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Thermodynamic and mutational studies of L-*N*-carbamoylase from *Sinorhizobium meliloti* CECT 4114 catalytic centre

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Abstract

Purified site-directed mutants of *Sinorhizobium meliloti* CECT 4114 L-*N*-carbamoylase (SmLcar) in which Glu132, His230, Asn279 and Arg292 were replaced have been studied by kinetic methods and isothermal titration calorimetry (ITC). The importance of His230, Asn279 and Arg292 residues in the recognition of *N*-carbamoyl-L-α-amino acids has been proved. The role of Glu132 has been confirmed in substrate hydrolysis. ITC has confirmed two Ni atoms per monomer of wild type enzyme, and two equal and independent substrate binding sites (one per monomer). Homology modelling of SmLcar supports the importance of His87, His194, His386, Glu133 and Asp98 in metal binding. A comprehensive reaction mechanism is proposed on the basis of binding experiments measured by ITC, kinetic assays, and homology of the active centre with β-alanine synthase from *Saccharomyces kluyveri* and other enzymes.

Keywords: L-*N*-carbamoylase; Mutagenesis; L-Amino acids; Modelling; Isothermal titration calorimetry

1. Introduction

Carbamoyl amidohydrolases acting on an *N*-carbamoylated α- or β-amino acid substrate have been classified as a group of enzymes based on the comparison of their amino acid sequence, suggesting that they possess a single ancestral gene [1]. This group is made up of three subfamilies: the first comprises bacterial *N*-carbamoyl-L-amino acid amidohydrolases (L-*N*-carbamoylases) [2–6] and eukaryotic β-alanine synthase from *Saccharomyces kluyveri* [1]; the second subfamily includes bacterial and archeal *N*-carbamoyl-D-amino acid amidohydrolases (D-*N*-carbamoylases) [7,8]; and the third one is formed by mammalian and other eukaryotic putative β-alanine synthases [9,10].

L-*N*-carbamoylases are thought to be detoxifying enzymes for the *N*-carbamoyl-L-α-amino acids formed between active carbamoyl groups, such as carbamoyl phosphate, and amino acids present in the cell [1]. Also, L-*N*-carbamoylases from *Pseudomonas* sp. strains BS and ON-4a have been described as involved in the synthesis of L-cysteine in association with an L-2-amino-Δ^2-thiazolin-4-carbonic acid hydrolase (L-ATC hydrolase) [11,12]. However, the most common application of these enzymes is for the production of optically pure natural and non-natural L-amino acids using the "hydantoinase process" [13].

Recently, the L-*N*-carbamoylase enzyme from *Sinorhizobium meliloti* (SmLcar) has been cloned in our laboratory and over-expressed in *E. coli* [14], enabling us to demonstrate that the presence of divalent metal ions such as Ni^{2+}, Mn^{2+} or Co^{2+} are essential for enzyme activity. Likewise, five amino acids corresponding to His87, His194, His386, Glu133 and Asp98 in the SmLcar sequence were found to probably be involved in the metal binding capability of the enzyme. Amino acid sequence alignment of L-*N*-carbamoylases from different sources, together with β-alanine synthase enzyme from *S. kluyveri* (Skβas), could reveal that the residues involved in the proposed catalytic mechanism of the latter are highly conserved in L-*N*-

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