SUBSTRATE AND STERIC SPECIFICITY OF HYDROPYRIMIDINE HYDRASE

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1. Introduction

Eadie et al. [1] partially purified animal and plant hydantoinases which catalyze the hydrolysis of hydantoin to hydantoic acid. These authors have also found that dimethylhydantoin is a competitive inhibitor of the enzymes.

Later Wallach and Grisolia [2] prepared from calf liver an enzyme, termed hydropyrimidine hydrase (EC 3.5.2.2.), which plays an important role in the metabolism of pyrimidines. The same enzyme rapidly hydrolyzes hydantoin to hydantoic acid and is inactive with disubstituted hydantoins and most probably is identical with the rat liver hydantoinase of Eadie et al. [1]. Dudley et al. [3] studied the metabolism of the racemic forms of N-substituted 5-phenylhydantoins which undergo N-dealkylation as an initial reaction and then are hydrolyzed giving the R-form of phenyl hydantoic acid as a major metabolic product. On the basis of these findings Dudley and Bius [4] formulated the hypothesis that only the R-isomer of 5-phenylhydantoin could undergo the ring-opening reaction and that the residual S-isomer undergoes spontaneous and/or enzymatic in vivo racemization.

In this communication, we report results of a study designed to gain insight into the specificity of the enzyme hydropyrimidine hydrase. It will be shown that this enzyme rapidly hydrolyzes the R-isomer of several hydantoins including 5-phenyl-hydantoin and is inactive with the S-isomer. The latter can undergo spontaneous racemization under the conditions of the hydrolysis.

2. Materials and methods

2.1. *Chemicals*

The amino acids and the other reagents were obtained from Fluka AG (Buchs, Switzerland) except *p*-hydroxy-phenylglycine which was received as a gift from Chimica Bulciago (Milan, Italy). The hydantoins were prepared according to the procedure of Suzuki [5]. The N-carbamoyl amino acids were prepared by the method of Stark and Smyth [6].

2.2. *Enzyme source and activity assay*

Hydropyrimidine hydrase from calf liver was prepared and purified according to the method of Wallach and Grisolia [2]. A partially purified preparation with a specific activity of 4 units/mg protein was used.

Enzyme activity was determined by measuring the rate of liberation of *N*carbamoyl-α-alanine in a reaction mixture which contained per ml: 200 μmol of R,S-5-methyl hydantoin, 100 μmol of sodium pyrophosphate, and 0.1–0.5 mg of protein. The pH was 8.5 and temperature 30°C. Under these conditions the rate of hydrolysis of 5-methyl hydantoin was constant until 10% of conversion. The reaction was stopped by adding trichloroacetic acid to a final concentration of 40 mg/ml. The deproteinized mixture was centrifuged. To the supernatant (2 ml), 1 ml of a 10% (w/v) solution of *p*-dimethylaminobenzaldheyde in 12 N HCl was added and optical density at 438 nm measured. The absorbance was proportional to the *N*-carbamoyl-α-alanine concentration up to about 2 mM.

One unit of activity was defined as the amount of enzyme required to produce 1 μmol of *N*-carbamoyl-α-alanine per min under assay conditions.

3. Results and discussion

3.1. *Substrate specificity*

The relative rates of hydrolysis of some hydantoin

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Table 1
Hydrolytic activity of hydropyrimidine hydrase on various hydantoins

R,S-Amino acid hydantoins	Relative rate of hydrolysis
α-Phenyl-glycine	100
Leucine	84
α-Alanine	32
Methionine	20
Glycine	16
Valine	12
Phenylalanine	3
p-OH-phenylglycine	3
Glutamic Acid	0
Aspartic Acid	0
Tryptophan	0
2-Thiohydantoin	0

The rate of carbamoyl amino acids release from hydantoins was determined spectrophotometrically as described in Materials and methods. The reaction mixture contained 20 μmol/ml of 5-substituted hydantoins in 0.2 M sodium phosphate buffer, pH 8.5, and 0.1–0.2 enzyme units/ml. The incubation temperature was 30°C. The arbitrary value, 100, was assigned to the rate obtained with 5-phenyl hydantoin, the substrate most rapidly hydrolyzed.

derivatives were determined by incubating the enzyme with a 20 mM hydantoin solution. This substrate concentration was chosen because it corresponds to the lowest solubility in water of the hydantoins tested.

The results are summarized in table 1. Certain hydantoins were hydrolyzed most readily, with 5-phenyl and 5-isobutyl hydantoin being most rapidly hydrolyzed. Those hydantoins having a charged group in the amino acid residue were totally resistant to hydrolysis. The length and branching of the carbon chain also altered the susceptibility to enzymic ring-opening. It is interesting to note the great difference in the hydrolysis rates of 5-phenyl and 5-benzyl hydantoins and the complete resistance to hydrolysis of tryptophan hydantoin.

3.2. *Steric specificity*

The stereospecificity of hydropyrimidine hydrase was investigated. The enzyme was incubated with several R,S-hydantoins for a long period of time. The carbamoyl derivatives formed and the unreacted fraction of the hydantoins were separated and isolated from the reaction mixtures. The physical properties of the isolated products are shown in table 2. The results of table 2 clearly demonstrate that only the R-isomer is susceptible to enzyme-catalyzed hydrolysis. In fact, in every cases only the R-carbamoyl derivative was isolated and the rotation sign of the unreacted hydantoin was that of the S-isomer. The value of this rotation was the closer to the specific rotation of S-hydantoin the lower was the rate of hydantoin racemization under hydrolysis conditions.

Analysis of the reaction products of the sample

Table 2
Steric specificity of hydropyrimidine hydrase

Amino acid hydantoin	$[\alpha]_D^{25}$ R-carbamoyl derivative (deg)		$[\alpha]_D^{25}$ Not hydrolized hydantoin (deg)	
	Found	Literature	Found	Literature for *S*-hydantoins
α-Phenylglycine	−137 (c = 1; 1 N NH_4OH)	−136.3 [7] (c = 1; 1 N NH_4OH)	–	−112 [4] (c = 0,2 ethanol)
α-Alanine	+9.6 (c = 1; water)	+9.6 [8] (c = 1.3; water)	−38	−48 [5] (ethanol)
Methionine	−11.8 (c = 1; 1 N NH_4OH)	[a]	−56 (c = 1. ethanol)	−62 [5] (ethanol)
Valine	−15.4 (c = 1; 1 N NH_4OH)	−14.1 [9] (c = 10; 1 N NaOH)	−95.5 (c = 1; ethanol)	−134 [5] (ethanol)
p-OH-phenylglycine	−175 (c = 0.5; ethanol–water 1:1)	−175 [10] (c = 0.5; ethanol–water 1:1)	–	–

[a] The specific rotation of R-carbamoyl-methionine synthesized according to the method of Stark and Smyth [6] was −11.8 (c = 1; 1 N NH_4OH)

containing R,S-5-phenyl hydantoin showed that the whole amount of substrate was hydrolyzed to R-carbamoyl phenylglycine and this is in agreement with the high rate of racemization observed for 5-phenyl hydantoin at pH 8.5. Although the results of this study do not permit a complete understanding of the physiological significance of hydropyrimidine hydrase towards amino acid hydantoins, from these data it appears safe to conclude that the enzyme is responsible for the selective in vivo hydrolysis of the R-isomer of substituted hydantions. In addition, it must be stressed that hydropyrimidine hydrase might find application both for the preparation of optically active amino acids and as a tool for the hydrolysis of hydantoins in the method of Stark and Smyth [6] for the determination of NH_2-residue in proteins.

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