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Molecular Cloning, Purification, and Biochemical Characterization of Hydantoin Racemase from the Legume Symbiont *Sinorhizobium meliloti* CECT 4114

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Hydantoin racemase from *Sinorhizobium meliloti* was functionally expressed in *Escherichia coli*. The native form of the enzyme was a homotetramer with a molecular mass of 100 kDa. The optimum temperature and pH for the enzyme were 40°C and 8.5, respectively. The enzyme showed a slight preference for hydantoins with short rather than long aliphatic side chains or those with aromatic rings. Substrates, which showed no detectable activity toward the enzyme, were found to exhibit competitive inhibition.

The enzymatic reaction known as the hydantoinase process (1) is responsible for the production of optically pure D- and L-amino acids, which are valuable intermediates in the synthesis of antibiotics, sweeteners, pesticides, pharmaceuticals, and biologically active peptides (3, 16). The great advantage of this process is that, potentially, any optically pure amino acid can be obtained from a wide spectrum of D,L-5-monosubstituted hydantoins used as substrate, and this method of production is cheaper and less contaminating than the chemoenzymatic process (7). In this cascade of reactions, the chemically synthesized D,L-5-monosubstituted hydantoin ring is first hydrolyzed by a stereoselective hydantoinase enzyme. Further hydrolysis of the resulting enantiospecific *N*-carbamoyl α-amino acid to the corresponding free D- or L-amino acid is catalyzed by highly enantiospecific *N*-carbamoyl α-amino acid aminohydrolase (*N*-carbamoylase). At the same time that the hydantoinase hydrolyzes the enantiospecific 5-monosubstituted hydantoin in the first step, the chemical and/or enzymatic racemization of the remaining nonhydrolyzed 5-monosubstituted hydantoin begins.

Chemical racemization of the 5-monosubstituted hydantoins proceeds via keto-enol-tautomerism under alkaline conditions (18). The velocity of racemization is highly dependent on the bulkiness and electronic factors of the substituent in position 5 (13) and is usually a very slow process (14). High rates of chemical racemization have been observed only for D,L-phenyl and D,L-5-*p*-hydroxy-phenylhydantoin because of the resonance stabilization by the 5 substituent. All other hydantoins take many hours to racemize (9). Increased racemization rates are obtained at alkaline pH values and with rising temperatures (17). Several microorganisms have shown total conversion and production of optically pure amino acids from racemic mixtures of hydantoin with very slow velocity of racemization, suggesting the existence of an enzymatic racemization process of D,L-5-monosubstituted hydantoins at physiological conditions catalyzed by a hydantoin-specific racemase (2, 11, 12).

Genetic organization and genomic localization of the three genes involved in the production of optically pure D- and L-amino acids have been reported previously (6, 19, 22). Likewise, the hydantoin racemase enzyme involved in the production of L-amino acids from *Pseudomonas* sp. strain NS671 (20) and *Arthrobacter aurescens* DSM 3747 (21), and more recently the one involved in the production of D-amino acids from *Agrobacterium tumefaciens* (8, 10), have been purified and biochemically characterized. *Sinorhizobium meliloti* is an α-proteobacterium of the family *Rhizobiaceae*, as is *Agrobacterium tumefaciens*, which forms agronomically important N_2-fixing root nodules in legumes (4). There is no information about the production of optically pure amino acids from racemic 5-monosubstituted hydantoins by *S. meliloti*. However, since the complete sequence of this organism was reported (5), several gene products which are putatively involved in amino acid production have been listed. Here we report the first heterologously expressed hydantoin racemase from *S. meliloti*, its purification, and its biochemical characterization. These properties have been compared with those of hydantoin racemase enzymes from *Arthrobacter aurescens*, *Pseudomonas* sp., and *Agrobacterium tumefaciens*.

Cloning and sequence analysis of *S. meliloti* hydantoin racemase. *S. meliloti* CECT 4114 genomic DNA was extracted as previously described (15), and the gene encoding hydantoin racemase was amplified by PCR. The primers used were designed based on the sequence for GenBank accession number NC003047 (4, 5). The primers were SmRac5 (5′-AGAATTCATGACAGGAAATTCATCATGCATATTCATCTC-3′) and SmXaRac3 (5′-AAGGTACCTTAATGATGATGATGATGATGTCTTCCTTCGATAGCACAAACCTTGTGACC-3′). The SmRac5 primer included a methionine (ATG) at position 1 of the hydantoin racemase gene instead of the original valine (GTG). Additionally, in order to avoid the creation of a fusion protein between the hydantoin racemase gene

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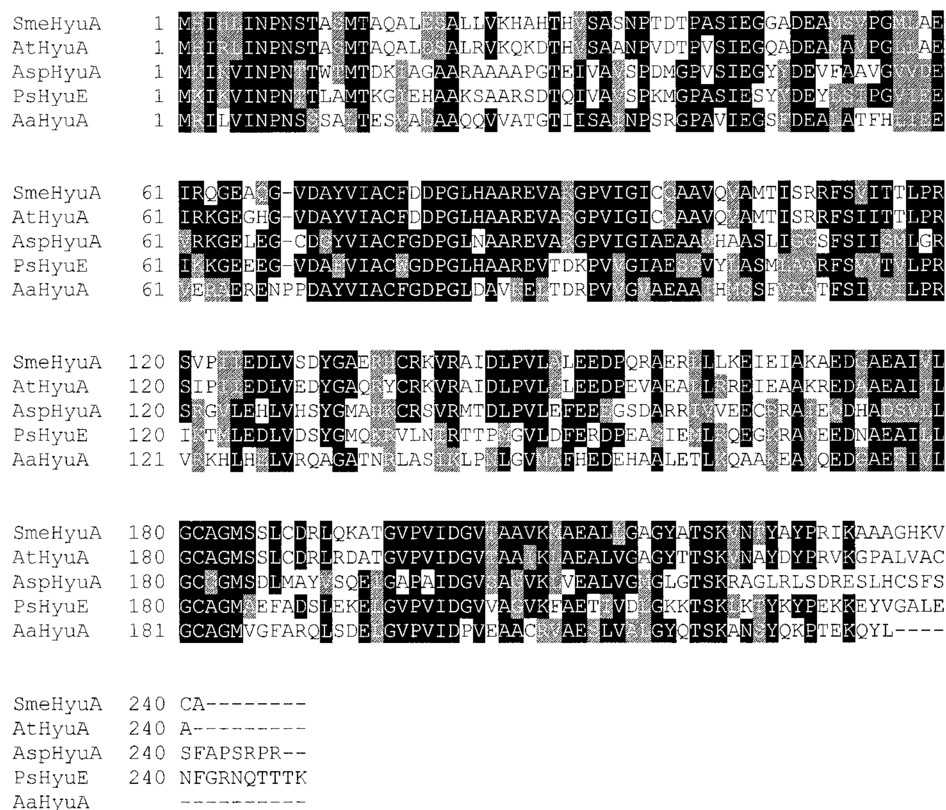

FIG. 1. Multiple alignment of the amino acid sequences of hydantoin racemases. Shown are the sequences for hydantoin racemase from *S. meliloti* (SmeHyuA), GenBank accession no. AY393697; hydantoin racemase from *Agrobacterium tumefaciens* C58 (AtHyuA), GenBank accession no. NP_35596; putative hydantoin racemase from *Agrobacterium* sp. strain IP-671 (AspHyuA), GenBank accession no. AF335479; hydantoin racemase from *Pseudomonas* sp. strain NS761 (PsHyuE), GenBank accession no. M84731; and hydantoin racemase from *Arthrobacter aurescens* DSM 3747 (AaHyuA), GenBank accession no. AF146701. Overall homology is highlighted in black, and partial homology is highlighted in grey.

and the N-terminal end of the β-galactosidase gene present in the pBluescript II SK(+) plasmid (pBSK; Stratagene Cloning Systems), a TGA codon was included upstream of the ribosome binding site sequence and the beginning of the gene in the SmRac5 primer. The SmXaRac3 primer included the factor Xa recognition sequence (Ile-Glu-Gly-Arg) and a polyhistidine tag (His_6 tag) before the stop codon. The *Eco*RI- and *Kpn*I-digested 768-bp fragment from the fusion protein was ligated into pBSK plasmid, which was cut with the same enzymes to create plasmid pSER27. *S. meliloti* hydantoin racemase showed significant amino acid sequence identity with hydantoin racemase amino acid sequences from different sources in GenBank (Fig. 1). Moreover, two cysteine residues at positions 76 and 181, which are probably involved in the catalytic center of the protein (21), were highly conserved within the studied hydantoin racemases. The highest sequence identity was presented between *S. meliloti* hydantoin racemase and *Agrobacterium tumefaciens* C58. Surprisingly, this identity was almost twofold higher than that shown previously between *Agrobacterium* sp. strain IP I-67 and *Agrobacterium tumefaciens* C58, which would be expected to show higher sequence identity as they belong to the same genus.

Functional expression and purification of hydantoin racemase. The hydantoin racemase gene was functionally expressed in *E. coli* BL21, and its activity was measured by chiral high-performance liquid chromatography, as previously described for *Agrobacterium tumefaciens* C58 hydantoin racemase (8). A one-step purification procedure of the recombinant hydantoin racemase fused to the His_6 tag was employed by using immobilized cobalt affinity chromatography followed by proteolytic digestion with factor Xa. Sodium dodecyl sulfate-poly-

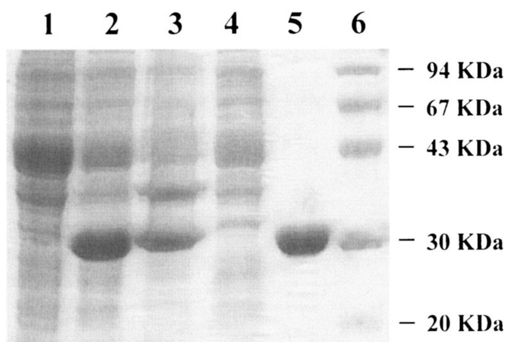

FIG. 2. SDS-PAGE analysis of each purification step of *S. meliloti* hydantoin racemase from *E. coli* BL21 harboring the pSER27 plasmid. Lane 1, *E. coli* BL21 containing pBSK plasmid without insert; lanes 2 and 3, supernatant and pellet of the resuspended crude extract after *E. coli* BL21(pSER27) cell sonication; lane 4, eluate after adding the sonicated supernatant to the metal affinity column; lane 5, purified enzyme; lane 6, low-molecular-mass markers.

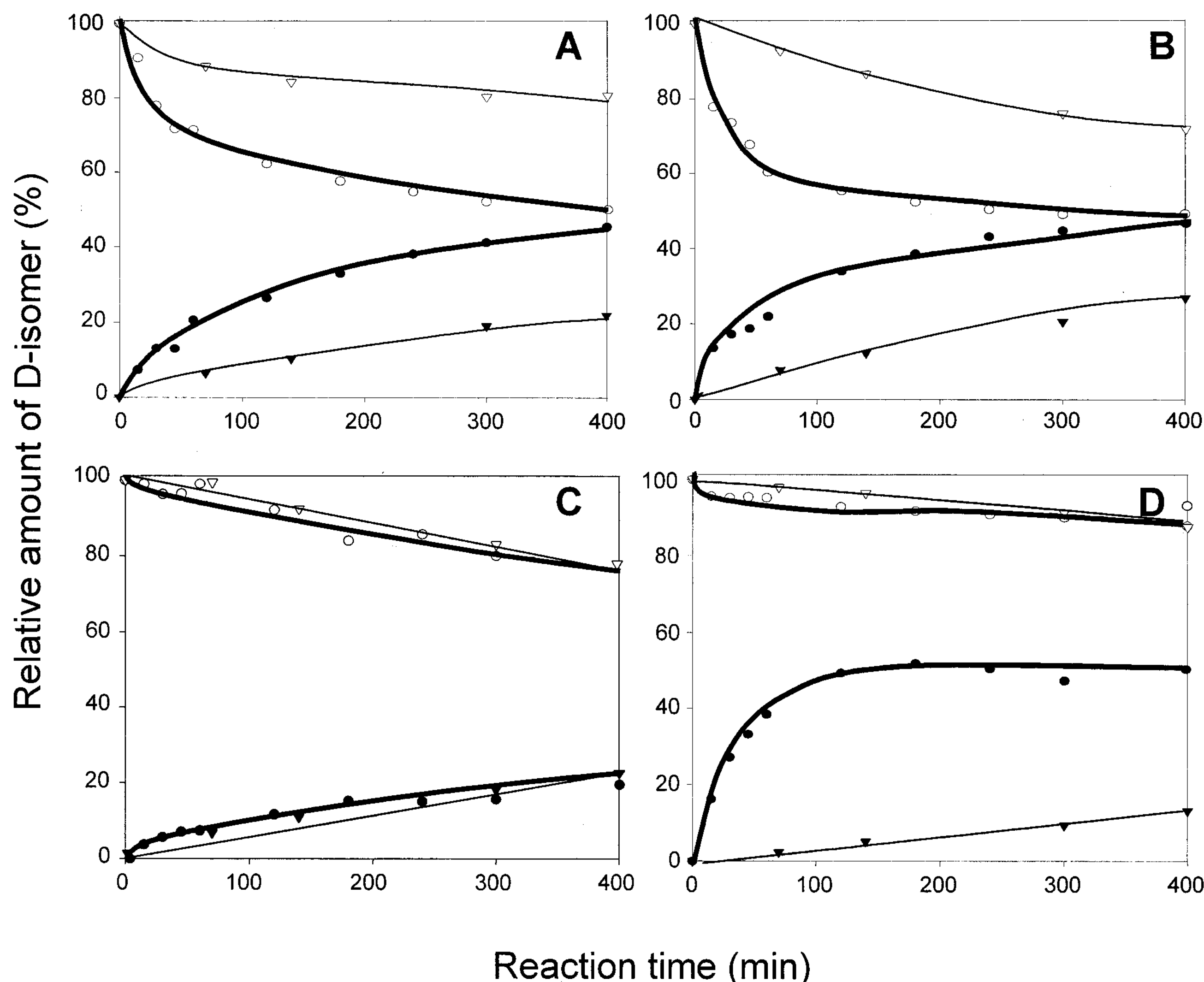

FIG. 3. Enzymatic racemization of different 5-monosubstituted hydantoins by *S. meliloti* hydantoin racemase. The hydantoin racemase activity of the D-isomer (○) and L-isomer (●) was measured at 40°C at pH 8.5 by chiral high-performance liquid chromatography at the points shown in the graphs. Chemical racemization of the D-isomer (▽) and L-isomer (▼) of each substrate was also measured at the same intervals; data for 5-benzylhydantoin (A), 5-isobutylhydantoin (B), 5-ethylhydantoin (C), and 5-methylthioethylhydantoin (D) are shown.

acrylamide gel electrophoresis (SDS-PAGE) analysis indicated that the purified enzyme was more than 95% pure after elution of the affinity column (Fig. 2). Specific activity was calculated for the purified enzyme. In 0.1 M Tris buffer (pH 8.5), the enzyme was stable at 4°C for 10 weeks; in the same buffer with 20% glycerol, the purified enzyme could be stored at −20°C for more than 3 months without noticeable loss of activity. The purified enzyme was active after 10 freeze-thawing cycles.

TABLE 1. Kinetic parameters of *S. meliloti* hydantoin racemase[a]

Substrate[b]	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)
L-EH	17.32 ± 1.25	6.42 ± 0.53	0.37 ± 0.06
L-IBH	6.41 ± 1.11	2.12 ± 0.27	0.33 ± 0.10
D-IBH	3.76 ± 0.96	3.24 ± 0.47	0.86 ± 0.34
L-BH	8.30 ± 1.18	1.94 ± 0.26	0.23 ± 0.07
D-BH	13.89 ± 1.99	2.29 ± 0.34	0.16 ± 0.05

[a] The kinetic parameters of *S. meliloti* hydantoin racemase were determined at 40°C for 15 min at pH 8.5. The k_{cat} value was defined as the number of millimoles of D- or L-5-monosubstituted hydantoin racemized per second and millimoles of enzyme at 40°C.

[b] L-EH, L-5-ethylhydantoin; D- and L-IBH, D- and L-5-isobutylhydantoin; D- and L-BH, D- and L-5-benzylhydantoin.

Molecular mass and subunit structure of hydantoin racemase. The apparent molecular mass of the *S. meliloti* purified enzyme subunit (31 kDa) after loading on SDS-PAGE (Fig. 2) was very similar to those of *Pseudomonas* sp. strain NS671 (32 kDa), *Arthrobacter aurescens* DSM 3747 (31 kDa), and *Agrobacterium tumefaciens* C58 (31 kDa) (8, 20, 21). In all cases, these apparent molecular masses were greater than those calculated from the amino acid sequence (25 to 27 kDa). The relative molecular mass of the *S. meliloti* hydantoin racemase (100 kDa) was measured by size exclusion chromatography on a Superdex 200 HR column. Consequently, the *S. meliloti* hydantoin racemase presents the same tetrameric structure as that previously described for *Agrobacterium tumefaciens* C58 (8), while the *Pseudomonas* sp. strain NS671 hy-

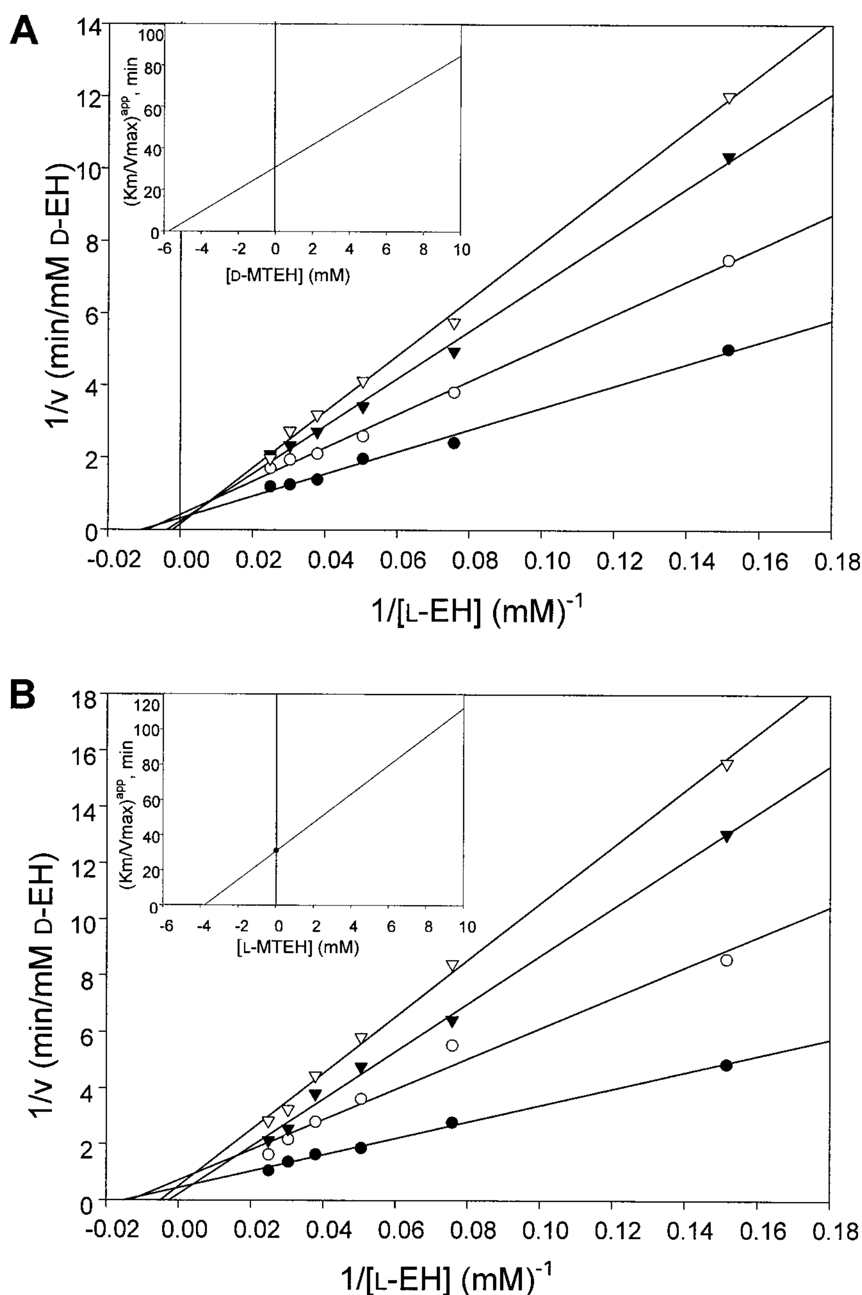

FIG. 4. D-5-Methylthioethylhydantoin (D-MTEH) (A) and L-5-methylthioethylhydantoin (L-MTEH) (B) inhibition pattern with L-5-ethylhydantoin (L-EH) as the variable substrate. D- and L-MTEH concentrations were varied in the presence of a constant concentration of L-EH. ●, no MTEH added; 3 mM (○), 6 mM (▼), and 9 mM (▽) MTEH. *S. meliloti* hydantoin racemase activity was assayed at 40°C for 15 min at pH 8.5. The insets show the plot of apparent K_m/V_{max} [$(K_m/V_{max}{}^{app})$] versus inhibitor concentration (D- or L-MTEH), giving K_i values of 5.75 mM and 3.76 mM for panels A and B, respectively. Reactions were performed in triplicate and yielded kinetic constants with less than 10% standard error from the mean.

dantoin racemase enzyme has been described as hexameric (20) and the *Arthrobacter aurescens* DSM 3747 hydantoin racemase enzyme has been classified as hexameric, heptameric, or octameric (21).

Physical characterization and effects of temperature and metal ions on hydantoin racemase activity. The *S. meliloti* hydantoin racemase showed an optimum reactivity at pH 8.5 when examined in 100 mM phosphate, Tris, or glycine-NaOH buffer at several pHs. This pH value was similar to that previously described for *Arthrobacter aurescens* DSM 3747 but was higher than that of *Agrobacterium tumefaciens* C58 (pH 7.5) and lower than that of *Pseudomonas* sp. strain NS671 (pH 9.5). Together with *Pseudomonas* sp. strain NS671, *S. meliloti* hydantoin racemase showed the lowest optimum temperature reactions (45°C), whereas *Arthrobacter aurescens* DSM 3747 and *Agrobacterium tumefaciens* C58 hydantoin racemases have

shown maximum activity at 55 and 60°C, respectively. When the thermal stability of the hydantoin racemase enzyme was measured after 30 min of preincubation at temperatures ranging from 4 to 80°C in 0.1 M Tris buffer (pH 8.5), the activity was gradually lost at temperatures of more than 30°C. This temperature is the lowest of the hydantoin racemases studied thus far (45°C for *Arthrobacter aurescens* DSM 3747 and *Pseudomonas* sp. strain NS671 and 55°C for *Agrobacterium tumefaciens* C58). Activity of the purified *S. meliloti* hydantoin racemase enzyme was assayed in the presence of 2 mM concentrations of different metal ions (Hg^{2+}, Ni^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Ca^{2+}, Fe^{2+}, Mg^{2+}, and Pb^{2+}) and a 0.1 M concentration of dithiothreitol and EDTA. Dithiothreitol and most of the metal ions studied had no significant effect on the enzyme, with the exception of Mn^{2+}, Co^{2+}, and Ni^{2+}, with which the activity of the enzyme was twofold lower. However, the insignificant effect of the metal chelating agent EDTA on the hydantoin racemase activity would indicate that it is not a metalloenzyme. The inhibitory effect of Cu^{2+}, Hg^{2+}, and Zn^{2+} on the hydantoin racemase enzyme has also been reported in previous works (8, 20, 21), but this is the first work in which Pb^{2+} shows the same inhibitory effect on the enzyme as the three aforementioned cations.

Substrate enantioselectivity and kinetic analysis of hydantoin racemase. The ability of the purified hydantoin racemase enzyme to racemize different 5-monosubstituted hydantoins with very low chemical racemization was examined (Fig. 3). The D- and L-isomers of 5-benzylhydantoin and 5-isobutylhydantoin were completely racemized (Fig. 3A and B). The D-isomer of 5-ethylhydantoin had no detectable racemization under the same conditions as those for the two above-mentioned substrates. However, its L-isomer (L-5-ethylhydantoin) showed the highest rate of racemization of all hydantoins analyzed (Fig. 3C). Neither the D- nor the L-isomer of 5-methylthioethylhydantoin showed any racemization in the presence of the purified hydantoin racemase enzyme (Fig. 3D). Upon analysis of hydantoin racemase activity for the five positive reactions, a slightly higher rate was detected for the aliphatic hydantoins (D- and L-5-isobutylhydantoin and L-5-ethylhydantoin) compared to D- and L-5-benzylhydantoin. Previously studied hydantoin racemase enzymes from *Pseudomonas* sp. strain NS671 and *Agrobacterium tumefaciens* C58 have shown better rates of racemization for hydantoins with aliphatic substituents, whereas the *Arthrobacter aurescens* DSM 3747 hydantoin racemase enzyme racemizes faster than aromatic hydantoins (8, 20, 21). *S. meliloti* hydantoin racemase showed higher activity with aliphatic substituents than with aromatic ones at position 5, and for the former, the highest racemization rate corresponded to the shortest length in the substituent chain.

Kinetic parameters for the two D- and L-isomers of 5-monosubstituted hydantoins were obtained from hyperbolic saturation curves by least-squares fit of the data to the Michaelis-Menten equation (Table 1). Reactions were carried out with different concentrations of D- or L-5-monosubstituted hydantoins at 40°C for 10 min with a constant enzyme concentration of 0.3 μM. The apparent K_m values obtained for *S. meliloti* hydantoin racemase were higher than those for *Agrobacterium tumefaciens* C58 when the same substrates were used, indicating a lower affinity for these substrates in the case of the enzyme from *S. meliloti*. There are no kinetic data for these substrates for the *Pseudomonas* sp. strain NS671 and *Arthrobacter aurescens* DSM 3747 hydantoin racemases. Substrates toward which the enzyme had no detectable activity (D-5-ethylhydantoin and D- and L-5-methylthioethylhydantoin) were studied as possible inhibitors of the hydantoin racemase. No inhibition effect was detected by D-5-ethylhydantoin for the substrate L-5-ethylhydantoin. However, inhibition by D- and L-5-methylthioethylhydantoin was studied at various concentrations of L-5-ethylhydantoin. Double-reciprocal plots of the data indicated that both D- and L-5-methylthioethylhydantoin are competitive inhibitors of L-5-ethylhydantoin as variable substrate (Fig. 4A and B). Substrate inhibition processes have been described for *Pseudomonas* sp. strain NS671 and *Arthrobacter aurescens* DSM 3747 hydantoin racemases, even at low substrate concentrations (20, 21). However, this phenomenon has been detected neither for *Agrobacterium tumefaciens* C58 hydantoin racemase in a previous study (8) nor for *S. meliloti* in this one. Hydantoin racemase from *S. meliloti* showed no activity with D- and L-5-methylthioethylhydantoin substrate, as was previously reported for *Pseudomonas* sp. strain NS671 hydantoin racemase toward either isomer of 5-isopropyl-hydantoin (20). However, this is the first time that substrates that the enzyme is not capable of racemizing have been studied as inhibitors of hydantoin racemase activity.

Nucleotide sequence accession number. The nucleotide sequence of the *S. meliloti* hydantoin racemase gene (726 bp) has been deposited in the GenBank database under accession number AY393697.

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