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ALLANTOIN RACEMASE: A NEW ENZYME FROM *PSEUDOMONAS* SPECIES

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Summary

1. Allantoin racemase is a novel enzyme which catalyzes the conversion of S(+)- and R(—)-allantoin into the racemate.

2. The enzyme is present in *Pseudomonas testosteroni*, *Pseudomonas putida* and five biotypes of *Pseudomonas fluorescens*, but absent in a number of other *Pseudomonas* species.

3. The enzyme of *Ps. testosteroni* was purified 133-fold and exposes optimal activity at pH 8.0—8.2 and 50° C. The enzyme is stable on heating for 15 min at 70° C.

4. The enzyme appeared to be specific for the optical isomers of allantoin and no cofactors are involved in the reaction.

5. The optical aspecificity of allantoinase of *Proteus rettgeri* was reaffirmed.

Introduction

The natural optical isomer of allantoin is (+)- or S-allantoin [1], according to the nomenclature of Cahn-Ingold-Prelog. This form is produced by uricase (urate : oxygen oxidoreductase, EC 1.7.3.3) [2], is found in plants [3] and in the urine of cattle [4], and is degraded by the allantoinases (allantoin amidohydrolase, EC 3.5.2.5) of soy bean [5,6], various animals [7] and a number of bacteria [7,8].

Some bacteria can be used to prepare R-allantoin from the racemic mixture [1], but other ones use both optical forms of allantoin either due to the presence of an aspecific allantoinase [7,8] or to the presence of allantoin racemase (Fig. 1), whose isolation, purification and properties are reported here for the first time.

Since the previously observed aspecificity of some allantoinases might be caused by the presence of allantoin racemase, which was unknown at that time, the allantoinase of *Proteus rettgeri* will be reinvestigated in this study.

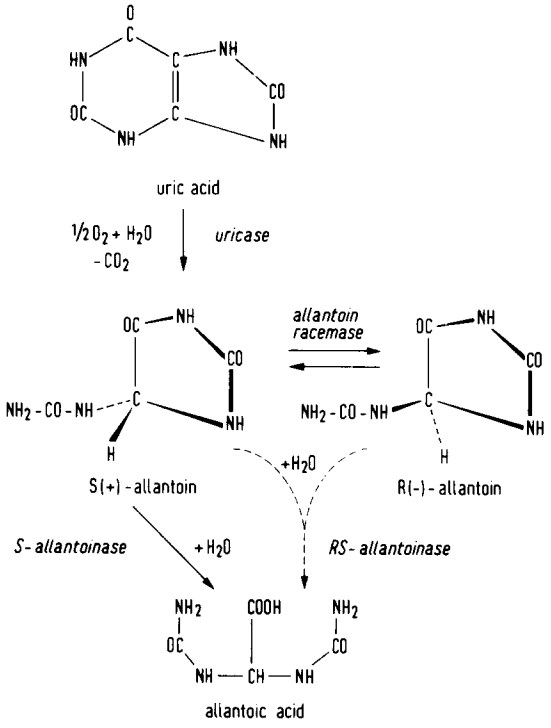


Fig. 1. Metabolic reactions involving allantoin.

Materials and Methods

Bacterial strains

The following strains from the laboratory collection were used: *Pseudomonas aeruginosa* V3003, V3004, V3006, V3007, V3008, V3010 and ATCC 17434, *Ps. aeruginosa* V3001, an achromogenic spontaneous mutant of V3003, *Pseudomonas fluorescens*, biotype A, ATCC 17397 and 17553, *Ps. fluorescens*, biotype B, ATCC 17467 and 17815, *Ps. fluorescens*, biotype C, ATCC 948 and 17400, *Ps. fluorescens*, biotype D, ATCC 9466 and 17809, *Ps. fluorescens*, biotype E, ATCC 13985 and 17419, *Ps. fluorescens*, biotype F, ATCC 17458 and 17513, *Pseudomonas putida* ATCC 12633, 17390, 17391, 17392, 17393, 17428, 17430 and 17484, *Pseudomonas alcaligenes* ATCC 14909, *Pseudomonas pseudoalcaligenes* ATCC 17440, *Pseudomonas stutzeri* ATCC 17588, 17759 and 17832, *Pseudomonas maltophilia* ATCC 13637, *Pseudomonas testosteroni* ATCC 11996, 15666, 15667 and 17510, *Pseudomonas acidovorans* ATCC 9355, 15668, 17406, 17455, 17479 and 17501, *Proteus rettgeri* V3522, which was previously named *Arthrobacter allantoicus* [7,8], and *Bacillus fastidiosus* SMG 83. In this study the nomenclature of Stanier et al. [9] will be followed, which implies that *Ps. acidovorans* ATCC 15667, strain 21 of Den Dooren de Jong [10], is called *Ps. testosteroni*. This strain was used in our previous studies on the allantoin metabolism of *Pseudomonas* species [7,11,12].

Cultivation of the bacteria

The bacteria were grown overnight at 30° C with rotatory shaking at 300 rev./min (New Brunswick Scientific gyratory shaker) in an allantoin-containing medium [13]. This medium was supplemented with 0.5% sodium fumarate on growing *Ps. stutzeri*, *Ps. alcaligenes*, *Ps. pseudoalcaligenes* and *Ps. maltophilia*, which cannot use allantoin as the sole source of carbon. Moreover, in case of *Ps. maltophilia*, the medium was supplemented with L-methionine (20 mg/l), which is essential for growth of this organism [9]. Growth of *Pr. rettgeri* was in a standing culture, since this organism is known to use allantoin best under anaerobic condition [7]. Cells were harvested by centrifugation (10 000 × *g* for 10 min at 4° C), washed with 0.9% NaCl solution and suspended in 50 mM phosphate buffer, pH 6.5. The cells were disrupted at 0° C by use of a MSE 150-W ultrasonic disintegrator at maximum output in six periods of 1 min. The broken-cell suspension was centrifuged at 4° C for 45 min at 100 000 × *g*. The supernatant fluid was used as crude extract.

Assay of allantoinase

A mixture containing 39 μmol RS- or R-allantoin, 0.08 μmol $MnSO_4$, 5 μmol dithiothreitol, 200 μmol Tris · HCl buffer, pH 7.4, and an appropriate amount of the enzyme was incubated at 30° C. Allantoate was measured by its conversion to glyoxylate at 100° C under acidic conditions and subsequent analysis of glyoxylate [14].

Assay of allantoin racemase

0.2 ml enzyme solution (10—250 μg protein depending on purity) were mixed with 1 ml 50 mM phosphate buffer, pH 6.5, containing 22 μmol R-allantoin. The optical rotation at 365 nm was followed at 30° C as a function of time with a Perkin-Elmer polarimeter, model 141, in a 10-cm cell. Observed rotations were always corrected for non-enzymic racemization of R-allantoin, which amounts to 0.07% per min [15]. A linear relation was obtained on plotting the logarithm of the optical rotation against time. The enzyme solutions were heated before use in order to inactivate allantoinase which interferes in the racemase assay by converting the product of the racemase reaction, viz. S-allantoin, into allantoate. Allantoinase activity was destroyed completely by a 15-min heating period at 70° C [7].

One unit of racemase activity was defined as the amount of enzyme which catalyzes the conversion of 1 μmol R-allantoin into the racemic form, i.e. 0.5 μmol R-allantoin into the optical antipode, per min at 30° C and pH 6.5. Specific activity is given in units per mg protein. Protein was measured according to Lowry et al. [16], using bovine serum albumin as a standard.

Purification of allantoin racemase from Ps. testosteroni

Cultivation of *Ps. testosteroni* ATCC 15667 (5 l allantoin-containing medium was used) and preparation of the crude extract are described above. Crude extract (35 ml) was heated for 15 min at 60° C and the precipitate was removed by centrifugation for 15 min at 10 000 × *g*. The precipitate was washed once at 60° C with 10 ml 50 mM phosphate buffer, pH 6.5. The supernatant fractions were combined and contained all activity. This fraction was applied to a column (50 cm × 2.5 cm) of DEAE-cellulose (Whatman DE-52)

which had been equilibrated with 50 mM phosphate buffer, pH 7.0. The protein was eluted at 4° C with a 1-l linear gradient of NaCl (0—0.3 M) in 50 mM phosphate buffer, pH 7.0. The racemase was eluted between 0.1 and 0.12 M NaCl. The active fractions were combined, concentrated by vacuum dialysis at 4° C and applied at 4° C to a column (90 cm × 2.7 cm) of Sephadex G-200, which had been equilibrated with 50 mM phosphate buffer, pH 7.0. Protein was eluted with the same buffer and 3.5-ml fractions were collected. Fractions 49—57, which contained most of the racemase activity were pooled.

This pool was treated for 15 min at 70° C and the supernatant fluid obtained after centrifugation for 15 min at 10 000 × *g* was used as purified enzyme.

Fluorescence

The fluorescence spectrum of purified allantoin racemase was measured at 30° C in a 1-cm cuvette in an Aminco-Bowman spectrophotofluorimeter. Protein concentration was 0.19 mg/ml in 50 mM phosphate buffer, pH 6.5. For comparison the spectrum of bovine serum albumin (1 mg/ml, 50 mM phosphate buffer, pH 6.5) was taken.

Materials

RS-Allantoin was obtained from Merck. R-Allantoin was prepared as given previously [1].

A solution of S-allantoin was prepared by incubation of 2400 units of uricase (specific activity: 8 units/mg protein) from *B. fastidiosus* with a suspension of 3 g uric acid in 150 ml 50 mM phosphate buffer, pH 6.5. This pH value, at which uricase exposes only about 3% of its optimal activity, was chosen to suppress racemization of the S-allantoin formed [15]. The incubation mixture was shaken vigorously at 30° C and the optical rotation of samples was measured to follow the course of the reaction. After 9 h an excess of 50 μmol/ml of S-allantoin was present. The reaction mixture was acidified to pH 3 to suppress racemization to a very low level and to remove most of the excess of uric acid. The residual uric acid was removed by chromatography of the mixture on PEI-cellulose and elution with an ethanol/water (4 : 1, v/v) mixture. In the fractions containing allantoin, the total amount was measured according to the differential analysis of glyoxylate derivatives [14] and the excess S-allantoin by testing the optical rotation. The mixture (300 ml) contained per ml 26 μmol S-allantoin and 3 μmol R-allantoin. Further studies will be made to improve the synthesis of S-allantoin.

Carbamoyl- and acetylamino acids and their hydantoins were prepared according to the method of Stark and Smyth [17]. 1-Acetylallantoin and 1,3-diacetylallantoin were prepared according to Biltz and Loewe [18], 3-methylallantoin according to Biltz and Robl [19], homoallantoin according to Simon [20], 5-aminohydantoin and 5-acetylaminohydantoin according to Biltz and Giesler [21] and allantoate according to Hermanowicz [22].

Results

Occurrence of allantoin racemase

The presence of the enzyme was demonstrated in all strains tested of the

TABLE I

PURIFICATION OF ALLANTOIN RACEMASE FROM *Ps. TESTOSTERONI* ATCC 15667

	Total protein (mg)	Total activity (units)	Specific activity	Recovery (%)	Times purified
Crude extract	892	1385	1.55	100	1
Heat treatment at 60° C	284	1360	4.8	98	3.1
DEAE-cellulose chromatography	40	1245	31	90	20
Sephadex G-200 gelfiltration	7.8	1150	148	83	95
Heat treatment at 70° C	5.6	1150	206	83	133

following *Pseudomonas* species and the average specific activities in the crude extract are given in parentheses: *Ps. fluorescens* biotypes A through E (ten strains: 3.6), *Ps. testosteroni* (four strains: 1.5) and *Ps. putida* (eight strains: 1.6). The specific activity was similar for exponentially growing and stationary cells. No trace of activity could be detected in the eight strains of *Ps. aeruginosa*, the six strains of *Ps. acidovorans*, the three strains of *Ps. stutzeri*, the two strains of *Ps. fluorescens* biotype F, in *Ps. alcaligenes*, *Ps. pseudoalcaligenes* or *Ps. maltophilia.*

Purification of the enzyme

Table I shows a typical result of the purification procedure for the enzyme from *Ps. testosteroni.* This enzyme was purified 133-fold by successive heat treatments, DEAE-cellulose chromatography and gelfiltration on Sephadex G-200. Under optimal conditions for temperature (50° C) and pH (8.0) the specific activity is 531.

The purified enzyme material did possess neither allantoinase activity nor activity of other uricolytic enzymes [8].

pH optimum

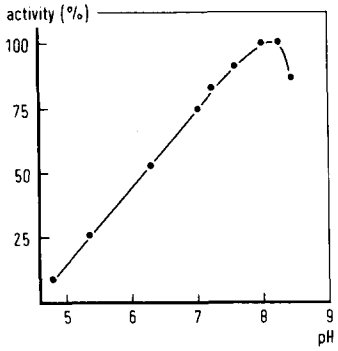
Fig. 2 shows the pH vs activity curve for allantoin racemase. The values are corrected for non-enzymic racemization of R-allantoin [15]. The initial reaction rate was linearly proportional with pH over a broad range (pH 4.8—7.5). Optimal activity was found at pH 8.0—8.2. Because of the very rapid chemical racemization of R-allantoin at pH values above 8, enzymic measurements were less accurate at these values. Measurements of the specific activity of the enzyme were performed at pH 6.5, where the racemization rate is about 100 times lower [15].

No effect of buffer ions (acetate, phosphate, triethanolamine, diethanolamine) on the reaction rate was observed.

Effect of temperature

The optimal temperature for allantoin racemase is 50° C (Fig. 3). The activity at 30° C is 67% of this value. The activation energy calculated from the activity at different temperatures was 11.2 kcal for the enzymic reaction, whereas 23.5 kcal was found for the non-enzymic reaction.

The crude enzyme was stable for at least 15 min at temperatures up to



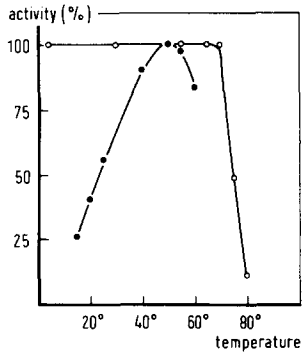


Fig. 2. pH vs activity curve of allantoin racemase from *Ps. testosteroni*. The incubation mixtures contained 84.5 μg partially purified racemase (from the DEAE-step). The specific activity of the enzyme was 31. Buffers used were acetate, phosphate, triethanolamine and diethanolamine (all 50 mM).

Fig. 3. Effect of temperature on the activity of allantoin racemase from *Ps. testosteroni*. To test the effect of heat pretreatment (○———○), 1 ml crude extract (7.86 mg protein; specific activity 1.5) was treated for 15 min at the indicated temperatures, then diluted 5 times and the precipitate was removed by centrifugation at 10 000 × *g* for 15 min. Activity was then tested in the standard assay. To test the effect of temperature during incubation 0.2 ml of the enzyme pretreated at 70° C (160 μg protein; specific activity 4.8) was assayed at the indicated temperatures (●———●).

70° C at pH values between 6.5 and 7 (Fig. 3). A similar curve was obtained for the purified enzyme of *Ps. testosteroni* and the enzyme in crude extracts of the other *Pseudomonas* species.

The enzyme could be stored for several weeks at 4° C without loss of activity, but repeated freezing and thawing inactivated the enzyme.

Reaction rate as function of the substrate concentration

A hyperbolic response of the initial reaction rate to increasing concentrations of R-allantoin was observed and a K_m of 4.4 mM was extrapolated from a Lineweaver-Burk plot measured at pH 6.5 in 50 mM phosphate buffer. A similar experiment performed in 0.3 M acetate buffer, pH 5.1, gave a K_m value of 2.6 mM.

In spite of these familiar kinetics, the reaction course follows first-order kinetics at different initial substrate concentrations (S_0). The reaction is followed by measurement of the optical rotation which gives the difference between the amounts of the optical antipodes. Plots of the logarithm of the optical rotation against time show a linear course while the reaction proceeds to residual substrate concentrations below 0.01 S_0. This holds for S_0 values ranging from 35 to 0.5 times K_m.

This result is most obviously explained by assuming that this racemase catalyzes a reversible interconversion of the R- and S-form. If both antipodes are bound equally well to the enzyme and if the reaction rates are equal in both directions, one must expect pseudo first-order kinetics, since the amount of enzyme available for the conversion of R-allantoin decreases continually during the reaction by a factor (R—S)/(R+S), in which R and S represent the concentrations of R- and S-allantoin, respectively.

Assumptions as made here do not apply generally for racemases since the

reaction rates and K_m-values are different for the optical antipodes in the case of alanine racemase of *Escherichia coli* [23] and the K_m-values of the L- and D-isomers are different for hydroxyproline 2-epimerase of *Ps. putida* [24] and proline racemase of *Clostridium sticklandii* [25].

Substrate specificity

Although S-allantoin was only available in dilute solutions containing also RS-allantoin as a result of racemization during the preparation, S-allantoin appeared to be a substrate as effective as R-allantoin under similar conditions.

N-Carbamoyl-L-asparagine, *N*-acetyl-L-glutamine and the hydantoins of *N*-carbamoyl-D-asparagine, *N*-carbamoyl-D- and -L-aspartate, *N*-carbamoyl-L-glutamine and *N*-carbamoyl-L-methionine were not detectably racemized by the enzyme.

Other allantoin derivatives could not be tested, since they were not available as optical isomers.

Cofactor requirement

A number of compounds known to be effective as coenzymes or cofactors of other racemases were tested. Addition of pyridoxal 5′-phosphate, pyridoxine, FAD and FMN (all 1 mM) or of NAD and ATP (both 20 mM) to the incubation mixture or preincubation of the enzyme for 30 min at 30° C with these compounds did not stimulate the activity. In the case of the first four compounds optical rotation was measured at 546 nm and activity was calculated from the molar rotation of allantoin at this wavelength [1] and the observed optical rotation in a similar way as in the standard assay.

The fluorescence spectrum of the purified enzyme did not indicate the presence of one of these coenzymes. The test conditions would allow the detection of 1 ng of pyridoxine or pyridoxal 5′-phosphate per 0.2 mg of protein. An intrinsic fluorescence was observed with excitation and emission maxima at 285 and 340 nm, respectively. Under similar conditions the fluorescence intensity of bovine serum albumin (excitation 285 nm; emission 350 nm) was identical.

Metal ions were not required for enzymic activity. No effect was exerted by Ca^{2+} and Mg^{2+} (both 8 mM), while Zn^{2+}, Co^{2+} and Mn^{2+} (all 0.8 mM) inhibited the enzymic activity 79%, 28% and 10%, respectively. EDTA (3 mM) had neither effect during incubation nor after a 30 min preincubation with the enzyme at 30° C. *o*-Phenanthroline (2 mM) had no influence when present in the incubation mixture.

No effect was exerted by cysteine, glutathione, thioglycolate, β-mercaptoethanol or dithiothreitol (all 20 mM) added to the incubation mixture. Preincubation for 30 min at 30° C with dithiothreitol (20 mM) stimulated the enzymic activity 1.2-fold.

Inhibitors of allantoin racemase

A series of allantoin derivatives (10 mM) were tested for their action on the enzyme. No effect was exerted by 1-acetylallantoin, 1,3-diacetylallantoin, homoallantoin, 3-methylallantoin and 5-acetylaminohydantoin. Moreover, allantoate, urate, L-tryptophan, L-proline, L-methionine and the hydantoins of

TABLE II

DEGRADATION OF R- AND RS-ALLANTOIN BY ASPECIFIC ALLANTOINASE OF *Pr. RETTGERI*

Substrate	Incubation time (min)	Change in optical rotation* (μmol/ml)	Allantoate formed (μmol/ml)	R-Allantoin degraded (μmol/ml)	S-Allantoin degraded (μmol/ml)
R-Allantoin	15	4.3	4.6	4.5	—
	30	8.5	8.7	8.6	—
RS-Allantoin	15	0.5	5.3	2.9	2.4
	30	1.0	10.6	5.8	4.8

* Equivalent to the difference between the amounts of R- and S-allantoin degraded.

N-carbamoyl-L-glutamine, *N*-carbamoyl-D-asparagine and *N*-carbamoyl-D- and -L-aspartate were also without effect on the enzymic activity. Hydantoin, 5-aminohydantoin and the hydantoin of *N*-carbamoyl-L-methionine inhibited the enzymic activity 20%, 25% and 34%, respectively.

Iodoacetate (3 mM), NaCN (30 mM) and $NaBH_4$ (30 mM) did not affect the enzymic activity, while addition of NH_2OH (30 mM) to the incubation mixture gave an inhibition of 30%. Preincubation of the enzyme for 30 min at 30°C with iodoacetate (8 mM) or *o*-phenanthroline (2 mM) decreased the enzymic activity 63% or 17%, respectively.

Incubation of the enzyme for 30 min at 30°C with NH_2OH (10 mM) resulted in an almost complete loss of activity. After dialysis against 50 mM phosphate buffer, pH 6.5, the activity was regained completely. This result suggested that a functional carbonyl-group is required for catalytic activity, but the study on the fluorescence spectrum seems to exclude pyridoxal 5'-phosphate as a candidate for this group.

Aspecific allantoinase of Pr. rettgeri

The aspecificity of the allantoinase of *Pr. rettgeri*, *E. coli* and *Streptococcus allantoicus* was reported previously [7]. The observed aspecificity might be caused by the concerted action of allantoin racemase and S-allantoinase. Therefore, the aspecificity of allantoinase of *Pr. rettgeri* was reinvestigated.

The enzyme was incubated with R- and RS-allantoin under the conditions given for the allantoinase assay. The amount of allantoate formed was measured and simultaneously the change of the optical rotation was recorded. The latter values were corrected for chemical racemization. The results are represented in Table II and confirm that the enzyme degrades both R- and S-allantoin without the participation of allantoin racemase, since the change in optical rotation, observed in the incubation mixture with R-allantoin, accounts for the production of allantoate. Consequently no S-allantoin is formed from R-allantoin. R-allantoin is degraded 1.2-fold faster than S-allantoin.

Discussion

Allantoin racemase is present in *Ps. testosteroni*, *Ps. putida* and *Ps. fluorescens*, biotypes A through E. The enzyme is not common to all fluorescent

pseudomonads since *Ps. aeruginosa* and *Ps. fluorescens*, biotype F, are devoid of it. Moreover, the enzyme appeared to be a taxonomic tool to differentiate between *Ps. acidovorans* and *Ps. testosteroni*. The enzyme was absent in representatives of the other groups of aerobic pseudomonads described by Stanier et al. [9].

Allantoin racemase enables the bacteria to use both optical antipodes of allantoin. Although the S(+)-form is the naturally occurring one, the rate of racemization is high enough, especially in alkaline conditions [15], to justify the supposition that allantoin is present in the racemic form in dead materials, such as decaying plant materials or excretion products of lower animals. Moreover, uric acid occurring in such materials is degraded generally under alkaline conditions and the allantoin formed will be racemized very rapidly. *Ps. testosteroni*, *Ps. putida* and most of the *Ps. fluorescens* strains solve this problem by synthesis of allantoin racemase, which together with S(+)-allantoinase present in these bacteria, makes both optical antipodes available for growth. Another group of bacteria, viz. *E. coli*, *Str. allantoicus* and *Pr. rettgeri* form an aspecific allantoinase to overcome this problem.

Several cofactors have been demonstrated to be present and/or active in racemases, viz. pyridoxal 5'-phosphate [26—30], FAD [27] and ATP [31]. None of these compounds affected the reaction of allantoin racemase or could be detected in the purified enzyme preparation.

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