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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, ΔG, ΔH and ΔS 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

experiments with mutants were carried out at 25 °C. The temperature of the cuvette holder was controlled with a temperature controlled circulating water bath. Enzymes were excited at 280 nm in order to obtain the intrinsic fluorescence spectra (no tryptophans are present in the enzyme sequence) [16]. The binding of D- and L-MTEH to the enzymes was monitored using the decrease of fluorescence emission at 304 nm. Excitation and emission bandwidths were 5 nm. Fluorescence measurements were corrected for dilution.

The saturation fraction, *Y*, can be expressed as:

$$Y = \frac{K[\text{Ligand}]}{1 + K[\text{Ligand}]} \tag{1}$$

where *K* is the characteristic microscopic association constant and [Ligand] is the free concentration of D- or L-MTEH and can be expressed as

$$[\text{Ligand}] = [\text{Ligand}]_T - nY[\text{Enzyme}] \tag{2}$$

where $[\text{Ligand}]_T$ is the total concentration of inhibitor, *n* is the number of active sites and [Enzyme] is the concentration of hydantoin racemase.

Moreover, the saturation fraction, *Y*, can be calculated as:

$$Y\frac{\Delta F_{\text{corr}}}{\Delta F_{\text{corr}}^{\max}} = \frac{F(\text{Ligand}) - F(0)}{F(\infty) - F(0)} \tag{3}$$

where $F(0)$, $F(\text{Ligand})$ and $F(\infty)$ are the corrected fluorescence intensities for the protein solution without ligand, at a concentration of ligand equal to D- or L-MTEH and at saturating ligand concentration, respectively.

3. Results and discussion

3.1. Molecular mass analysis of the wild type and mutant enzymes

SEC-HPLC experiments have shown that hydantoin racemase is a tetramer (100 kDa) at pH 7.5. The aggregation state of the enzyme does not change after binding of D- or L-MTEH at saturation conditions. Similar experiments were conducted for C76S, C76A, C181S and C181A obtaining the same result of aggregation state (data not shown).

3.2. ITC studies of wild type SmeHyuA

Calorimetry isotherms were fitted to a model of one type of independent sites. Total heat evolved in the titration after *i* titrations, $Q(i)$, is given by

$$Q = \frac{\text{nM}_\text{t}\Delta\text{HV}_0}{2} \times \left[1 + \frac{X_\text{t}}{\text{nM}_\text{t}} + \frac{1}{\text{n}K\text{M}_\text{t}} - \sqrt{\left(1 + \frac{X_\text{t}}{\text{nM}_\text{t}} + \frac{1}{\text{n}K\text{M}_\text{t}}\right)^2 - \frac{4X_\text{t}}{\text{nM}_\text{t}}}\right] \tag{4}$$

where *K* is the binding constant, n the number of sites, V_o the active cell volume, M_t the bulk concentration of macromolecule in V_o, and X_t the bulk concentration of ligand.

The parameter of greatest interest for comparison with the experiment, however, is the heat change from the completion of injection *i*-1 to the completion of injection *i*. The correct expression then for heat released, $\Delta Q(i)$ from the *i*th injection is

$$\Delta Q(i) = Q(i) + \frac{\text{d}V_i}{V_0}\left[\frac{Q(i) - Q(i-1)}{2}\right] - Q(i-l) \tag{5}$$

A typical ITC profile for the binding of L-MTEH to hydantoin racemase is shown in Fig. 1A. Fig. 1B includes a plot of the total heat evolved per mole of the inhibitor versus the relation inhibitor concentration over enzyme concentration. The smooth solid line represents the best fit of the experimental data to a model of four equal and independent sites (one per monomer).

The total heat for the saturation of the enzyme is the sum of the heats of each injection. The binding of a ligand to a protein is usually accompanied by proton release or uptake and the standard enthalpy changes derived from binding isotherms contain contributions from ionization enthalpy of the buffer species. In order to obtain the binding enthalpy of the complex form, the enthalpy contribution due to the protonation–deprotonation of the buffer must be subtracted

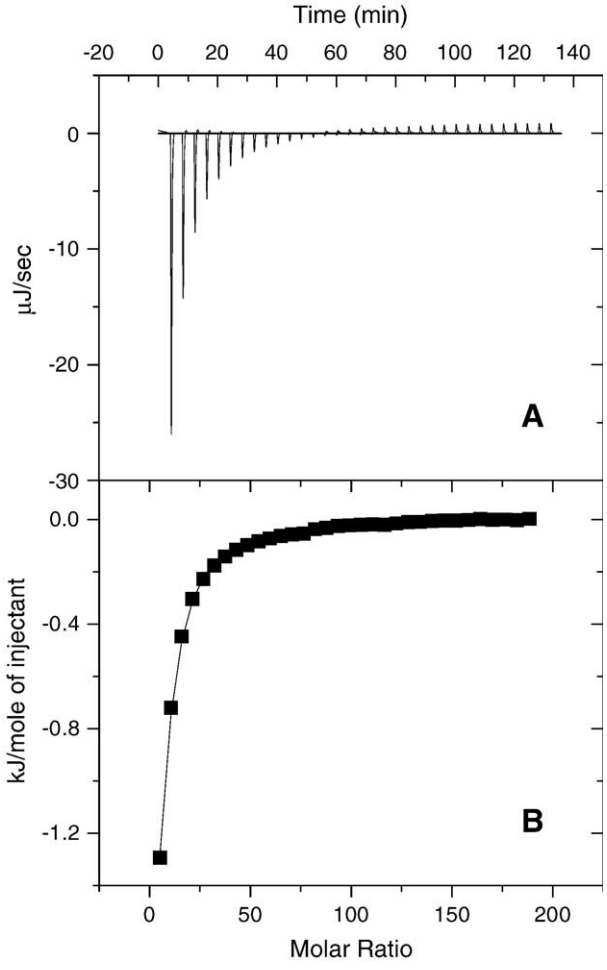

Fig. 1. Calorimetric titration of hydantoin racemase witht L-MTEH in 50 mM HEPES, 10 mM NaCl and 2 mM DTT at pH 7.5 and 35 °C. (A) 75.00-mM stock solution of L-MTEH was injected into a sample cell containing 72.02 µM of hydantoin racemase. (B) Heat evolved per mol of inhibitor injected versus ratio inhibitor concentration/enzyme concentration.

Table 1
Binding enthalpies for D- and L-MTEH, number of protons exchanged and ΔC_p

Inhibitor	Temperature (K)	ΔH_b (J/mol)	n	ΔC_p (J/kmol)
L-MTEH	293	−1641.1±77.3	−0.03	−228.2±17.1
	298	−2538.5±60.6	−0.03	
	303	−3641.6±76.1	−0.04	
	308	−5076.2±71.9	−0.03	
D-MTEH	293	−1195.5±52.7	−0.01	−268.8±21.3
	298	−2089.2±59.8	−0.03	
	303	−3746.5±63.5	−0.02	
	308	−5123.0±62.7	−0.03	

from the enthalpy change observed. To determine the number of protons released or taken up on complex formation, ITC experiments were carried out using three different buffers with different ionization enthalpies at each pH. The data obtained were analyzed using the following relationship:

$$\Delta H_{obs} = \Delta H_b + n \cdot \Delta H_{ioniz} \quad (6)$$

where n is the number of protons exchanged with the buffer media to stabilize the complex and ΔH_{ioniz} is the ionization enthalpy of the buffer.

The results obtained for the binding of D- and L-MTEH to SmeHyuA at pH 7.5 and different temperatures are shown in Table 1. The number of protons exchanged in ligand–enzyme binding is approximately zero, indicating that no protons are liberated or taken up in the association.. The binding of either D- or L-MTEH to SmeHyuA is enthalpically favourable at all temperatures studied. Van der Waals interactions and hydrogen bondings are usually considered to be the major potential sources of negative ΔH values [29–31]. Thus, we suggest that Van der Waals forces and hydrogen bondings play an important role in the interaction between hydantoin racemase and D- and L-MTEH.

Heat capacity changes involved in protein–ligand binding originate from changes in the degree of surface hydration in the free and complex molecules, and to a lesser extent from changes in molecular vibrations [32,33]. In the association of a protein to a ligand a substantial fraction of polar and nonpolar surface is buried, and some semiempirical methods have been developed to calculate ΔC_p from the molecular surface buried in the complex. Murphy et al. have suggested

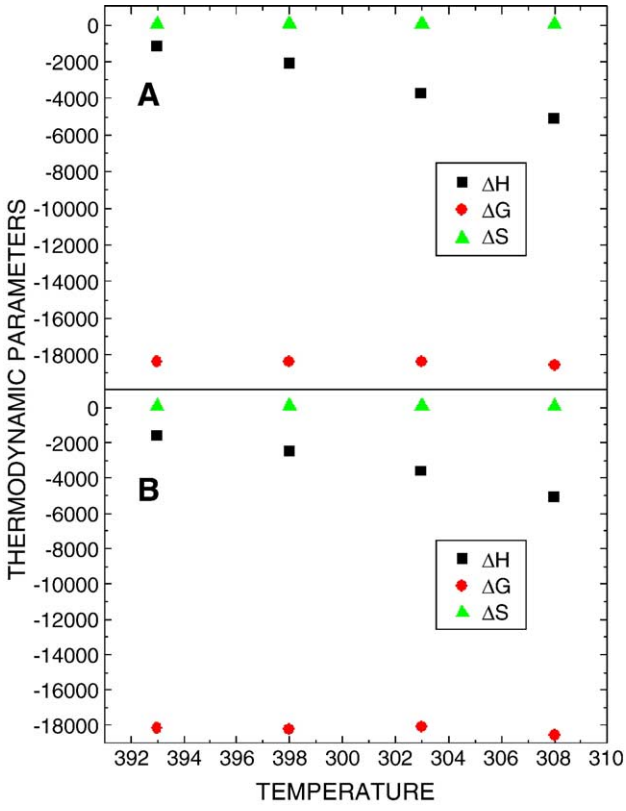

Fig. 2. Temperature dependence of the thermodynamic parameters for the binding of D- (A) and L-MTEH (B) to hydantoin racemase at pH 7.5. The parameters have been corrected for effects of buffer ionization. ΔG and ΔH are expressed in J/mol and ΔS in J/kmol.

the following equations for ΔC_p and $\Delta H60$ (enthalpy change at 60 °C):

$$\Delta C_p = 1.88\Delta ASA_{np} - 1.09\Delta ASA_p \quad (7)$$

$$\Delta H^0_{60} = -35.3\Delta ASA_{np} - 131\Delta ASA_p \quad (8)$$

where ΔC_p, ΔH_{60} and ΔASA are in $J \times K^{-1} \cdot mol^{-1}$, $J \times mol^{-1}$ and $Å^2$ units respectively [32,34]. ΔASA_{np} and ΔASA_p represent the changes in nonpolar and polar areas exposed to the solvent (accessible surface area) that take place upon protein ligand binding. The temperature of 60 °C in the expression is the mean value of the denaturation temperatures

Table 2
Thermodynamic parameters and binding constants determined by ITC and fluorescence for D- and L-MTEH to hydantoin racemase at different temperatures

Inhibitor	T (K)	K (M^{-1}) (ITC)	ΔH_b (J/mol)	ΔG (kJ/mol)	ΔS (J/kmol)	K (M^{-1}) (Fluorescence)
D-MTEH	293	1915.0±78.3	−1195.5±52.7	−18.4±0.1	58.7±0.4	1103.8±72.5
	298	1665.0±67.8	−2089.2±59.8	−18.4±0.1	54.8±0.4	915.6±68.3
	303	1477.0±59.0	−3746.5±63.5	−18.4±0.1	48.5±0.4	825.7±63.8
	308	1308.0±55.7	−5123.0±62.7	−18.6±0.1	43.1±0.5	815.9±55.8
L-MTEH	293	1757.0±72.1	−1641.1±77.3	−18.2±0.1	56.5±0.5	1225.2±58.6
	298	1584.3±62.6	−2538.5±60.6	−18.2±0.1	52.6±0.4	998.9±63.2
	303	1332.0±52.4	−3641.6±76.1	−18.1±0.1	47.7±0.5	975.7±59.8
	308	1427.0±55.7	−5076.2±71.9	−18.6±0.1	43.9±0.5	859.8±53.9

Table 3
Thermodynamic parameters and binding constants determined by ITC and fluorescence for the binding of D- and L-MTEH to hydantoin racemase mutants at 298 K

Enzyme	Inhibitor	K (M^{-1}) (ITC)	ΔH (J/mol)	ΔG (kJ/mol)	ΔS (J/kmol)	K (M^{-1}) (Fluorescence)
C76S	D-MTEH	$6.2\cdot10^3\pm0.1\cdot10^3$	−2317.6±219.9	−21.6±0.1	64.8±1.1	$5.3\cdot10^3\pm0.3\cdot10^3$
C76A		$6.1\cdot10^3\pm0.2\cdot10^3$	−2150.4±158.5	−21.6±0.1	65.2±0.9	$5.0\cdot10^3\pm0.2\cdot10^3$
C181S		$1.4\cdot10^3\pm0.1\cdot10^3$	−2884.2±141.9	−17.9±0.2	50.3±1.1	$1.3\cdot10^3\pm0.2\cdot10^3$
C181A		$1.6\cdot10^3\pm0.1\cdot10^3$	−2547.2±183.0	−18.2±0.2	52.6±1.3	$1.2\cdot10^3\pm0.1\cdot10^3$
C76S	L-MTEH	$5.8\cdot10^4\pm0.6\cdot10^4$	−2980.7±86.5	−27.2±0.3	81.1±1.3	$3.8\cdot10^4\pm0.4\cdot10^4$
C76A		$2.2\cdot10^4\pm0.1\cdot10^4$	−2571.3±58.6	−24.8±0.1	74.7±0.6	$9.8\cdot10^3\pm0.5\cdot10^3$
C181S		$1.8\cdot10^3\pm0.1\cdot10^3$	−3129.8±114.0	−18.6±0.1	51.8±0.7	$1.5\cdot10^3\pm0.3\cdot10^3$
C181A		$1.6\cdot10^3\pm0.1\cdot10^3$	−3080.2±106.5	−18.2±0.2	50.7±1.0	$1.2\cdot10^3\pm0.2\cdot10^3$

of the model proteins used in the analysis. Using ΔH_{60} calculated assuming a constant ΔC_p and the experimental heat capacity change, the changes in accessible surface areas were calculated and the results were 59.9 and 60.2% for ΔASA_{np}, 40.1 and 39.8% for ΔASA_p for D- and L-MTEH, respectively. Changes in apolar and polar solvent-accessible surface areas upon complex formation have been estimated by those relationships mentioned above. On the basis of the X-ray crystallographic data of several proteins, the changes in the water-accessible surface areas of both nonpolar and polar residues on protein folding have been calculated. Such calculations reveal that the ratio $\Delta ASA_{np}/\Delta ASA_p$ varies between 1.2 and 1.7 [32]. This range is comparable with the mean value for the ratio of $\Delta ASA_{np}/\Delta ASA_p$ of ~1.5 (1.51 and 1.49 for D- and L-MTEH respectively), calculated for the interactions described in this study. The application of Murphy's approach [32] to the experimentally determined values indicates that the surface areas buried on complex formation comprise 60% of the nonpolar surface. Therefore, as Spolar and Record [35] indicate, these values, together with a low value of ΔC_p, can be taken as evidence of the "rigid body" interactions, and therefore, no large conformational changes take place as a consequence of the association with these ligands.

Negative ΔC_p values suggest changes in hydrophobic accessible surface areas buried upon ligand binding, with liberation of water molecules from both the protein and the ligand and so a reduction of solvent accessibility to nonpolar surfaces accompanies protein-ligand binding. This suggests that hydrophobic interactions play a role in the binding of the enzyme to the inhibitors.

The entropy change was calculated from the ΔH_b, and the value of ΔG^0 from the microscopic binding constant at each temperature ($\Delta G^0 = -RT\ln K = \Delta H_b - T\Delta S^0$). The values obtained are shown in Table 2. The dependence of thermodynamic parameters on temperature for D- and L-MTEH are shown in Fig. 2. ΔS^0 values are positive at all temperatures studied,

Table 4
Relative activity of the Hydantoin racemase wild type and mutant enzymes

Enzyme	Relative activity (%)	
	D-IBH	L-IBH
Wild type	100	100
C76S	0.41	0.31
C76A	N.D. *	N.D. *
C181S	0.20	0.91
C181A	N.D. *	N.D. *

The relative activity is referred to the wild type enzyme with each substrate. Wild type enzyme activity was 1.94 mM min^{-1} mg^{-1} for D-IBH and 1.27 mM min^{-1} mg^{-1} for L-IBH.
* non detectable.

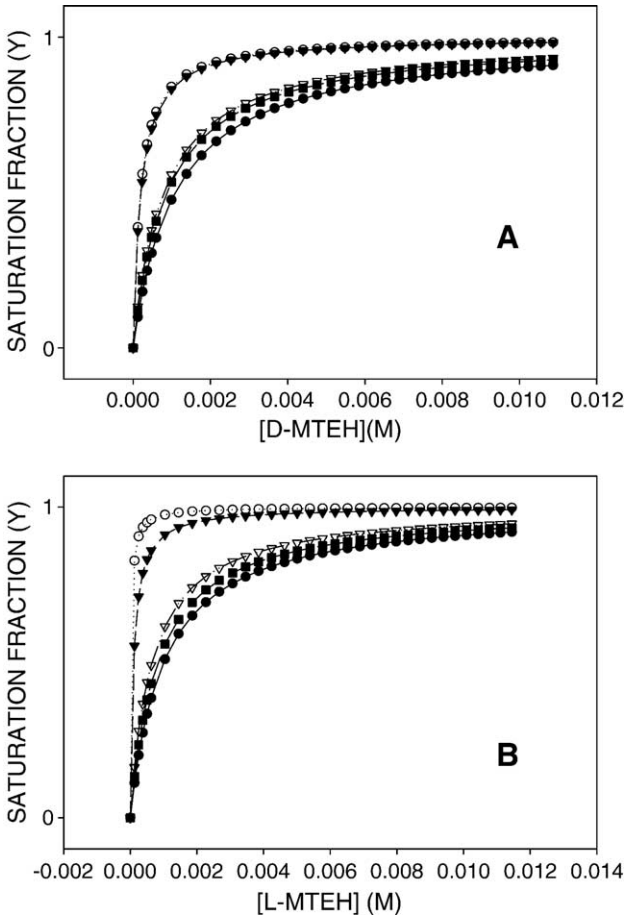

Fig. 3. Fluorescence titration of D-MTEH (A) and L-MTEH (B) binding to wild type hydantoin racemase and mutants. Titration was performed in 50 mM HEPES, 10 mM NaCl at pH 7.5 and 25 °C. Enzyme concentrations were in the range 0.77–0.81 μM and were titrated by addition of 3 and 10 μl of solution at a concentration of 100.03 mM of D-MTEH or 105.33 mM of L-MTEH.

probably due to the liberation of water molecules when hydrophobic surfaces of the enzyme and the inhibitors come into contact upon binding. Therefore, binding is both enthalpically and entropically driven in the temperature range of 20–35 °C. Free energy $\Delta G°$ dependence on temperature is weak due to the enthalpy–entropy compensation, a behaviour commonly reported in ligand–protein interactions [36,37].

3.3. ITC studies of Hydantoin racemase mutants

Enzymes such as glutamate racemase and diaminopimelate epimerase present two cysteines involved in the racemization/epimerisation of their corresponding substrate [23,24]. All the known hydantoin racemases present two conserved cysteines in their amino acid sequences [17], which may be the counterparts of the catalytic residues in the former enzymes and may be involved in the catalytic centre of the protein [14]. In order to study their implication in the enzyme inhibition, hydantoin racemase Cys76 and Cys181 where changed to alanine (C76A and C181A, respectively) and serine (C76S and C181S, respectively). Binding experiments of D- and L-MTEH with the four hydantoin racemase mutants and activity assays were carried out in order to study the role of these amino acids in binding and in catalysis (Table 3). C76S and C76A mutants increase the affinity of the enzyme to L-METH in one order of magnitude compared with hydantoin racemase wild type. C181S and C181A showed no changes in the affinity constant. These data would indicate that the presence of a smaller residue in 76 position makes the active site more accessible to the inhibitor. Results obtained in the binding of D-MTEH to hydantoin racemase mutants were similar to those obtained for L-MTEH, increasing the binding constant 5-fold for C76S and C76A with respect to the wild type, while no changes were observed with C181S and C181A. Thermodynamic parameters of D- and L-MTEH binding to hydantoin racemase mutants show that these processes are enthalpically and entropically driven in the conditions of this study. Activity assays with mutants show a drastic decrease in activity of the enzyme, indicating that both cysteines 76 and 181 are essential for catalysis. When cysteines are mutated to Alanine, the resulting mutated enzymes were devoid of detectable activity. Serine mutants retained a fraction of the activity (Table 4).

3.4. Fluorescence studies of wild type Hydantoin racemase and mutants

The binding of D- and L-MTEH to wild type hydantoin racemase and mutants was observed by intrinsic fluorescence. While the presence of DTT in buffer of ITC experiments is necessary to avoid precipitation of the enzyme at high concentrations, in fluorescence experiments DTT is not added to the buffer due to the quenching effect observed. Concentrations used in fluorescence experiments are very low and precipitation is not present. A decrease in fluorescence as a function of substrate concentration is obtained at different temperatures in the buffer (50 mM HEPES, 10 mM NaCl pH 7.5). Fig. 3 represents the saturation fraction, *Y*, versus concentration of D- or L-MTEH, and the data fit to a model of four equal and non-interacting sites (Fig. 3). The good fit of the experimental curve is evidence of the absence of cooperativity in the binding of D- and L-MTEH to hydantoin racemase in native and mutated state, and yields the binding constant values (K) in agreement with data obtained by ITC (Tables 2 and 3).

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