ± 0.4
Isobutyrate		1899.0	−3259.1 ± 35.1	−18.7 ± 0.1	51.8 ± 0.5
2-Phenylpropionate		100.6	−2303.2 ± 54.7	−17.4 ± 0.1	50.7 ± 0.5
Propionate	A359G	263.4	−11,382.9 ± 68.6	−13.8 ± 0.1	8.1 ± 0.6
Acetate		258.1	−9778.3 ± 79.0	−13.8 ± 0.1	13.5 ± 0.6
Isobutyrate		490.7	−12,979.3 ± 84.0	−15.4 ± 0.1	8.1 ± 0.6
2-Phenylpropionate		N.D.			
Propionate	W218F	N.D.			
Acetate		N.D.			
Isobutyrate		N.D.			
2-Phenylpropionate		N.D.			

4. Conclusions

Atβcar exhibits relaxed substrate specificity, which allows it to hydrolyze several different precursors, which makes it a useful tool for the obtention of α, β, γ and δ amino acids. On the other hand, the preliminary results carried out on this work on the enantioselectivity of the enzyme for substrates different to *N*-substituted-α-amino acids might be a drawback for the production of enantiomerically pure β-, γ- or δ-amino acids (but not L-α-amino acids). The use of proteins with substrate promiscuity allows old enzymes to be applied to industrial processes in novel ways.

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