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SPECIFICITY OF BINDING SUBSITES OF ALLANTOICASE

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SUMMARY

1. Allantoicase (allantoate amidinohydrolase, EC 3.5.3.4) converts allantoate into (−)-ureidoglycolate and urea. Moreover, (+)-ureidoglycolate is converted into glyoxylate and urea.

2. The reaction may be written as $E + S \leftrightharpoons ES$, $ES + H_2O \leftrightharpoons EP +$ urea, $EP \leftrightharpoons E + P$, where E is the enzyme, S is allantoate or (+)-ureidoglycolate and P is (−)-ureidoglycolate or hydrated glyoxylate. The dissociation constants of the first and third equilibrium are equal and amount to $2.5 \cdot 10^{-2}$ M. Urea is not bound directly to the enzyme, and the second reaction is rate limiting.

3. The absolute value of the heat of formation of the ES complex is lower than 0.9 kcal/mole, and the energy of activation of the complex is 12.7 kcal/mole.

4. At pH values between 6 and 9 both v_{max} and K_m are independent of the pH. Above pH 9, K_m decreases, while below pH 6, v_{max} decreases.

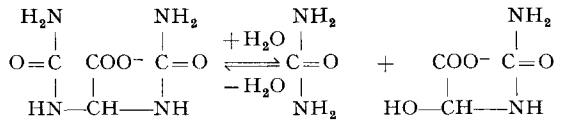
5. About 150 components were examined for competitive inhibition. From the results obtained with carboxylic, hydroxy, D-amino and *N*-carbamoyl-D-amino acids, a simple rule was derived for the calculation of the K_i values of most inhibitors and the K_m values of the substrates. The structure of the inhibitor must be read in a definite steric sequence. Each group of the inhibitor contributes to the K_m or K_i value in such a manner that the resulting value is the product of these contributions.

6. On the basis of the results obtained with the inhibitors, the specificity of the enzymic reaction was studied. No other reaction was found to be catalyzed by the enzyme.

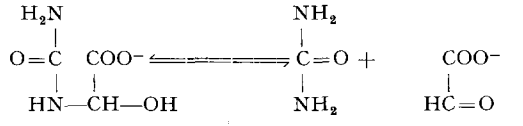
INTRODUCTION

Allantoicase (allantoate amidinohydrolase, EC 3.5.3.4) catalyzes the conversion of allantoate (diureidoacetate) into (−)-ureidoglycolate and urea (Eqn. 1) and the conversion of (+)-ureidoglycolate into glyoxylate and urea[1] (Eqn. 2).
Both reactions lead to the establishment of an equilibrium which can be approached easily from both sites. The nonenzymic hydrolysis of allantoate was studied previously[2].

Allantoicase was found in animals, fungi and various bacteria[3]. The present



allantoate urea (−)-ureidoglycolate (1)



(+)-ureidoglycolate urea glyoxylate (2)

communication deals with the specificity of the binding subsites of the enzyme from *Pseudomonas aeruginosa*, which could be prepared in a rather pure form[4]. Moreover, the binding strength of the substrates as a function of pH and temperature is under investigation.

EXPERIMENTAL

Materials

Sodium allantaote monohydrate was prepared by alkaline hydrolysis of allantoin[5]. Sodium ureidoglycolate (1-ureido,1-hydroxy acetate) was prepared from urea and sodium glyoxylate[6]. In a similar way sodium ureidolactate (1-ureido,1-hydroxy propionate) was synthesized from urea and sodium pyruvate. Oxonic or allantoxanic acid and hydroxonic or dihydroallantoxanic acid were prepared according to BILTZ AND GIESLER[7]. The chemical structure of these compounds was discussed by BRANDENBERGER AND BRANDENBERGER[8]. 5-Aminohydantoin was prepared according to BILTZ AND HANISCH[9]. Homoallantoin (5-ureido,5-methylhydantoin) and sodium homoallantoate (1,1-diureidopropionate) were prepared according to SIMON[10], alloxanic acid according to BILTZ AND KOBEL[11] and uroxanic acid according to BILTZ AND STROBL[12]. The *N*-carbamoylamino acids were isolated from the reaction mixtures of equimolar amounts of potassium cyanate and the amino acids at pH 7–8; the reaction mixtures were incubated for 20 h at room temperature. In this manner the *N*-carbamoyl derivatives of L- and D-alanine, of L- and D-aspartic acid, of L- and D-asparagine, of L-glutamine and of L-glutamic acid were prepared.

N-Carbamoylserine and *N*-carbamoylthreonine were products of the Sigma Chemical Co., St. Louis, calcium D-lactate was from Mann Research Laboratories, New York, and lithium L-lactate was from Serva Entwicklungslabor.

Enzymes

Leucine aminopeptidase (EC 3.4.1.1) was prepared according to HANSON *et al.*[13]. L-lactate:NAD oxidoreductase (EC 1.1.1.27) was obtained from C. F. Boehringer, Mannheim. Jack bean urease (EC 3.5.1.5) was from the Sigma Chemical Co.

Allantoicase was prepared from *Pseudomonas aeruginosa* cells grown in a medium containing allantoin as the sole source of carbon, nitrogen and energy[4]. The enzyme was purified 70 times[4], and the specific activity amounted to about 450

units/mg protein and did not diminish on storage for 8 months at −20° in the presence of 10^{-4} M $MnSO_4$. All experiments reported in this communication were performed with the same enzyme preparation.

Methods

K_m and K_i values of most compounds were measured in the following manner. The initial velocity of allantoate or (±)-ureidoglycolate degradation was determined at 30° in incubation mixtures containing per ml: 220 μmoles triethanolamine–HCl buffer (pH 7.9), 0.17 μmole $MnSO_4$, purified allantoicase, 157, 98, 61.3, 38.3 or 24 μmoles sodium allantoate or sodium (±)-ureidoglycolate, and an appropriate amount of the compound tested for inhibition. This substance was neutralized previously, if necessary. In tests with allantoate or (±)-ureidoglycolate as substrate, 1.68 or 7.0 μg purified allantoicase were used per ml, respectively. In an aliquot of the incubation mixture the amount of ureidoglycolate *plus* glyoxylate or of glyoxylate formed was measured according to the differential glyoxylate analysis using Methods D and C, respectively[14]. In the tests with allantoate as substrate, the values obtained with this analysis were corrected for the small amount of allantoate which was hydrolyzed during the analytical procedure according to Method D (ref. 1). In the tests with ureidoglycolate as substrate, the values obtained were corrected for nonenzymic hydrolysis of this compound during the incubation.

The K_i value of allantoate was measured by testing the inhibiting effect of allantoate in the incubation mixtures with (±)-ureidoglycolate as substrate.

The K_i value of glyoxylate was determined by measuring urea formation in the incubation mixtures given above. The substrate was allantoate, and the inhibiting effect of 0.152 and 0.038 M glyoxylate was tested. The incubation mixtures contained 1.28 mg jack bean urease per ml, and the amount of ammonia produced was determined according to a phenolate–hypochlorite test[15]. The triethanolamine–HCl buffer was replaced by 0.17 M Na_2HPO_4–KH_2PO_4 buffer (pH 7.4), since nitrogen containing buffers, *viz.* Tris, triethanolamine and diethanolamine, interfered with the determination of ammonia.

The K_m value of urea was determined by measuring the enzymic disappearance of urea at 30° in incubation mixtures containing per ml: 70.7, 50.5, 30.3 or 20.2 μmoles urea, 199 μmoles sodium glyoxylate, 190 μmoles Na_2HPO_4–KH_2PO_4 buffer (pH 7.4) and 29.5 μg allantoicase. At various time intervals the amount of urea remaining was determined. A large excess of jack bean urease was added and the amount of ammonia formed was measured[15]. The values obtained were corrected for nonenzymic condensation of glyoxylate and urea.

The K_m value of glyoxylate was determined by measuring the enzymic production of ureidoglycolate at 30° in incubation mixtures containing per ml: 292 μmoles urea, 73.1, 43.9, 26.4 or 15.8 μmoles sodium glyoxylate, 222 μmoles triethanolamine–HCl buffer (pH 7.9) and 5.4 μg allantoicase. The amounts of ureidoglycolate formed and glyoxylate which disappeared, were determined according to the differential glyoxylate analysis and corrected for the nonenzymic reaction. The K_i value of allantoate was measured by testing the inhibiting effect of allantoate (42 μmoles/ml) in the incubation mixtures.

K_i and K_m values were determined by plotting the results according to LINEWEAVER AND BURK[16] and are expressed in M/l. In most instances the negative

logarithms of these values, pK_m and pK_i, are given in this report. No correction was made for partial dissociation of the inhibitors, and the values refer to the sum of dissociated and undissociated molecules. V_{max} and v are expressed in μmoles converted/ml per min and refer to the standard incubation mixtures given above. Instead of v_{max} the maximal specific activity of the enzyme is given in this report.

Buffer molarities refer to the concentration of the buffering substances. One unit of enzyme activity is defined as the amount which will catalyze the transformation of 1 μmole of allantoate per min at 30° in the incubation mixture containing 157 μmoles sodium allantoate. The specific activity is expressed in units per mg protein.

RESULTS

K_m and K_i values of substrates and products

Allantoicase catalyzes the two reactions given in the INTRODUCTION. Both reactions lead to the establishment of an equilibrium and can be measured in either direction. The K_m values of the substrates and products thus determined, are given in Table I. Moreover, the competitively inhibiting effects of allantoate on the second

TABLE I

pK_m AND pK_i VALUES OF SUBSTRATES AND PRODUCTS OF ALLANTOICASE

pK_m and pK_i values are measured as mentioned in the EXPERIMENTAL section. Reactions (1) and (2) are given in the INTRODUCTION.

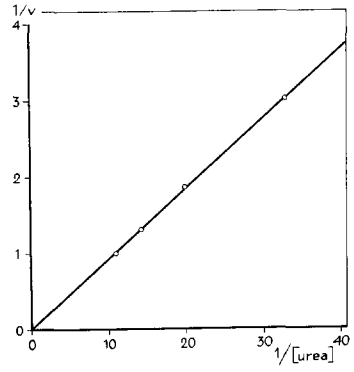
Substrate or product	*Reaction (1)*	*Reaction (2)*
Allantoate	$pK_m = 1.60$	$pK_i = 1.66$
(±)-Ureidoglycolate		$pK_m = 1.58$
Glyoxylate	$pK_i = 1.52$	$pK_m = 1.50$
Urea		$pK_m = -\infty$

reaction in both directions and of glyoxylate on the first reaction, were measured. Because (+)- and (−)-ureidoglycolate are not available at present, the K_m values refer to (±)-ureidoglycolate. The pK_m and pK_i values of allantoate were about equal, as expected, since both values refer to the binding strength of allantoate to the same active center. The same was true for glyoxylate. Moreover, the pK_m and pK_i values of all substrates and products, except urea, were about the same. The K_m value of urea was immeasurably high (Fig. 1), suggesting that urea is not bound directly to the enzyme but to the enzyme–glyoxylate or enzyme–(−)-ureidoglycolate complex; for example:

$$E + \text{glyoxylate} \rightleftharpoons E\text{-glyoxylate}, \quad E\text{-glyoxylate} + \text{urea} \rightleftharpoons E\text{-(+)-ureidoglycolate} \rightleftharpoons E + \text{(+)-ureidoglycolate}$$

K_m and v_{max} as a function of temperature

Fig. 2 shows the pK_m for allantoate as a function of temperature. The maximal specific activity of the enzyme which is also given was calculated from the v_{max} values determined at the various temperatures. The energy of activation of the ES complex was 12.7 kcal/mole.



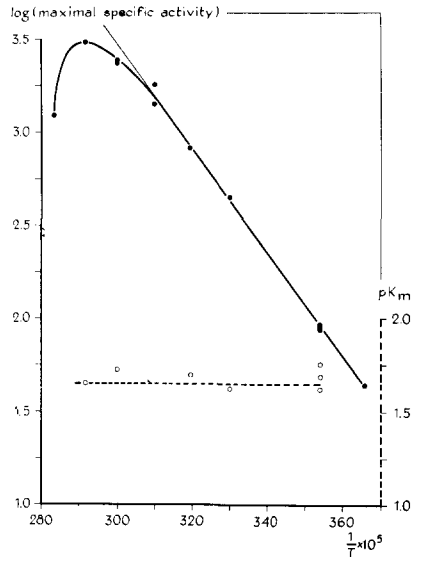


Fig. 1. Determination of K_m of urea. The catalytic effect of allantoicase on the reaction between glyoxylate and urea was determined under conditions given in the EXPERIMENTAL section.

Fig. 2. pK_m and maximal specific activity of allantoicase as a function of temperature. Allantoate was tested as substrate in the standard assay mixture (EXPERIMENTAL section) containing an appropriate amount of the enzyme.

The energy of activation of nonenzymic allantoate hydrolysis amounted to 21.5 kcal/mole; this value was calculated from data given previously[2]. From the dependence of K_m on the temperature it appeared that the heat of formation of the ES complex amounted to between +0.9 and −0.9 kcal/mole. Maximal enzymic activity was measured at 75°.

K_m and v_{max} as a function of pH

pK_m and maximal specific activity of allantoicase with allantoate or (±)-ureidoglycolate as substrate are given as a function of pH in Fig. 3. The pK_m values for both substrates were independent of pH below pH 8.5, and the maximal specific activity was constant above pH 6.0. Below pH 6.0 the logarithm of the maximal specific activity, and above pH 8.5, the values of pK_m obeyed rules of DIXON AND WEBB[17], and the slopes of the lines changed from 0 to +1 or −1.

In alkaline solutions hydroxyl ions behaved as competitive inhibitors of the enzyme. Since the substrates did not dissociate at these pH values, part of the enzyme with a pK value of about 9.4 caused the observed drop of pK_m. In acid solutions part of the ES complex was protonated and caused the decrease of enzymic activity. The pK value of this group appeared to depend on the substrate bound and amounted to 5.6 in the case of allantoate and 6.0 in the case of (±)-ureidoglycolate.

Above pH 6 the maximal specific activity for allantoate was about 5 times higher than that for (±)-ureidoglycolate. In the latter case, both optical antipodes were substrates because (+)-ureidoglycolate was transformed to urea and glyoxylate, and (−)-ureidoglycolate reacted with urea yielding allantoate. If both antipodes

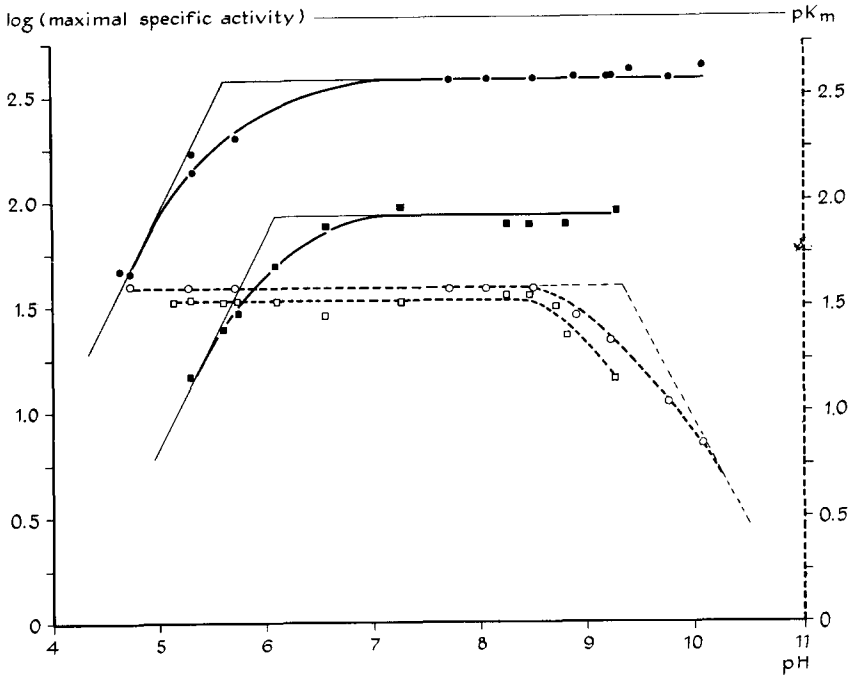


Fig. 3. pK_m and maximal specific activity of allantoicase as a function of pH. The enzyme was tested at 30° with allantoate (○, ●) and (±)-ureidoglycolate (□, ■) as substrate according to the standard assay (EXPERIMENTAL section). pK_m values (○, □) and maximal specific activity (●, ■) are given. The triethanolamine–HCl buffer was replaced by 0.083 M acetic acid–sodium acetate, succinic acid–sodium succinate, diethanolamine–HCl and Na_2CO_3–$NaHCO_3$ buffers in the pH regions 4.6–5.7, 5.8–6.5, 7.7–9.2 and 8.9–10.2, respectively. In tests with (±)-ureidoglycolate as substrate corrections were made for nonenzymic hydrolysis of this compound, which is particularly rapid at pH values below 5 and above 8.

were bound to the enzyme with the same strength as allantoate, glyoxylate and (±)-ureidoglycolate, then only half of the enzyme would be available for the degradation of (+)-ureidoglycolate to glyoxylate and urea. The latter reaction was measured and, therefore, the specific activity for this reaction was only 2.5 times smaller than that for allantoate conversion.

Competitive inhibitors of allantoicase

Besides hydroxyl ions (pK_i = 4.6) and glyoxylate (pK_i = 1.52), various other substances competitively inhibited the conversion of allantoate by allantoicase. In order to obtain more information on the specificity of allantoicase for competitive inhibitors, we tested about 150 compounds. The results are partly summarized in Table II.

Among the inorganic compounds tested, Cl^- (pK_i = −0.04) and F^- (pK_i = −0.28) inhibited only slightly, CNO^- (pK_i = 1.05) more strongly.

Various carboxylic acids inhibited to about the same extent, *viz.* acetate (pK_i = 0.4), monochloroacetate (pK_i = 0.3), monoiodoacetate (pK_i = 0.33), tartrate (pK_i = 0.52), tartronate (pK_i = 0.43) and carbonate (pK_i = 0.53). The carboxylate group appeared to be a requisite for inhibition because acetamide (pK_i < −0.6) had

TABLE II

INHIBITING EFFECT OF D-HYDROXY ACIDS, D-AMINO ACIDS AND *N*-CARBAMOYL-D-AMINO ACIDS ON ALLANTOICASE ACTIVITY

OH NH_2 $NH{-}CO{-}NH_2$ H–C–R COOH *R*	*pK_i-values*			
	D-*hydroxy acids* (1)	D-*amino acids* (2)	*N-carbamoyl-*D-*amino acids* (3)	*Difference* (3) − (2)
–H	2.59	1.2	2.51	1.3
$-CH_3$	2.42*	0.40	2.10	1.7
$-CH_2-CH_3$	2.99**			
$-CH_2-CH_2-CH_3$	2.96**			
$-CH-(CH_3)_2$	2.91**	0.36	3.0	2.6
$-CH(CH_3)-CH_2-CH_3$	2.51**			
$-CH_2OH$	2.41**	2.40**	3.4	1.0
$-CH_2-O-PO_3H_2$	< 0.8	< 0.3***		
$-CH(OH)-CH_3$		2.16**	3.4	1.2
$-CH_2-CH_2OH$		< 0.6***		
–COOH	0.43			
$-CH_2-COOH$	0.33***	< 0.5	1.5	
$-CH_2-CO-NH_2$		2.45	4.03	1.6
$-CH_2-CH_2-NH_2$		< 1.0***		
$-CH(OH)-COOH$	0.52***			
$-CH_2-CH_2-COOH$			0.94	
$-CH_2-CH_2-CO-NH_2$		1.82	3.2	1.4
$-CH(COOH)-CH_2-COOH$	< 0.3			
$-NH-CO-NH_2$	1.58****		1.66	

* Contains < 4% L-lactate, pK_i of L-lactate smaller than 1.9.
** The racemic mixture was tested; pK_i values refer to the D-antipode.
*** The racemic mixture was tested; pK_i values refer to the mixture.
**** pK_m of (±)-ureidoglycolate.

no such effect. Large ions, *e.g.* benzoate, *p*-hydroxy benzoate, nicotinate and 2-hydroxy pyridine-5-carboxylate, were either poor or non-inhibitors (pK_i < 0). The rather strong inhibiting effect of formate (pK_i = 1.25) was striking in this context.

In contrast to glyoxylate, pyruvate (pK_i < 0.4) was a poor or non-inhibitor.

Competitive inhibition of hydroxy acids

In general, α-hydroxy acids were rather strong inhibitors (Table II). The inhibiting effects were of the same order of magnitude: glycolate (pK_i = 2.59) D-lactate (pK_i = 2.42), 1-hydroxy butyrate (pK_i = 2.99), 1-hydroxy valerate (pK_i = 2.96), 1-hydroxy isovalerate (pK_i = 2.91) and 1-hydroxy isocapronate (pK_i = 2.51). The effects of the optical antipodes were tested in the case of lactate. Calcium D-lactate was examined with lactate dehydrogenase and appeared to contain less than 4% L-lactate. The pK_i value of this compound was 2.42. In contrast, the pK_i value of lithium L-lactate was 1.88. The last four hydroxy acids of the series mentioned above were tested in the racemic form, but the pK_i values refer to the D-antipodes.

Various derivatives of hydroxy acids were tested. The pK_i value of glycerate fitted the above values and was 2.41. Blocking of the carboxylate group canceled the inhibiting effect: ethyl ester of D-lactate (pK_i < −0.2) and glycolcyanide (pK_i < 0.8). The presence of a second anionic group in the molecule strongly affected the inhibiting

strength: *e.g.* 3-phosphoglycerate ($pK_i < 0.8$), tartronate ($pK_i = 0.43$), DL-malate ($pK_i = 0.33$), tartrate ($pK_i = 0.52$) and isocitrate ($pK_i < 0.3$). The second anionic group appeared to abolish the enhancing effect of the hydroxy group, and the inhibiting power dropped to the level of carboxylic acid. 2-Hydroxy butyrate ($pK_i = 1.0$) was less inhibitory than 1-hydroxy butyrate. *p*-Hydroxy benzoate and ascorbate showed no inhibiting effect ($pK_i < -0.15$). A simple alcohol function such as that present in ethanol ($pK_i < -0.2$) and glycol ($pK_i < -0.3$) did not provide an inhibiting effect.

Among the α-hydroxy acids, the substrate of allantoicase, (±)-ureidoglycolate ($pK_m = 1.58$) was bound only slightly.

Thioacids, *e.g.* thioglycolate, could not be tested because these compounds interfered with the glyoxylate analysis.

Competitive inhibition by amino acids

pK_i values equal to or lower than 0.4 were measured for L-α-amino acids, *viz.* L-alanine, L-valine, L-serine, L-threonine, L-aspartate, L-asparagine, L-glutamate, L-glutamine, L-citrulline and L-arginine.

pK_i values of glycine ($pK_i = 1.2$) and D-amino acids are given in Table II. D-serine ($pK_i = 2.40$), D-threonine ($pK_i = 2.16$), D-asparagine ($pK_i = 2.45$) and D-glutamine ($pK_i = 1.82$) were rather strong inhibitors. The latter two compounds contain a $-CO-NH_2$ grouping which is also present in the substrates allantoate, (±)-ureidoglycolate and urea. Succinamic acids ($pK_i < 0.2$), D-ornithine ($pK_i < 1.0$) and D-aspartate ($pK_i < 0.5$) were either bound slightly or not at all to the enzyme. Therefore, a definite steric position of the integral $-CO-NH_2$ grouping and the amino grouping was required for inhibition. In contrast to D-serine and D-threonine, DL-homoserine ($pK_i < 0.6$) and DL-serine *O*-phosphate ($pK_i < 0.3$) were weak inhibitors. The importance of the amino grouping for the inhibition could be seen by comparing the pK_i values of 2-hydroxy butyrate ($pK_i = 1.0$) and D-serine ($pK_i = 2.40$).

Effect of N-acylamino acids

N-Carbamoylamino acids are structurally related to the substrates of allantoicase. This is particularly true for *N*-carbamoylasparagine.

N-Carbamoyl derivates of L-alanine, L-valine, L-asparagine, L-aspartate, L-glutamine and L-glutamate exposed a slight inhibiting effect if any ($pK_i < 0.5$). In contrast, *N*-carbamoyl-D-amino acids were rather strong inhibitors, especially the derivatives of D-amino acids which inhibited themselves (Table II). In general, the pK_i values of *N*-carbamoyl-D-amino acids were 1.0–1.7 higher than those of the corresponding D-amino acids. *N*-Carbamoyl-D-valine ($pK_i = 3.0$), however, inhibited unexpectedly strongly.

The binding strength of the enzyme for allantoate ($pK_i = 1.66$) and (±)-ureidoglycolate ($pK_m = 1.58$) was rather low compared with that of the *N*-carbamoyl derivatives of D-serine ($pK_i = 3.4$), D-threonine ($pK_i = 3.4$), D-asparagine ($pK_i = 4.03$) and D-glutamine ($pK_i = 3.2$), and even lower than that of the *N*-carbamoyl derivatives of glycine ($pK_i = 2.51$) and D-alanine ($pK_i = 2.10$).

The ethyl ester of *N*-carbamoylglycine ($pK_i = 0.65$) showed a slight inhibitory effect, which could be due to contamination.

Various other *N*-acyl derivatives of amino acids showed a slight inhibiting

effect if any, *e.g.* *N*-formylglycine ($pK_i = 0.57$), *N*-acetylglycine ($pK_i = 0.60$), *N*-acetyl-D-asparagine ($pK_i = 0.35$), glycylglycine ($pK_i < 0.4$), and saturated solutions of *N*-benzoylglycine, glycocyamine and creatine. The inhibiting effect of the former compounds was similar to that of carboxylic acids.

Effect of miscellaneous substances

Allantoicase was not perceptibly inhibited by saturated solutions of allantoin, creatinine, uracil, dihydrouracil, alloxanate, uroxanate, hydantoin and the hydantoin derived from L-alanine, L-valine, D-valine, D-aspartate, L-glutamate and D-glutamate.

5-Aminohydantoin ($pK_i = 2.5$), oxonate ($pK_i = 2.6$), hydroxonate ($pK_i = 1.80$) and orotate ($pK_i = 1.0$) were competitive inhibitors.

α-Methyl derivatives of allantoate and ureidoglycolate, *viz.* homoallantoate and ureidolactate, were bound only slightly if at all ($pK_i < 0.4$). The same was true for *N*-formylurea, *N*-acetylurea, hydroxyurea, methylurea, thiourea, guanidine, biuret, urethane, semicarbazide, hydrazine, phenylhydrazine, hydroxylamine, carbamoylphosphate and ammonium chloride.

Competitive inhibition of allantoicase tested with ureidoglycolate as substrate

The inhibitors of allantoicase discussed so far were examined with allantoate as substrate. Ureidoglycolate degradation was inhibited in a similar way. D-asparagine, *N*-carbamoyl-D-asparagine and *N*-carbamoyl-D-alanine inhibited ureidoglycolate degradation to the same extent as allantoate degradation. The L-derivatives of these compounds were either poor or non-inhibitors.

Specificity of the enzymic reaction of allantoicase

The study on the competitive inhibitors revealed some information on the specificity of the binding subsites of the enzyme. With this knowledge in view, a number of compounds were examined for substrate activity.

The enzyme produced urea from allantoate and (+)-ureidoglycolate. The specific activity tested with allantoate as substrate was about 450 units/mg. No urea or ammonia was formed from *N*-carbamoyl derivatives of glycine, L-alanine, D-alanine, L-aspartate, L-asparagine, D-asparagine and D-glutamine, or from hydroxyurea, oxonate, hydroxonate or oxamate. The specific activity of the enzyme tested with these compounds was less than 0.1 unit/mg.

No glyoxylate or acid-labile glyoxylate derivatives were produced from oxonate or hydroxonate. The specific activity was less than 0.1 unit/mg.

The enzyme catalyzed the condensation of urea with glyoxylate or (−)-ureidoglycolate. No perceptible disappearance of urea or ammonia occurred as a result of the action of the enzyme in the presence of glycolate, oxalate, pyruvate, L-lactate, tartrate, malate or DL-threonine. Moreover, allantoicase did not catalyze the oxidation of NADH or the reduction of NAD^+ in the presence of glyoxylate or glycolate.

Allantoicase did not catalyze a reaction between glyoxylate and guanidine, formylurea, pyruvate, α-ketoglutarate, succinate, malate or tartrate, nor a condensation of glyoxylate with itself as observed for glyoxylate carboligase, not even in the presence of Mg^{2+}, thiamine pyrophosphate and FAD.

Allantoicase resembles leucine aminopeptidase to some extent, especially in its metallo–enzymic character, which will be discussed separately. However, leucine

naphthylamide did not act as a substrate for allantoicase (specific activity smaller than 0.01 unit/mg), and leucine aminopeptidase did not act on allantoate and was either not at all or only slightly inhibited by carbamoyl-D-asparagine.

DISCUSSION

Allantoicase catalyzes the conversion of allantoate into (−)-ureidoglycolate and urea and of (+)-ureidoglycolate into glyoxylate and urea. The K_m values of allantoate, (±)-ureidoglycolate and glyoxylate are about equal. These compounds appear to compete for the same active site of the enzyme and are bound with about equal strength. Although no direct evidence could be presented, it seems likely that (+)- and (−)-ureidoglycolate are bound with the same strength. Urea is not bound directly to the enzyme, since the K_m value is immeasurably high. These results fit the following reaction sequence best:

$$E + S \underset{-1}{\overset{+1}{\rightleftharpoons}} ES, \quad ES + H_2O \underset{-2}{\overset{+2}{\rightleftharpoons}} EP + \text{urea}, \quad EP \underset{-3}{\overset{+3}{\rightleftharpoons}} E + P$$

in which E is the enzyme, S is allantoate or (+)-ureidoglycolate and P is (−)-ureidoglycolate or the hydrated form of glyoxylate. Both S and P are bound reversibly to the enzyme, and in this bound form the equilibrium $S + H_2O \leftrightharpoons P +$ urea is established either *via* a one-step or a two-step mechanism[2]. In the latter instance, a dehydrated form of P is an intermediate. The enzyme cannot be saturated with urea, and the reverse reaction appears to be infinitely accelerated by increasing amounts of urea. Therefore, the reaction $ES + H_2O \leftrightharpoons EP +$ urea must be rate limiting.

The dependence of v_{max} and K_m on the temperature indicates that the equilibrium position of the first reaction in the above equation is almost independent of the temperature. The equilibrium position of the second reaction shifted to the right when the temperature was enhanced; ΔH of the second reaction to the right was calculated from data previously reported[2] and amounted to +3.5 kcal/mole.

The dependence of v_{max} and K_m on the pH indicates that hydroxyl ions compete with the substrate for the active site of the enzyme. A group in the active site is deprotonated or loaded with hydroxyl ions; the pK of this group is 9.4. Moreover, a group of the ES complex with a pK value of about 6.0 seems to play a part in the catalytic action of the enzyme. Allantoicase contains a strongly bound manganous ion in the active center (C. VAN DER DRIFT AND G. D. VOGELS, in preparation). Perhaps, these pK values refer to the formation of a Mn^{2+}–OH^- complex and the breaking of one of the Mn^{2+}–enzyme bonds of the ES complex. The logarithm of the stability constant of $[Mn(OH)^+]/[Mn^{2+}][OH^-]$ is reported[18] to be 4.52 at 20°. The pK value of 9.4 agrees well with this value.

The measurements of the K_i values of competitive inhibitors of the allantoicase reaction reveal the following:

(1) All inhibitors are anions, and the most potent inhibitors contain a carboxylate group. If the inhibitor is not too large and contains no other groups which can be bound to the enzyme, the pK_i value is about 0.4. Formate is bound rather strongly (pK_i = 1.25).

(2) α-Hydroxy acids are strongly bound (pK_i from 2.4 to 3.0). The D-antipode is bound much better than the L-antipode. A second negative group present in the molecule of the inhibitor cancels the extra binding strength resulting from the presence of the hydroxyl group. β-Hydroxy acids are poor inhibitors.

(3) In contrast to L-amino acids, glycine and some D-amino acids are bound rather strongly. These D-amino acids contain a hydroxyl or $-CO-NH_2$ function. One of these functions and the presence of the α-amino group are required for strong binding, and the steric orientation must be similar to that of D-amino acids.

(4) In contrast to the *N*-carbamoyl derivatives of L-amino acids, those of D-amino acids are strong inhibitors. Other *N*-acyl derivatives exposed no inhibiting effect exceeding that of carboxylic acids.

The above rules are given schematically and quantitatively in Fig. 4. Following the dashed arrow one can calculate the pK_i or pK_m values by adding the values indicated for the groups separately. If the compound concerned does not contain one of the indicated groups at the right position, further calculation should be stopped, and the pK_i or pK_m value calculated at that moment is the right value.

A few compounds do not comply with the above rules. The second hydroxyl group present at the β-carbon atom of glycerate does not contribute to the binding strength. The effect of the amino group in D-amino acids is not observed in the case of D-alanine and D-valine. 5-Aminohydantoin ($pK_i = 2.5$) and formate are bound unexpectedly strongly.

These rules lead to the following conclusions:

(1) The pK_i or pK_m values can be calculated by adding or subtracting the pK_i or pK_m values of the particular groups in the molecule; in other words the K_i or K_m

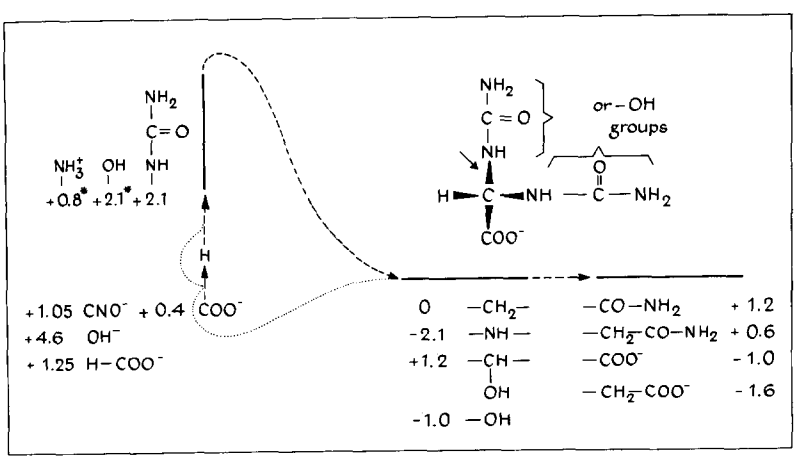


Fig. 4. Specificity of the binding subsites of allantoicase. The surface of the enzyme is thought to be located in the left and lower part of the figure. The steric formulas of allantoate and the other substrates are given in the right upper part, and an arrow indicates the bond which is most probably cleaved. The steric arrangement of the subsites is similar to that given for allantoate. The figures given refer to the contribution of each group to the pK_i and pK_m values of the molecule (see text). If ^-OH is present at this position the dashed arrow must not be followed further. Moreover, the effect of this group is canceled by the presence of a second anionic group in the molecule. The contribution of the amino group at this position in only valid if H or one of the indicated groups is present in the right lower part of the figure.

value of a compound is the product of the K_i and K_m values of the particular groups.

(2) The effect of the groups must be read in a definite direction. Most probably binding of the substrate proceeds in the same direction.

It is important to note that the above rules do not imply that the substrate is bound to five separate interaction sites of the enzyme. Most probably three such sites suffice to explain the results.

Binding of the inhibitor in the upper part of Fig. 4 proceeded most specifically. The inhibitor is not bound when the ureido group is replaced by $-CH_2-CO-NH_2$, $-NH-CO-H$, $-NH-CO-CH_3$, $-NH-CO-C_6H_5$, $-NH-CO-CH_2-NH_2$, $-NH-C(=NH)-NH_2$. A $-NH_3^+$, $-OH$ or $-NH-CO-NH-$ present at this site enhances the binding strength of the inhibitor. The latter group is present in oxonate and hydroxonate.

The relatively low K_m values of the substrates allantoate, ureidoglycolate and glyoxylate seemed to be caused by a repulsive effect in the right part of the molecule. This part exhibited a less marked specificity.

The high specificity of the enzyme is a result of the specificity of the two places at which the ureido or hydroxy groups attach. Moreover several of the compounds tested cannot be split along a reaction mechanism similar to that of allantoate which involves the formation of a double bond at the α-carbon atom[2].

Because the absolute configurations of (+)- and (−)-ureidoglycolate are not known, it is not possible to decide which of the ureido groups is split off by the enzyme. Work is in progress at present on the isolation of the product of allantoate degradation *viz.* (−)-ureidoglycolate and on the determination of the chiro-optical properties of this compound. The results indicate that (−)-ureidoglycolate is *S*-ureidoglycolate according to the nomenclature of CAHN *et al.*[19] (E. J. 'S-GRAVENMADE AND G. D. VOGELS, unpublished results). This would mean that the ureido group of allantoate indicated by an arrow in Fig. 4 is split off.

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