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On the mechanism of hydrolysis of hydantoins by D-hydantoinase from *Vigna angularis*: inhibition studies

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Abstract

Inhibition studies were performed with D-hydantoinase from *Vigna angularis*. These studies were based on the fact that 5,5-di-substituted hydantoins are not recognizable substrates for the enzyme. *Rac*-5-methyl-5-phenylhydantoin was shown to be an efficient competitive inhibitor of this D-hydantoinase (K_i = 1.28 mM). (*R*)-5-mono-substituted hydantoins were identified as the proper substrates of the enzyme. It is proposed that this reaction is constituted by a prior fast racemization step that provides the necessary and constant feeding of (*R*)-5-mono-substituted isomer and a latter (*R*)-specific enzymatic hydrolysis. The enzyme must present a hydrophobic environment in the pro-*R* face and a basic residue in the pro-*S* face. The feedstock configuration, its molecular volume and the presence of hydrogen in position 5 of the hydantoin are the main factors responsible for the substrate specificity showed by this enzyme.
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1. Introduction

Chiral amino acids and its derivatives are important starting materials in the production of pharmaceuticals, such as semisynthetic β-lactam antibiotics, peptides and enzymatic inhibitors [1–9]. They can be efficiently synthesized by biocatalytic conversion of substituted hydantoins using D-hydantoinase (dihydropyrimidine amidohydrolase EC 3.5.2.2), which is widespread in nature [8–10]. This enzyme is able to catalyze the enantiospecific hydrolysis (and ring opening) of *rac*-5-mono-substituted hydantoins to the corresponding *N*-carbamoyl-D-amino acid. These compounds can be easily converted into the respective D-amino acid either by diazotation or by a second hydrolytic enzymatic step by using *N*-carbamoyl amino acid amidohydrolase (EC 3.5.1.6) [3].

D-Hydantoinase from *Vigna angularis* was firstly reported by Morin [10] as a good catalyst for the synthesis of D-amino acids derivatives and it has been used in our laboratory for the production of carbamoyl-D-phenylglycine with 100% conversion and enantiomeric excess (EE) higher than 98% [8,9].

In order to increase our knowledge concerning the catalytic properties of this enzyme several mono- and di-substituted hydantoins were tested as substrates. In the present paper it is reported that 5-mono-substituted-hydantoins carrying aromatic or aliphatic side chains are recognized as substrates but hydantoins harboring charged groups, such as hydantoin-5-acetic acid and 5,5-di-substituted-hydantoins, are not. Based on inhibition studies performed

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with these latter compounds, a model explaining the substrate specificity showed by D-hydantoinase from *V. angularis* is proposed.

2. Experimental

2.1. Materials

D-Hydantoinase from *V. angularis*, hydantoin, hydantoin-5-acetic acid, *p*-dimethylaminobenzaldehyde and bovine serum albumin (fraction V) were obtained from Sigma. Diazald® kit (for CH_2N_2) generation and the aldehydes and ketones used for the synthesis of hydantoins were purchased from Aldrich. All other chemicals were of analytical grade and obtained from Merck, Darmstadt.

2.2. Methods

2.2.1. Synthesis of rac-5-hydantoins

Rac-5-phenylhydantoin and *rac*-5-methyl-5-phenylhydantoin were synthesized according to the method described by Henze and Speer [11] by using the corresponding aldehyde and ketone and $(NH_4)CO_3/NH_4CN$. Hydantoin-5-acetic acid methyl ester was prepared by methylation of the free acid with CH_2N_2/Et_2O according to Blank et al. [12].

2.2.2. Assay of D-hydantoinase activity

Hydantoinase activity was determined in reaction mixtures containing 100 mM H_3BO_3/KCl buffer, pH 9.0, 10 mM of substrate and an adequate concentration of enzyme to determine the initial velocity after 15 min of incubation at 30 °C. The reaction was started by addition of the enzyme and stopped by addition of TCA 12%. The concentrations of the products were determined in aliquots (10 μl) of the supernatant fluid by addition of *p*-dimethylaminobenzaldehyde, essentially according to Morin [10] and by Chiral HPLC using a Nucleosil Chiral-1 column (4.6 mm × 250 mm) (Macherey-Nagel, Germany) and $CuSO_4$ 1 mM as the mobile phase at a flow rate of 10 ml/min, with detection at 235 nm.

2.2.3. Kinetic studies

Time-course curves for the hydrolysis of different *rac*-hydantoins (hydantoin, hydantoin-5-acetic acid, hydantoin-5-acetic acid methyl ester, 5-phenylhydantoin and 5-methyl-5 phenylhydantoin) were determined by removing at different times 100 μl aliquots of the reaction mixtures corresponding to each *rac*-hydantoin (10 mM). The composition of the reaction mixtures as well as other experimental conditions was described under Section 2.2.2. Reaction was stopped with the addition of 200 μl of 12% trichloroacetic acid (w/v) and the samples were centrifuged at 12,000 × *g* for 1 min in a micro centrifuge (Incibras, São Paulo, Brazil) in order to eliminate precipitated proteins. The concentration of each *N*-carbamoyl-D-amino acid was determined by injection of 10 μl of the resulting supernatant fluid into the chiral HPLC column described under Section 2.2.2.

For inhibition studies, reaction mixtures contained, in a total volume of 0.3 ml: 100 mM H_3BO_3/KCl, pH 9.0, different concentrations of hydantoin (10–100 mM), a constant concentration of 5-methyl-5-phenylhydantoin (0–10 mM) and adequate concentration of enzyme to determine the initial velocity after 15 min incubation at 30 °C. Reaction rate was calculated by determination of the concentration of product formed with *p*-dimethylaminobenzaldehyde essentially as described by Morin [10]. The initial velocity of the reaction was calculated in terms of mM of *N*-carbamoylglycine produced per minute by using an appropriated standard curve.

2.2.4. Kinetic data processing

Estimates of parameters and of their asymptotic standard errors were obtained by fitting Eq. (1) to data using a nonlinear least-squares computer program, developed in our laboratory and specifically devised for steady-state studies of enzyme kinetics [13].

2.2.5. Molecular calculations

Theoretical calculations of molecular volumes of *rac*-5-methyl-5-phenyl-hydantoin and *rac*-5-phenylhydantoin were performed by using the AM1 (semi empirical method) program of PC SPARTAN PRO.

2.2.6. Determination of protein concentration

Protein concentration was determined according to Hartree [14] by using bovine serum albumin as standard.

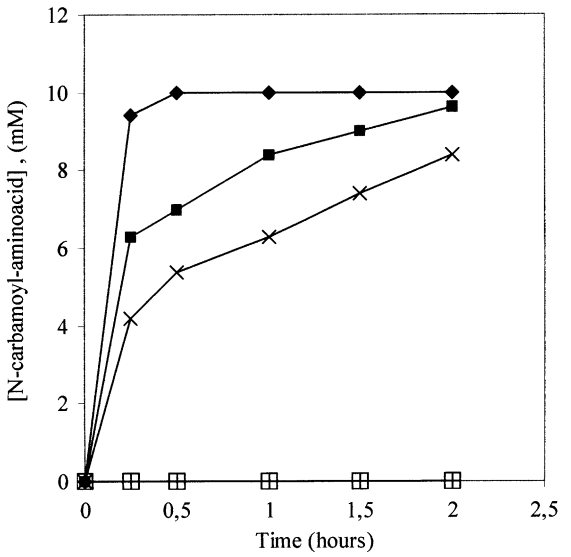

Fig. 1. Time-course curves for the hydrolysis of some hydantoins catalyzed by *V. angularis* D-hydantoinase. The course of reaction was followed by chiral HPLC determination of concentration of the respective *N*-carbamoyl-D-amino acid at the times indicates in the figure as described in Section 2.2.3: (◆) *N*-carbamoyl-glycine; (■) *N*-carbamoyl-D-phenylglycine; (×) *N*-carbamoyl-D-aspartic acid methyl ester; (+) *N*-carbamoyl-D-5-methyl-5-phenylglycine; (□) *N*-carbamoyl-D-aspartic acid.

3. Results and discussion

The time-course curves for the hydrolysis of different hydantoins catalyzed by D-hydantoinase of *V. angularis* are shown in Fig. 1. As depicted in Fig. 1 hydantoin, the standard substrate of this enzyme was the best one with the reaction achieving 100% of conversion in 30 min. Total conversion was also achieved with *rac*-5-phenylhydantoin but after 2 h of reaction at 30 °C and a reaction time significantly higher was required by *rac*-hydantoin-5-acetic acid methyl ester (Fig. 1). However as shown in Fig. 1 *rac*-hydantoin-5-acetic acid was not recognized by the enzyme as substrate as well as *rac*-5-methyl-5-phenylhydantoin. The result obtained with the former compound when compared with that of the corresponding methyl ester suggest that mono-substituted hydantoins carrying a charged group in 5-position greatly decrease binding of the substrate at the active site of D-hydantoinase. Moreover, considering that *rac*-5-phenylhydantoin is a good substrate of the enzyme and that *rac*-5-methyl5phenylhydantoin is not recognized by the biocatalyst (Fig. 1), the possibility exists that the presence of a methyl group in 5-position instead of the hydrogen atom present in this position of the corresponding substrate also decreases significantly the binding affinity of 5,5-di-substituted hydantoins.

In order to obtain experimental support for these interpretations, *rac*-hydantoin-5-acetic acid and *rac*-5-methyl-5-phenylhydantoin were tested as potential inhibitors of D-hydantoinase from *V. angularis*. The enzymatic activity was measured with 40 mM hydantoin and different concentrations (0–40 mM) of each one of the above mentioned hydantoins. While *rac*-hydantoin-5-acetic acid did not affect the activity of the enzyme, *rac*-5-methyl-5-phenylhydantoin produced total inhibition of D-hydantoinase at the highest concentration tested (Results not shown). Fig. 2 shows that the di-substituted hydantoin behaved as a linear competitive inhibitor of the enzyme supported by Eq. (1),

$$v_i = \frac{V_m S}{K_M(1 + I/K_i) + S} \quad (1)$$

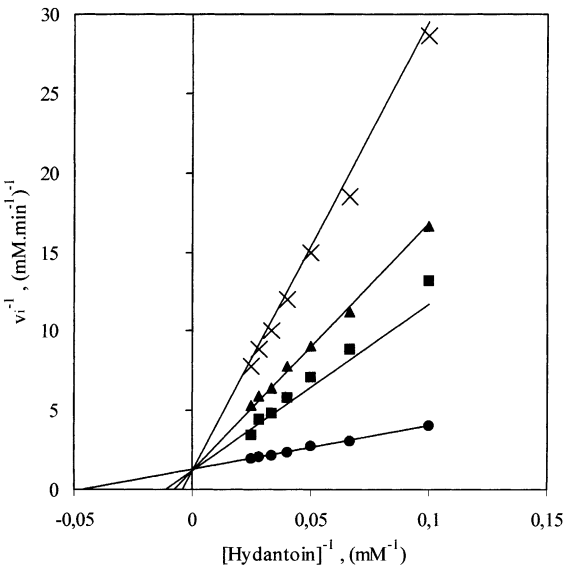

Fig. 2. Inhibition of the hydrolysis of hydantoin by *rac*-5-methyl-5-phenyl-hydantoin catalyzed by D-hydantoinase from *V. angularis*. The concentration of hydantoin was varied between 10 and 40 mM. The concentrations of inhibitor were: (●) zero; (■) 3.0 mM; (▲) 5.0 mM and (×) 10.0 mM. Other experimental details are given in Sections 2.2.3 and 2.2.4.

Table 1
Competitive inhibition of D-hydantoinase by 5-methyl-5-phenyl-hydantoin

Parameter value	Estimate ± S.E.[a]
V_m (mM min^{-1})	0.83 ± 0.04
K_M (mM)	32.0 ± 2.60
K_i (mM)	1.28 ± 0.06
Syx[b] (mM min^{-1})	0.01066

Estimates of kinetic parameters of Eq. (1).
[a] Asymptotic standard error of parameter. Estimates of parameters obtained by nonlinear regression of experimental data depicted in Fig. 1.
[b] Residual standard error of parameter.

where v_i, initial rate; V_m, maximum velocity; S, hydantoin concentration; K_M, Michaelis constant of hydantoin; I, concentration of *rac*-5-methyl-5-phenylhydantoin; K_i, dissociation constant (inhibition constant) of D-hydantoinase-5-methyl-5-phenylhydantoin complex.

Estimates of the three parameters of Eq. (1) and of their asymptotic standard errors are displayed in Table 1. A good fitting of Eq. (1) to the experimental data was obtained as shown by the magnitude of the value of the asymptotic standard error of each parameter estimate and by the size of the residual standard error of the fitting. Therefore, our results showed that 5-methyl-5-phenyl-hydantoin is a strong linear competitive inhibitor of this enzyme with a K_i value of 1.28 mM.

Based on our results shown in Figs. 1 and 2, in previous results recently published by us [8,9] concerning the substrate specificity of this D-hydantoinase and in Morin article [10], also dealing with this subject, in this paper it is suggested that D-hydantoinase activity is extremely dependent on the configuration and on the chemical character of the substituent group in the 5-position of the substrate. For instance Fig. 1 shows that hydantoins carrying a charged group are not recognized as substrate by this enzyme and do not bind at the active site (hydantoin-5-acetic acid was not and inhibitor). However, elimination of this charge by forming *rac*-hydantoin-5-acetic acid methyl ester transforms the former compound into a good substrate. In addition, the fact that just 5-monosubstituted hydantoins harboring an aromatic or aliphatic group act as substrates of this hydantoinase [8–10] and the observation that *rac*-5-methyl-5-phenylhydantoin is a potent

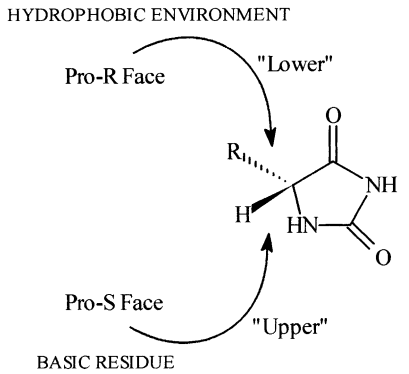

Fig. 3. Substrate faces (Cahn, Ingold & Prelog Convention) and enzyme environment. R = H or nonpolar substitutent.

competitive inhibitor (Fig. 2), suggest that this enzyme must carry a hydrophobic region at its catalytic site lining the lower (pro-*R* face) of the substrate (Fig. 3).

Overwhelmingly, the great difference between a substrate and an inhibitor is then the presence of a group in the lower (pro-*S* face) of the substrate (Fig. 3). Therefore, the enzyme is able to recognize a substrate carrying a hydrogen in pro-*S* face, having, according to Cahn, Ingold and Prelog's rule, a *R*-configuration. According to these findings, the hydrolysis mechanism would only be operative with 5-(*R*)-mono-substituted hydantoins thus producing enantiospecifically (*R*)-*N*-carbamoyl-α-amino acids. In this case, very fast chemical racemization reaction occurs prior to the enzymatic hydrolysis step. The racemization step, thereby, works as a pump, keeping the 1:1 equilibrium between the (*S*) and (*R*) configurations, while the (*R*)-enantiomeric form, specifically (and properly), feeds the enzyme, giving rise to very high conversions and enantiomeric excesses [8].

However, whenever a substitutent other than a H atom is present in the 5-position, the racemization can not occur and the either (*S*)- or (*R*)-hydantoins can occupy the active site, but not allowing the reaction to proceed acting, thereby, as competitive inhibitors of the enzyme (Table 1).

The general catalytic mechanism for hydrolytic enzymes are generally regarded as involving a concerted acid–basic mechanism, consisting of a *triad* Hys–Asp–Ser [15] for serine proteases [16], a tandem Hys–Cys [17] for cysteine proteases or a *N*-terminal Cys, Thr or Ser [18] in the catalytic site for other

hydrolases. As a formal consequence of the results presented hereby, it is proposed that very closed to the catalytic amino acid residues of the enzyme active site [15–19], a basic residue (amine, guanidyl or imidazoyl group), able to interact with the (*R*)-5-acid hydrogen present in the hydantoinic ring, must be present in the upper substrate face, the pro-*S* face (Fig. 3).

The presence of any group other than hydrogen, regardless of substrate configuration not only cannot produce the desirable interactions [20,21] but also presents much higher van der Waals radii. In addition, conformational changes in the enzyme combined with local pH changes would provide the product release from the enzymatic pocket [22]. The need of a basic residue to anchor the hydantoin ring can be related either to the mobility of a Hys [18] or to the presence of other amino acid residues [19] capable to anchor the substrate, analogously to what it has been proposed for acetylcholinoesterase in the prior case [18], and to *Pseudomonas* glutaminase–asparaginase in the latter [19].

To support this view the molecular volumes for *rac*-5-methyl-5-phenylhydantoin and *rac*-5-phenylhydantoin were calculated (AM1, semi-empirical method). The former compound showed a molecular volume of 213.3 $Å^3$ and the latter 193.8 $Å^3$. The greater molecular volume and the lack of hydrogen in the proper spatial position are the main responsible factors for the action of *rac*-5-methyl-5-phenylhydantoin as competitive inhibitor for D-hydantoinase from *V. angularis*.

Based on kinetic experiments involving substrate specificity and inhibition studies the present work give the first information concerning the necessary interactions that must exist at the active site of D-hydantoinase from *V. angularis* for the productive binding of substrates (5-monosubstituted hydantoins harboring an hydrophobic or uncharged group). Moreover, the binding mechanism proposed gives a satisfactory explanation for the formation of non-productive complexes in the presence of di-substituted hydantoins (linear competitive inhibitors). These findings can be considered as the first contribution for the elucidation of the catalytic mechanism of this enzyme.

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