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# Binding studies of hydantoin racemase from *Sinorhizobium meliloti* by calorimetric and fluorescence analysis

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**Abstract**

Hydantoin racemase enzyme together with a stereoselective hydantoinase and a stereospecific D-carbamoylase guarantee the total conversion from D,L-5-monosubstituted hydantoins with a low velocity of racemization, to optically pure D-amino acids. Hydantoin racemase from *Sinorhizobium meliloti* was expressed in *Escherichia coli*. Calorimetric and fluorescence experiments were then carried out to obtain the thermodynamic binding parameters, $\Delta G$, $\Delta H$ and $\Delta S$ for the inhibitors L- and D-5-methylthioethyl-hydantoin. The number of active sites is four per enzyme molecule (one per monomer), and the binding of the inhibitor is entropically and enthalpically favoured under the experimental conditions studied. In order to obtain information about amino acids involved in the active site, four different mutants were developed in which cysteines 76 and 181 were mutated to Alanine and Serine. Their behaviour shows that these cysteines are essential for enzyme activity, but only cysteine 76 affects the binding to these inhibitors.


*Keywords:* Binding; Mutagenesis; Hydantoin racemase; Inhibition; Thermodynamic parameter

## 1. Introduction

Hydantoin racemase catalyzes the transformation of both D- and L-isomers of 5-monosubstituted hydantoins to the corresponding racemic mixtures. This racemization ability has made it the key enzyme for the production, via the enzymatic reaction known as hydantoinase process [1], of optically pure D- and L-amino acids, valuable intermediates for the synthesis of antibiotics, sweeteners, pesticides, pharmaceuticals and biologically active peptides [2,3]. In this cascade of reactions, the chemically synthesized D,L-5-monosubstituted hydantoin ring is hydrolyzed by a stereoselective hydantoinase enzyme. The resulting enantiospecific *N*-carbamoyl α-amino acid is transformed into the corresponding free D- or L-amino acid in a reaction catalyzed by highly enantiospecific *N*-carbamoyl α-amino acid aminohydrolase (*N*-carbamoylase). However, total conversion and 100% optically pure D- or L-amino acid are only obtained when a hydantoin racemase racemizes the remaining non-hydrolyzed 5-monosubstituted hydantoin [4].

Hydantoin racemase enzyme allows the racemization of the 5-monosubstituted hydantoins under physiological conditions where chemical racemization is not favoured. Chemical racemization of the 5-monosubstituted hydantoins proceeds via keto-enol-tautomerism under alkaline conditions [5]. The velocity of racemization is highly dependent on the bulkiness and electronic factors of the substituent in 5-position [6] and is usually a very slow process [7]. High velocities of chemical racemization have only been observed for D,L-phenyl and D,L-5-p-hydroxy-phenylhydantoin because of the resonance stabilization by the 5-substituent. The racemization of all other hydantoins is a very long process [8]. Increased racemization rates are obtained at alkaline pH values and higher temperatures [9].

Since several microorganisms have produced total conversion of optically pure amino acids from racemic mixtures of

*Abbreviations:* ITC, Isothermal titration calorimetry; MTEH, Methylthioethyl hydantoin; SmeHyuA, hydantoin racemase from *Sinorhizobium meliloti* CECT 4114; ASA, Accessible surface area; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; ACES, *N*-(2-Acetamido)-2-aminoethanesulfonic acid

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hydantoins [10–12], hydantoin racemase enzymes from different sources involved in the production of optically pure D- and L-amino acids have been purified and biochemically characterized [13–17]. Likewise, genetic organization and genomic localization of the genes involved in the production of these amino acids have been reported with a hydantoinase, a carbamoylase and a hydantoin racemase gene, together with a putative hydantoin transport protein [18–20]. However, very little is known about the thermodynamic basis of the ligand-binding site.

Our laboratory has recently characterized the hydantoin racemase from *Sinorhizobium meliloti* CECT 4114, and substrates to which the enzyme had no detectable activity (D- and L-5-methylthioethyl-hydantoin, D- and L-MTEH) were found to exhibit competitive inhibition [16]. In the present work, the energetics of the binding of non-substrates (D- and L-MTEH) to hydantoin racemase have been determined by isothermal titration calorimetry (ITC) and fluorescence. Although there are no structural data of the enzyme, a calorimetric analysis of these binding properties should provide information about the thermodynamics for the molecular recognition process for ligands. In the racemization process cysteins may be involved [21,22]. This enzyme has two cysteines in positions 76 and 181. Enzymes involved in the racemization/epimerization of different substrates such as glutamate racemase and diaminopimelate epimerase present two cysteines involved in the catalytic centre [23,24]. These enzymes share a common mechanism that employs two active site cysteine residues in catalysis. In a given reaction direction, one cysteine serves to deprotonate the substrate at the -position and the other reprotonates the resulting carbanionic intermediate on the opposite face, generating the enantiomeric product [24] In order to study the role of hydantoin racemase, Cys76 and Cys181 in racemization we made mutations with Serine and Alanine, obtaining four mutants (C76S, C76A, C181S, C181A) whose activity has been analyzed. We have also studied the binding of these mutants to inhibitors L-MTEH and D-MTEH in order to clarify the role of these amino acids in the inhibition process.

## 2. Materials and methods

### 2.1. Materials

All chemicals were of analytical grade, and were used without further purification. Mutagenesis kits were purchased from Stratagene. TALON™ metal affinity resin was purchased from Clontech Laboratories, Inc. Buffer reagents HEPES, ACES and potassium phosphate were purchased from Sigma Aldrich Quimica (Madrid, Spain). The 5-monosubstituted hydantoins used in this work, D- and L-5-methylthioethyl-hydantoin (D- and L-MTEH) and D-L-5-isobutyl-hydantoin (D- and L-IBH) were synthesized according to the literature [5].

### 2.2. Site-directed mutagenesis

Mutagenesis 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 pSER27 harbouring the wild type *SmehyuA* gene was used as template [16]. The following sense primers were used along with their antisense counterparts, and their substitution sites are underlined:

C76S: 5′-CTATGTCATAGCCTCTTTCGACGACCCG -3′;
C76A: 5′-CTATGTCATAGCCGCTTTCGACGACCCG -3′;
C181S: 5′-GATCGTCCTTGGCTCCGCGGGAATGTCAT -3′;
C181A: 5′-GATCGTCCTTGGCGCCGCGGGAATGTCAT -3′;

Mutations were confirmed by using the dye dideoxy nucleotide sequencing method in an ABI 377 DNA Sequencer (Applied Biosytems).

### 2.3. Expression and purification of the wild type and mutant enzymes

The hydantoin racemase genes were functionally expressed in *E. coli* BL21. A one-step purification procedure of the recombinant hydantoin racemase fused to $His_6$ tag was employed by using immobilized cobalt affinity chromatography followed by proteolytic digestion with factor Xa. Enzyme concentrations were measured according to Lowry et al. [25]. Purity of the proteins was checked by SDS-PAGE by the Laemmli method [26].

### 2.4. Enzyme assays

The enzyme reaction was carried out with each purified enzyme together with 3–5 mM of D- or L-IBH dissolved in 100 mM phosphate buffer (pH 7.5) in a final reaction volume of 200 μl. The mixture was incubated at 35 °C for 15 min and the reaction was stopped by adding 400 μl of 1M HCl. After centrifugation, the supernatant was analyzed by high performance liquid chromatography (HPLC) as previously described [15].

### 2.5. Size exclusion chromatography-HPLC

Size exclusion chromatography-HPLC (SEC-HPLC) analysis was performed in an HPLC System (Breeze HPLC System, Waters, Barcelona) using a Superdex 200 HR 10/30 column (Amersham Biosciences, Barcelona, Spain) previously equilibrated at pH 7.5. Protein elution was monitored at 280 nm. Molecular mass was determined using protein standards: β-amylase (200 kDa); Alcohol dehydrogenase (148 kDa); Ovalbumine (44 kDa) and Ribonuclease A (14 kDa).

### 2.6. Isothermal titration calorimetry (ITC)

Titrations were performed using the MCS high-sensitive microcalorimeter manufactured by Microcal Inc. (Microcal, Northhampton, MA, USA). This instrument has been described elsewhere [27,28]. A circulating water bath was used to stabilize the temperature. The instrument was allowed to equilibrate overnight. The SmeHyuA enzyme was dialyzed extensively against ITC buffers (50 mM HEPES, potassium phosphate or ACES, 10 mM NaCl, 2 mM DTT, pH 7.5) prior to all titrations. The D- and L-MTEH 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 35.30 to 78.68 μM, while concentrations of L- and D-MTEH ranged from 38.46 to 75.00 mM.

The system was allowed to equilibrate and a stable baseline was recorded before initiating an automated titration. The titration experiment consisted of 33 injections of 7 μl each into the sample cell. The injections were carried out at 4-min intervals. The sample cell was stirred at 400 rpm. Dilution experiments were performed by identical injections of both ligands 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.

### 2.7. Fluorescence studies

Fluorescence emission spectra were measured from 20 °C to 35 °C in a Perkin Elmer LS55 spectrofluorimeter for wild type hydantoin racemase, while