Site-directed mutagenesis indicates an important role of cysteines 76 and 181 in the catalysis of hydantoin racemase from *Sinorhizobium meliloti*

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Abstract

Hydantoin racemase enzyme plays a crucial role in the reaction cascade known as “hydantoinase process.” In conjunction with a stereoselective hydantoinase and a stereospecific carbamoylase, it allows the total conversion from D,L-5-monosubstituted hydantoins, with a low rate of racemization, to optically pure D- or L-amino acids. Residues Cys76 and Cys181 belonging to hydantoin racemase from *Sinorhizobium meliloti* (SmeHyaA) have been proved to be involved in catalysis. Here, we report biophysical data of SmeHyaA Cys76 and Cys181 to alanine mutants, which point toward a two-base mechanism for the racemization of 5-monosubstituted hydantoins. The secondary and the tertiary structure of the mutants were not significantly affected, as shown by circular dichroism. Calorimetric and fluorescence experiments have shown that Cys76 is responsible for recognition and proton retrieval of D-isomers, while Cys181 is responsible for L-isomer recognition and racemization. This recognition process is further supported by measurements of protein stability followed by chemical denaturation in the presence of the corresponding compound.

Keywords: mutagenesis; hydantoin racemase; reaction mechanism; isothermal titration calorimetry; fluorescence; circular dichroism

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Abbreviations: ITC, isothermal titration calorimetry; CD, circular dichroism; MH, 5-methyl-hydantoin; EH, 5-ethyl-hydantoin; IPH, 5-isopropyl-hydantoin; BH, 5-benzyl-hydantoin; IBH, 5-isobutyl-hydantoin; SmeHyaA, hydantoin racemase from *Sinorhizobium meliloti* CECT 4114; PhoAR, aspartate racemase from *Pyrococcus horikoshii* OT3; GdnHCl, guanidinium hydrochloride; SEC, size exclusion chromatography; RMS, root mean square.

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As biological processes are predominantly stereospecific, little attention has been paid to racemization processes. Racemases are a small group of enzymes which have been biochemically classified as a subgroup of the isomerases (EC 5.1.X.X) (Schnell et al. 2003). Hydantoin racemase enzyme catalyzes the transformation of both D- and L-isomers of 5-monosubstituted hydantoins to the corresponding racemic mixtures. This racemization ability has been useful in the enzymatic reaction known as hydantoinase process, by which optically pure natural and non-natural D- or L-amino acids are obtained (Wilms et al. 2001; Martinez-Rodriguez et al. 2002). These compounds are valuable intermediates for the synthesis

of antibiotics, sweeteners, pesticides, pharmaceuticals, and biologically active peptides (Syldatk et al. 1990; Bommarius et al. 1998). In this procedure a D,L-5-monosubstituted hydantoin is hydrolyzed by a stereoselective hydantoinase enzyme, yielding an *N*-carbamoyl- α -amino acid, which is then transformed into the corresponding free D- or L-amino acid by a highly enantiospecific *N*-carbamoyl- α -amino acid amidohydrolase (*N*-carbamoylase). The remaining nonhydrolyzed 5-monosubstituted hydantoin can undergo a racemization process via keto-enol tautomerism. High velocities of chemical racemization have only been observed for D,L-phenyl and D,L-5-*p*-hydroxy-phenylhydantoin due to the resonance stabilization by the 5-substituent (Lazarus 1990). The racemization of other hydantoins is usually a very slow process (Pietzsch et al. 1992) and is highly dependent on the bulkiness and electronic factors of the substituent in the 5-position, the pH, and the temperature (Syldatk et al. 1992; Pietzsch and Syldatk 2002). Hydantoin racemase enzyme allows the racemization of the 5-monosubstituted hydantoins under physiological conditions where chemical racemization is not favored. Total conversion and 100% optically pure D- or L-amino acids are only obtained when a hydantoin racemase racemises the remaining nonhydrolyzed 5-monosubstituted hydantoin (Martínez-Rodríguez et al. 2002).

Hydantoin racemase enzymes from several sources have been purified and biochemically characterized (Watabe et al. 1992b; Wiese et al. 2000; Las Heras-Vazquez et al. 2003; Martínez-Rodríguez et al. 2004a, b; Suzuki et al. 2005a). Genomic localization and genetic organization of these genes have been reported together with a hydantoinase, a carbamoylase, and a putative hydantoin transport protein (Watabe et al. 1992a; Hils et al. 2001; Wiese et al. 2001; Suzuki et al. 2005b). Sequence homology has shown two highly conserved cysteine residues in the studied hydantoin racemases (Wiese et al. 2000; Martínez-Rodríguez et al. 2004a; Suzuki et al. 2005b), and they have been shown to be involved in the catalytic activity of these enzymes (Andujar-Sanchez et al. 2006). In this work, binding studies of several substrates to C76A and C181A active site mutants of the hydantoin racemase from *Sinorhizobium meliloti* CECT 4114 (SmeHyaA) have been carried out to establish the role of Cys76 and Cys181 in catalytic activity. Based on our previous studies (Andujar-Sanchez et al. 2006), the wild-type and mutant SmeHyaA were a tetramer with a molecular mass of ~ 100 kDa. Although no structural data of the enzyme are available, a homology-based model has been obtained using the structure of aspartate racemase from *Pyrococcus horikoshii* OT3 (PhoAR) (Liu et al. 2002). Together with structural, kinetic, and binding studies, information on the importance of these residues in recognition and racemization of the substrates is provided in this work.

Results

Sequence analysis

SmeHyaA presented a high amino acid sequence similarity with other hydantoin racemases as described elsewhere (Martínez-Rodríguez et al. 2004b). When analyzing SmeHyaA sequence with BLAST, similarity percentages of $>30\%$ were found with putative aspartate/glutamate racemases from different sources. Indeed, hydantoin racemases have been grouped in a protein family together with aspartate racemase, glutamate racemase, and arylmalonate decarboxylase (the so-called family pfam01177) due to putative conserved domains (Marchler-Bauer and Bryant 2004). However, lower similarity percentages ($<10\%$) were found when compared with aspartate or glutamate racemases of well-known activity. Despite the low sequence similarities, two completely conserved cysteines (Fig. 1) have been shown to be involved in the racemization process for glutamate and aspartate racemases (Tanner et al. 1993; Liu et al. 2002), and thus belong to the active site. Other residues have been studied for their implication in substrate binding or in assisting catalytic acid/base for a two-base mechanism in glutamate racemase (Hwang et al. 1999; Glavas and Tanner 2001). However, the sequence comparison of SmeHyaA with these enzymes has not shown conservation of any amino acids at these positions.

Isothermal titration calorimetry (ITC) studies

In order to obtain reliable estimates of the binding affinity of a ligand to a protein by isothermal titration calorimetry (ITC), the product of the binding constant and the concentration of macromolecule binding sites must be between 0.1 and 1000 (Wiseman et al. 1989; Andujar-Sanchez et al. 2004). In the present work, the values of binding constants obtained are $\sim 10^2 \text{ M}^{-1}$. In a recent study, Turnbull and Daranas (2003) reported that binding isotherms using ITC can be determined for low-affinity systems if four criteria are met: (1) a sufficient portion of the binding isotherm is used for analysis, (2) the binding stoichiometry is known, (3) the concentrations of ligand and receptor are known with accuracy, and (4) adequate signal-to-noise is observed during the titration. Binding studies of SmeHyaA wild type and mutants fulfill all four criteria.

ITC experiments were conducted at pH 7.5 to determine the thermodynamic parameters of the binding of hydantoin, D-IPH, L-IPH, D-MH, L-MH, and L-EH to C76A and C181A active site mutants of SmeHyaA. The previously used compound (Martínez-Rodríguez et al. 2004b) D-EH could not be employed because it is not accepted as substrate; furthermore, neither D- nor L-BH and IBH could be used for binding experiments, due to

StaHGR	67	MLVIACNTATA	77	StaHGR	180	IILGCTHYPLL	190
BsubGR	69	MLVIACNTATA	79	BsubGR	181	LILGCTHYPLL	191
LacBGR	69	ALVIACNTATA	79	LacBGR	181	LVVGCTHEPLL	191
PedPGR	68	ALVIACNTATN	78	PedPGR	180	LILGCTHEPLL	190
LacfGR	68	MMVVACNTATA	78	LacfGR	180	LIMGCTHEPLL	190
BreLGR	86	MIVVIACNTASA	96	BreLGR	197	LVVGCTHYPLL	207
AquPGR	65	IIVVACNTASA	75	AquPGR	174	LILGCTHYPLL	184
EscCGR	87	LAVVACNTAST	97	EscCGR	200	VVLGCTHEPLL	210
MicaAR	71	MVIMACNTSSA	81	MicaAR	182	LVYGCTHYRHL	192
BifbAR	80	FIIVLTCHN-TAH	89	BifbAR	200	LMLGCTELSVLL	210
StrTAR	79	FIIVLTCHN-TAH	88	StrTAR	193	VILGCTELSLM	203
DessAR	79	FIIMPCN-TAH	88	DessAR	191	IIAGCTEVSVA	201
PhAspRac	77	FIIMPCN-TAH	86	PhAspRac	190	IIAGCTEVSVV	200
SmeHyuA	71	AYVIACF--DD	79	SmeHyuA	177	IVLGCAGMSSL	187

Figure 1. Alignment of different glutamate (GR) and aspartate racemases (AR) of proven activity and SmeHyuA. The GenBank accession number of each sequence appears in parentheses. The completely conserved cysteines involved in the catalytic mechanism are shown. ShaGR, GR from *Staphylococcus haemolyticus* (P52974); BsuGR, GR from *Bacillus subtilis* (IZUW_A); LbrGR, GR from *Lactobacillus brevis* (BAA06106); PpeGR, GR from *Pediococcus pentosaceus* (ZP_00323817); LfeGR, GR from *Lactobacillus fermentum* (Q03469); BlaGR, GR from *Brevibacterium lactofermentum* (BAA78374); ApyGR, GR from *Aquifex pyrophilus* (1B74_A); EcoGR, GR from *Escherichia coli* (P22634); MaeAR, AR from *Microcystis aeruginosa* (CAD70220); BbiAR, AR from *Bifidobacterium bifidum* (BAD82810); SthAR, AR from *Streptococcus thermophilus* (CAA43598); DspAR, AR from *Desulfurococcus* sp. (D84067); PhoAR, AR from *Pyrococcus horikoshii* (1JFL_A); SmeHyuA, hydantoin racemase from *Sinorhizobium meliloti* (AAQ93382).

the lower solubility of these compounds. Calorimetry isotherms were fitted to a model of one type of independent sites. Microscopic binding constants were obtained from the best fit of the experimental data to a model of four equal and independent sites (one per monomer) (Table 1). No binding was detected for L-IPH, L-EH, or L-MH with the C181A mutant; further, no binding to D-IPH or D-MH was observed with the C76A mutant.

Positive values of ΔH_b were obtained for the binding of L-IPH to C76A and D-IPH to C181A (Table 1). Under experimental conditions, therefore, binding of mutants of hydantoin racemase to D- and L-IPH is endothermic. For hydantoin, D- and L-MH, and L-EH binding enthalpy is negative (Table 1).

Binding fluorescence studies of substrates to C76A and C181A active site mutants of SmeHyuA

The binding of D- and L-IPH to the C76A and C181A active site mutants of hydantoin racemase was observed by intrinsic fluorescence. A decrease in fluorescence as a function of substrate concentration was obtained. Figure 2 represents the saturation fraction, Y (Equation 3), versus concentration of D-IPH, L-IPH, and L-EH, and the fitting to a model of four equal and noninteracting sites. The fits of the experimental data indicate the absence of cooperativity in the binding of D- and L-IPH to C76A and C181A mutants of SmeHyuA and yield the binding constant values (K) showed in Table 1, which is arranged based on the types of mutants and enantiomeric compounds.

Table 1. Binding thermodynamic parameters determined by ITC and fluorescence of different substrates to C76A and C181A mutants of SmeHyuA at 25°C

Substrate	Enzyme	$K(M^{-1})$ (ITC)	$K(M^{-1})$ (Fluorescence)	ΔH_b (kcal/mol)
Hydantoin	C76A	78.04 ± 8.74	Nondetected	-1355.03 ± 24.41
Hydantoin	C181A	77.17 ± 7.66	Nondetected	-1933.14 ± 28.53
L-MH	C76A	107.6 ± 13.12	Nondetected	-375.53 ± 9.62
L-MH	C181A	No binding	Nondetected	—
D-MH	C76A	No binding	Nondetected	—
D-MH	C181A	58.52 ± 9.53	Nondetected	-326.14 ± 14.12
L-EH	C76A	455.40 ± 37.68	362.61 ± 23.56	-166.74 ± 7.76
L-EH	C181A	No binding	No binding	—
L-IPH	C76A	186.01 ± 21.69	234.76 ± 49.52	319.12 ± 23.06
L-IPH	C181A	No binding	No binding	—
D-IPH	C76A	No binding	No binding	—
D-IPH	C181A	173.30 ± 17.05	152.82 ± 32.05	461.11 ± 27.91

Figure 2. Binding titration of L- and D-IPH to C76A and C181A mutants of hydantoin racemase and L-EH binding to C76A mutant. Fluorescence titration was performed in 50 mM potassium phosphate, 10 mM NaCl (pH 7.5) at 25°C. Enzyme concentrations were in the range 0.8–0.9 μ M. Titrations were carried out by addition of either 3 or 10 μ L of stock solutions at concentrations of 100 mM of D-IPH, 103.5 mM of L-IPH, or 102.3 mM of L-EH. The raw data of L- and D-IPH were similar, and after normalization yielded superimposed Y values in the graph.

Guanidinium hydrochloride (GdnHCl) unfolding of wild type and active site mutants of *SmeHyaA*

The emission fluorescence spectrum of *SmeHyaA* had a maximum at 308 nm; since the protein has no tryptophan residues, the fluorescence spectrum arises from the different contributions of the five tyrosines located at positions 72, 131, 218, 226, and 228. The GdnHCl-denaturation of hydantoin racemase was followed by monitoring the changes in fluorescence at the maximum of the spectrum (the excitation was set at 280 nm). Profiles of fluorescence intensity versus denaturant concentration were obtained for wild type and C76A and C181A *SmeHyaA* mutants.

The data were fitted to a two-state model in which the fluorescence of the folded and unfolded states is dependent on denaturant concentration (Pace 1986; Sinha et al. 2005). Size exclusion chromatography (SEC) experiments at different GdnHCl concentrations showed that the sole significantly populated species in equilibrium during the denaturation process were the tetramer and the unfolded monomer (data not shown). The conformational transition induced by increasing the GdnHCl concentration and monitored by the decrease in fluorescence intensity had a sigmoidal shape (Fig. 3). The values of m , $C_{1/2}$, and ΔG_w are shown in Table 2. Mutants are slightly more stable (although the values are within the experimental uncertainty) than wild-type enzyme (with a difference of 0.3 and 0.4 kcal/mol for C76A and C181A, respectively), whereas C76A bound to L-IPH and C181A binding to D-IPH are stabilized by 4.8 ± 0.3 and 4.7 ± 0.5 kcal/mol, respectively. Stabilization by substrate binding is also indicated by the increase in $C_{1/2}$ values.

As it was observed, C76A was not stabilized in the presence of D-IPH, neither was C181A with L-IPH. These facts support our previous results in Table 1, suggesting that both substrates did not bind to the mutants. We can compare the difference in the values of the free energies in the presence and in the absence of the substrate with those obtained with the so-called free energy of binding (Creighton 1993), defined as: $\Delta G = -RT\ln(K)$, where K is the dissociation constant from fluorescence in Table 1. Both values compare reasonably well for both active site mutants: in the order of 4.6 ± 0.6 kcal/mol (see above), for the difference in the free energies (Table 2), and 3.2 ± 0.4 kcal/mol for the free energy of binding (Table 1). The differences between both values are probably due to the errors in the fitting procedure and the absence of a very long baseline in the mutant proteins. Taken together, these facts suggest that the stabilization observed in the presence of substrate is mainly due to its binding.

Circular dichroism (CD) measurements

Far-UV CD was used in the structural analysis of wild type and active site mutants of *SmeHyaA* as a spectroscopic probe that is sensitive to changes in protein secondary structure (Woody 1995; Kelly and Price 2000). The CD spectrum of the wild-type and mutants

Figure 3. Changes in the intrinsic fluorescence intensity of wild-type and C76A and C181A mutants of *SmeHyaA* as a function of GdnHCl concentration. Intrinsic fluorescence intensity spectra of wild type, C76A, and C181A (0.8–1.0 μ M, expressed as monomer concentration) were obtained after equilibrium had been achieved (24 h) at the indicated denaturant concentrations in 50 mM potassium phosphate, 10 mM NaCl (pH 7.5) at 25°C. Normalized fluorescence intensity (N.F.I.) was calculated using $(F(d) - F_U)/(F_N - F_U)$, where $F(d)$ is the fluorescence value at a concentration, d , of GdnHCl, and F_N and F_U are the values for the native and unfolded *SmeHyaA*, respectively. The lines through the experimental data are the fitting to Equation 6. The inset shows the fluorescence spectra of both mutant enzymes at 0 M GdnHCl (native) and at 5 M GdnHCl (unfolded).

Table 2. Thermodynamic parameters of the GdnHCl-induced denaturation of wild type and C76A and C181A mutants of hydantoin racemase at 25°C

Enzyme	<i>m</i> (kcal/mol M)	<i>C</i> _{1/2} (M)	Δ <i>G</i> _w (kcal/mol)
Wild type	4.99 ± 0.09	1.25 ± 0.03	29.15 ± 0.26
C76A	5.13 ± 0.05	1.27 ± 0.04	29.42 ± 0.27
C181A	5.08 ± 0.06	1.31 ± 0.02	29.56 ± 0.18
C76A with L-IPH	3.82 ± 0.08	2.41 ± 0.03	34.18 ± 0.31
C76A with D-IPH	5.10 ± 0.05	1.28 ± 0.02	29.51 ± 0.17
C181A with D-IPH	3.65 ± 0.08	2.59 ± 0.03	34.43 ± 0.32
C181A with L-IPH	5.03 ± 0.07	1.32 ± 0.05	29.61 ± 0.34

of SmeHyaA at 298 K showed two intense minima at 208 and 222 nm, which are characteristic of proteins with α -helical structure (Woody 1995; Kelly and Price 2000) (Fig. 4). The estimated population of α -helix from Equation 8 for the wild-type protein and mutants is 69%. The similarity of the far-UV CD spectra among the different SmeHyaA variants (Fig. 4) suggests that none of the mutations significantly altered the secondary structure of the protein. However, we cannot rule out, at this structural characterization level, that the removal of the SH group upon Ala mutation might cause a local, but functionally important, structural perturbation at the active site region. It is conceivable that the two active site cysteines might act in a well-orchestrated manner within a local hydrogen-bonded environment, which can enhance the acidity of the cysteine side chains, and which could be altered upon mutations. Further experiments are being carried out in our laboratories to exclude this possibility.

Modeling studies

Even though the overall similarity of SmeHyaA was higher with other racemases of known structure, the automatic modeling server ModWeb used the structure of PhoAR. The program produced a homology-based model, including 209 residues out of a total of 247. The omitted residues belong to the N (five residues) and C (33 residues) termini. The quality of the model, measured as model score, has a value of 0.92, higher than the prespecified cut-off of 0.7 considered as good, with a probability of a correct fold of >95% (Melo et al. 2002). Quality of the models was assessed using PROCHECK and WHATIF programs (see Materials and Methods section). An overall average G factor of -0.09 was recorded (G factor scores should be above -0.5). Ramachandran plot statistics indicated that 92.9% of the main chain dihedral angles are found in the most favorable regions. Ideally, one would hope to have >90% of the residues in these “core” regions. Bearing in mind the flaws of any model, residues Cys76 and Cys181 were found to be located at opposite positions. Interestingly, the model predicts a 50% helical structure, which is similar to that

obtained experimentally by CD (69%), taking into account that at 222 nm aromatic residues also absorb and, then, it could lead to erroneous estimations (Woody 1995; Kelly and Price 2000). The root mean square (RMS) with the C α chain obtained when comparing the catalytic center was 0.21 (with chain A of PhoAR, PDB 1FJLA) and 1.61 Å (with chain A of glutamate racemase from *Aquifex pyrophilus*, ApyGR, PDB 1B73A) (Fig. 5).

Discussion

Hydantoin racemases have been classified in a family (pfam01177) together with aspartate racemase, glutamate racemase, and arylmalonate decarboxylase, due to the presence of conserved domains (Marchler-Bauer and Bryant 2004). Glutamate and aspartate racemase enzymes act through a catalytic mechanism known as “two-base mechanism.” In this reaction, two residues that can either retrieve or donate a proton (usually two cysteines) very often appear located opposite one another (Hwang et al. 1999; Liu et al. 2002). The chirality of the stereocenter of the substrate is changed by the enzyme when the substrate is located between these residues. Apart from the above-mentioned enzymes, others such as *Trypanosoma cruzi* proline racemase (Buschiazzi et al. 2006), *Alcaligenes faecalis* maleate *cis-trans* isomerase (Hatakeyama et al. 2000), or *Haemophilus influenzae* diaminopimelate epimerase (Cirilli et al. 1998) have been found to present two cysteines involved in the racemization of their corresponding substrates, and it has been established that their catalysis occurs by a two-base mechanism.

In a previous work, Cys76 and Cys181 mutants of SmeHyaA showed a marked decrease in catalytic activity (Andujar-Sanchez et al. 2006). Substitution by a residue able to transfer a proton (serine) retained a small fraction of the activity, whereas substitution by an alanine resulted in no detectable activity, thus indicating that the presence of a proton donor group in that position is critical for the catalysis of this enzyme. Similar effects upon

Figure 4. Far-UV CD spectra of wild-type SmeHyaA and its mutants. Molar ellipticity of wild type (open circles), mutants C181A (filled squares), and C76A (open squares) were acquired at 298 K in 0.1 cm pathlength cells. The estimated content of helical secondary structure was 69% for the three protein species.

Figure 5. Superposition of active sites from the different proteins. The active site of SmeHyaA model (CPK) is compared with that of (A) aspartate racemase from *Pyrococcus horikoshii* (1FJLA, orange) and that of (B) glutamate racemase from *Aquifex pyrophilus* (1B73A, purple). The RMS obtained with the magic fit routine of Spdbv program for these residues was 0.21 Å for 1FJLA and 1.61 Å for 1B73A, using only the C α chain.

mutations have been observed in maleate *cis-trans* isomerase (Hatakeyama et al. 2000) and glutamate racemase from *Lactobacillus fermenti* (Glavas and Tanner 2001), other enzymes acting by a two-base mechanism.

For further insight into the role of each residue, the binding affinities of SmeHyaA C76A and C181A active site mutants were evaluated. Binding experiments conducted by fluorescence with C76A mutant and D- and L-IPH, and L-EH have shown that this mutant is unable to bind the D-isomers of the substrates (Table 1). The same experiments carried out with C181A mutant proved that this mutant was not able to bind the L-isomers (Table 1). The nondetectable binding of hydantoin and D- or L-MH by fluorescence may be a result of either the absence of a chain or a shorter chain in the 5-position of the substrate. Changes in the environment of the tyrosine residues responsible for the fluorescence spectrum, therefore, seem to require a bulkier chain.

The binding of the above substrates to both mutants was also measured by ITC. Even though the K values for these substrates are in the range of $\sim 10^2$ M $^{-1}$, according to the criteria of Turnbull and Daranas (2003), we were able to calculate them, with similar results for D-IPH, L-IPH, and L-EH to those found by fluorescence. As shown in Table 1, higher values of ΔH_b were obtained as the size of the chain at the 5-position of the substrate increased. Positive values of enthalpy change are commonly associated with electrostatic and hydrophobic interaction, while van der Waals interactions and hydrogen bonding are the major sources of negative values (Ross and Subramanian 1981; Ysern et al. 1994; Torigoe et al. 1995). Thus, as the size of the lateral chain increases, hydrophobic interactions with residues in the catalytic center are expected to appear. These results

would explain why binding measurements of hydantoin and MH could not be measured by fluorescence, as the lateral chain does not exist or it may not be bulky enough to interact with the residues involved in fluorescence changes. The binding of the nonsubstituted hydantoin to both mutants is clear evidence that the presence of a proton at the 5-position of the substrate, and not the lateral chain, is the critical factor for proper binding.

The addition of D-IPH to C76A or L-IPH to C181A increased the stability of the active site mutants when incubated in the presence of GdnHCl, as is shown by the increase in the values of $C_{1/2}$ and ΔG_w (Table 2). For other substrates (D-IPH with C181A and L-IPH with C76A) the stability is similar to that of the isolated protein, probably due to the lack of substrate binding to the enzyme. Since the structure of the mutant remained unaltered, as shown by SEC-HPLC (Andujar-Sanchez et al. 2006) and CD (Fig. 4), all these experiments suggest that Cys76 is a key residue for the recognition of D-isomers of the 5-monosubstituted hydantoins, whereas Cys181 is indispensable for recognition of L-isomers. The 3D-model obtained (Fig. 5) supports the hypothesis of Cys76 and Cys181 located opposite one another and further confirms the “two-base mechanism” proposed for SmeHyaA. Moreover, this catalytic behavior is inferred from the observations in a previous work with hydantoin racemase from *Arthrobacter aurescens* (Wiese et al. 2000), where reactions followed by NMR in D $_2$ O showed that the solvent isotope is efficiently incorporated into the product enantiomer, but not into the substrate enantiomer, regardless of which enantiomer served as substrate.

The results described in this work suggest two possible alternative interpretations. In the first one, the enzyme could adopt two forms, which likely would involve reorganization of several active site residues and hydrogen bonding patterns, but in any form, there would be one thiol and one thiolate whose roles could be reversed in the other form. Under this view, one form would bind only D-enantiomers, while the other would bind only the L-enantiomers. The active site mutation of cysteines 76 or 181 drives the free enzyme into one of the two forms (Fig. 6). The introduction of a neutral alanine residue into one site of the cysteines seems to cause the other site to assume the thiolate form. In the active site mutant C181A, the residue at position 181 is neutral, and therefore C76 may be most stable in its thiolate form. Since this mutant is shown to bind the L-enantiomer most tightly, it could be expected that C181 is the base that normally deprotonates the L-enantiomer. On the other hand, the results with C76A mutant point toward that C76 is the base that normally deprotonates the D-enantiomers. Similar protein forms have been observed in the mechanism of proline isomerase (Rudnick and Abeles 1975; Belasco et al. 1986; Fisher et al. 1986).

Figure 6. Proposed racemization mechanism of hydantoin racemase using a two-base mechanism. The hypothetical character of the intermediate structure is indicated by the dashed lines surrounding the figure.

In an alternative and simpler model of a two-base mechanism, when a D-isomer of a 5-monosubstituted hydantoin is available, Cys76 (in a thiolate form) would act as a base and retrieve a proton. A plane intermediate of the substrate could be formed, as has been proposed for the chemical racemization of the hydantoins (Lazarus 1990) and for the racemization of D- and L-glutamate by glutamate racemase (Glavas and Tanner 2001). Cys181 would act as an acid inserting a proton in the opposite side of the substrate, thus producing the L-5-monosubstituted hydantoin (Fig. 6). On the other hand, the isomerization of the L-isomer of the substrate would be carried out by the binding to Cys181 and the retrieval of a proton. In this case, this residue would act as the base, and Cys76 would donate a proton to the putative intermediate formed.

Materials and methods

Materials

All chemicals were of analytical grade and were used without further purification. TALON metal affinity resin was purchased from Clontech Laboratories, Inc. Hydantoin was purchased from Sigma. The 5-monosubstituted hydantoins used in this work,

D- and L-5-isopropyl-hydantoin (D- and L-IPH), D- and L-5-methyl-hydantoin (D- and L-MH), and L-5-ethyl-hydantoin (L-EH), were synthesized as described (Martinez-Rodriguez et al. 2006).

Sequence analysis

The sequence of SmeHyaA was aligned and compared with those of glutamate and aspartate racemases with proven activity described in the literature using CLUSTALW program (Jeanmougin et al. 1998), and sequences were manually edited with Microsoft Word 2003.

Expression and purification of the wild-type and mutant enzymes

Site-directed mutagenesis of Cys76 and Cys181 to alanine in the wild-type enzyme SmeHyaA and their purification by a one-step procedure have been previously described (Andujar-Sanchez et al. 2006). An additional gel filtration chromatography step was carried out using a Superdex 200 gel filtration column (Amersham Biosciences) to eliminate any DNA coeluting with the protein. The samples were dialyzed extensively against 50 mM potassium phosphate, 10 mM NaCl (pH 7.5), and stored at -80°C . The protein was further dialyzed in the corresponding buffer according to the technique used (see below). Enzyme concentrations were measured using a variation of the Lowry

method (Rodríguez-Vico et al. 1989). The presence of the His-tag at the C terminus did not modify the enzymatic or folding features of the protein (data not shown).

Isothermal titration calorimetry

Titration experiments were performed using the MCS high-sensitive microcalorimeter manufactured by Microcal Inc., which has been described elsewhere (Wiseman et al. 1989; Andujar-Sanchez et al. 2004). A circulating water bath (Neslab RTE-111) was used to stabilize the temperature. The instrument was allowed to equilibrate overnight. The SmeHyaA enzyme was dialyzed extensively against 50 mM potassium phosphate, 10 mM NaCl, 2 mM DTT, pH 7.5 prior to all titrations. The D- and L-substrates 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 50.2 to 57.3 μ M for the C76A mutant of SmeHyaA, and from 50.6 to 69.3 μ M for the C181A mutant of SmeHyaA, while concentrations of L- and D-substrates ranged from 95.25 to 127.7 mM.

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 μ L each into the sample cell, carried out at 4-min intervals at 25°C. 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.

Fluorescence studies

Determination of binding constants and intrinsic fluorescence

Fluorescence emission spectra were measured at 25°C in a Perkin Elmer LS55 spectrofluorimeter for mutants of hydan-toin racemase in 50 mM potassium phosphate, 10 mM NaCl (pH 7.5). The temperature of the cell holder was controlled with a circulating water bath. Enzymes were excited at 280 nm (0.8 μ M) in order to obtain the intrinsic fluorescence spectra (no tryptophans are present in the enzyme sequence). The binding of D- and L-substrates to the enzymes was monitored by using the decrease in fluorescence emission at 308 nm. Excitation and emission bandwidths were 5 nm. Fluorescence measurements were corrected for dilution.

The saturation fraction, Y , can be expressed as

$$Y = (K \times [\text{Ligand}]) / (1 + K \times [\text{Ligand}]) \quad (1),$$

where K is the characteristic microscopic association constant and $[\text{Ligand}]$ the free concentration of D- or L-substrates and can be expressed as

$$[\text{Ligand}] = [\text{Ligand}]_T - n \times Y \times [\text{Enzyme}] \quad (2),$$

where $[\text{Ligand}]_T$ is the total concentration of substrate, n the number of active sites, and $[\text{Enzyme}]$ the concentration of SmeHyaA.

The saturation fraction, Y , can be calculated as

$$Y = (F(\text{Ligand}) - F(0)) / (F(\infty) - F(0)) \quad (3),$$

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

GdnHCl unfolding experiments

Unfolding studies with GdnHCl as denaturant were performed at pH 7.5 (in 50 mM potassium phosphate, 10 mM NaCl). Protein concentration was 0.8–1.0 μ M (expressed as monomer concentration) and the concentration range of denaturant used was 0–5 M. Denaturant concentrations were determined by measuring the index of refraction with a refractometer (Refracto 30GS, Mettler Toledo) and applying the following equation (Pace 1986):

$$[\text{GdnHCl}] = 57.147 \times (n - n_0) + 38.68 \times (n - n_0)^2 - 91.60 \times (n - n_0)^3 \quad (4),$$

where n is the index of refraction of the buffer at a particular GdnHCl concentration and n_0 the index of refraction of the buffer in absence of GdnHCl.

Equilibrium times for the unfolding transition of wild-type and mutants of SmeHyaA were determined by following changes in intrinsic fluorescence. A 24-h preincubation time was used prior to the fluorescence measurements to ensure that the denaturation equilibrium was reached, as we have observed by variations in the λ_{max} of the spectra. Samples were then incubated at 25°C for 30 min just before measurements.

The profiles of fluorescence intensity versus denaturant concentration were analyzed according to a two-state denaturation model (Sinha et al. 2005). We have assumed that the tetramer and each of the monomeric species contributed equally to the fluorescence, that is, none of the tyrosines was involved at the tetrameric interface. The free energy of denaturation of proteins in the presence of denaturant, ΔG , is linearly related to the concentration of denaturant:

$$\Delta G = \Delta G_w - mC \quad (5)$$

Taking into account an equilibrium between tetramer and monomer, ΔG_w can be calculated from:

$$\Delta G_w = -R \times T \times \ln(0.5 \times [M]^3) + m \times C_{1/2} \quad (6),$$

where $C_{1/2}$ is the concentration of denaturant at which half of the protein is denatured, m the slope of the transition, and $[M]$ the monomer concentration (which was four times the tetramer concentration).

Reversibility

Samples containing wild-type or mutant SmeHyaA enzymes (15 μ M) were incubated for 24 h at 4°C with different GdnHCl concentrations up to 4 M, at which complete denaturation was

observed (see below). Renaturation was started by dilution of these samples with 50 mM potassium phosphate, 10 mM NaCl (pH 7.5) to 0.8 μ M of protein, and the corresponding final GdnHCl concentrations.

The mechanism of chemical denaturation of hydantoin racemase is completely unknown, but high levels of renaturation (~95%) were obtained in samples preincubated in concentrated GdnHCl (data not shown). Chemical denaturation followed by enzyme activity could not be obtained because C76A and C181A mutants of SmeHyaA were completely inactive (Martinez-Rodriguez et al. 2004b).

CD measurements

CD spectra were collected on a J810 spectropolarimeter (Jasco) fitted with a thermostated cell holder and interfaced with a Peltier. The instrument was periodically calibrated with (+) 10-camphorsulphonic acid. All experiments were made in 0.1 cm pathlength cells (Hellma).

Spectra were acquired at a scan speed of 50 nm/min with a response time of 2 sec and averaged over four scans at the desired temperature. Protein concentration was 4–6 μ M in 20 mM potassium phosphate (pH 7.5), 10 mM NaCl. All spectra were corrected by subtracting the corresponding baseline. The mean residue ellipticity, $[\Theta]$, was obtained from the raw ellipticity data, Θ , as:

$$[\Theta] = \Theta / (10 \times l \times c \times N) \quad (7)$$

where l is the cell pathlength (in cm), c is the protein concentration (in M), and N is the number of amino acids (Kelly and Price 2000). The helical content of wild-type SmeHyaA and its mutants was calculated from the $[\Theta]$ at 222 nm (Zurdo et al. 1997), according to

$$f_h = [\Theta]_{222} / [\Theta]_{222}^{\infty} (1 - k/n) \quad (8)$$

where f_h is the helical fraction of the protein, $[\Theta]_{222}$ the observed mean residue ellipticity, $[\Theta]_{222}^{\infty}$ the mean ellipticity for an infinite α -helix at 222 nm ($-34,500 \text{ deg cm}^2 \text{ dmol}^{-1}$), k a wavelength-dependent constant (2.57 at 222 nm), and n is the number of peptide bonds (in SmeHyaA and its mutants this should be 252 per monomer).

Modeling studies

A model of SmeHyaA was obtained by automatic modeling server ModWeb (Pieper et al. 2004), using the structure of aspartate racemase from *Pyrococcus horikoshii* (Liu et al. 2002) (PDB 1FJL). The stereochemical geometry of the final model was validated by PROCHECK and WHATIF programs at the Biotech Validation Suite for Protein Structures homepage (<http://biotech.ebi.ac.uk:8400/>). Manual model building of the structure was performed with Swiss PDB viewer (Spdbv) (Guex and Peitsch 1997) and RASMOL (Sayle and Milner-White 1995). The RMS was obtained with Spdbv program using only the C_{α} chain.

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