BBA 65554

ACTIVATION AND INACTIVATION OF ALLANTOATE AMIDOHYDROLASE

C. VAN DER DRIFT AND G. D. VOGELS
Department of Biochemistry, University of Nijmegen, Nijmegen (The Netherlands)

(Received June 24th, 1966)
(Revised manuscript received November 8th, 1966)

SUMMARY

Allantoate amidohydrolase from *Streptococcus allantoicus* has been purified 50-fold by acetone fractionation, DEAE-cellulose chromatography and gel filtration. Mn^{2+} was essential for catalytic activity; optimal activity was obtained at 10^{-4} M Mn^{2+}. The purified enzyme could be activated by pretreatment at pH values below 4.3 or by pretreatment with $2 \cdot 10^{-5}$ M EDTA at pH 6.0. Optimal enzymic activity was measured at pH 8.5. Activated enzyme could be inactivated completely by $2 \cdot 10^{-4}$ M Mn^{2+} at pH 6.0. This inactivation was not observed below pH 4.3 or above pH 8. Activation and inactivation were probably the result of a reversible dissociation of the Mn^{2+}–protein complex.

INTRODUCTION

The metabolism of allantoin and allantoate in several microorganisms has been communicated previously[1-3]. Allantoinase (allantoin amidohydrolase, EC 3.5.2.5) transformed allantoin to allantoate[4,5]. In *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Penicillium notatum* and *Penicillium citreo-viride* allantoate was degraded to urea and ureidoglycolate by the action of allantoicase[3] (allantoate amidinohydrolase, EC 3.5.3.4). In *Streptococcus allantoicus*, *Arthrobacter allantoicus*, *Escherichia coli*, *Escherichia coli* var. acidilactici, *Escherichia freundii* and *Pseudomonas acidovorans* allantoate amidohydrolase produced CO_2, NH_3 and ureidoglycine from allantoate, and ureidoglycine in turn was converted to ammonia and ureidoglycolate[1]. In both groups of organisms ureidoglycolate was degraded to glyoxylate and urea by the enzyme ureidoglycolase (ureidoglycolate amidinohydrolase)[3,6].

Allantoate amidohydrolase could be activated by pretreatment at low pH values and the activated enzyme could be inactivated by subsequent treatment at a pH of about 6 (ref. 1). The present report deals with the purification of the enzyme and the effect of Mn^{2+} on its activation and inactivation at various pH values.

EXPERIMENTAL

Materials and methods

The preparation of sodium allantoate and ureidoglycolate and of cell-free extracts from *S. allantoicus* and *A. allantoicus* were described earlier[1,2]. Glutamate dehydrogenase was obtained from C. F. Boehringer. GSH was purchased from Sigma.

Ammonia was determined by dehydrogenation of NADH with glutamate dehydrogenase in the presence of α-ketoglutarate[1]. Allantoate, glyoxylate and the sum of ureidoglycine and ureidoglycolate were determined by differential glyoxylate analysis[2]. Protein was measured according to LOWRY *et al.*[7] using bovine serum albumin as standard and nucleic acids were determined from the absorbances at 280 and 260 mμ.

Purification of allantoate amidohydrolase

Acetone was added dropwise to the crude cell-free extract at $-10°-15°$. The protein fractions obtained between 0–53% and between 53–58% final acetone concentration were separated at 10 000 $\times$ *g* for 25 min at $-20°$. The latter fraction was combined with the protein fraction obtained between 48–53% final acetone concentration by reprecipitation of the former fraction dissolved in 0.05 M Tris–HCl buffer (pH 7.5). The Tris–HCl buffer (pH 7.5) used in this and other purification steps contained $1.7 \cdot 10^{-4}$ M EDTA. The combined protein fractions, dissolved in the Tris buffer, were applied to a DEAE-cellulose column (32 cm $\times$ 1.8 cm), thoroughly equilibrated with 0.05 M Tris–HCl buffer (pH 7.5). The adsorbed material was eluted stepwise with the same buffer containing an increasing NaCl concentration. The fraction containing the allantoate amidohydrolase activity was eluted with 0.35 M NaCl in the buffer and was dialyzed overnight against 0.05 M Tris–HCl buffer (pH 7.5), lyophilized and dissolved in 2.5 ml of the same buffer. This solution was applied to a column (78 cm $\times$ 2.1 cm) of Sephadex G-200, packed according to ROTHSTEIN[8], and the protein was eluted with 0.05 M Tris–HCl buffer (pH 7.5). The active fractions were pooled and designated as purified enzyme.

Measurement of enzymic activity

Allantoate amidohydrolase activity was determined according to a modification of the method previously described[1]. To measure enzymic activity of activated enzyme 1 vol. of enzyme solution was mixed with 3 vol. 0.05 M sodium citrate–HCl buffer (pH 3.5); the pH of the mixture was 3.9. After 30 sec at 0° 10 vol. 0.13 M diethanolamine–HCl buffer (pH 8.6) were added containing per ml 23 μmoles sodium allantoate, 0.15 μmole $MnSO_4$ and 5.9 μmoles neutralized GSH. The resulting mixture, with a final pH of 8.6, was incubated at 30° and the amount of allantoate degraded was measured by differential glyoxylate analysis (Method D)[2]. Activation of purified enzyme was mostly performed by pretreatment of the enzyme solution with 3 vol. 0.1 M KH_2PO_4–Na_2HPO_4 buffer (pH 6.0) for 30 min at 30°.

The spontaneous activity of the untreated enzyme preparation was measured on addition of the buffered substrate solution to the enzyme followed by the citrate or phosphate buffer.

One unit of enzyme activity was defined as the amount which will catalyze the conversion of 1 μmole allantoate per min. The specific activity was expressed in units per mg protein.

RESULTS

Purification

Table I shows a typical result of the purification procedure for the enzyme from *S. allantoicus*. This enzyme was purified 50-fold (recovery 28%). The same procedure resulted in a 40-fold purification of the enzyme from *A. allantoicus*, but the recovery was only 10%. The amount of nucleic acid in the purified material was less than 0.5%,

TABLE I

PURIFICATION OF ALLANTOATE AMIDOHYDROLASE FROM *S. allantoicus*

	Total protein (mg)	*Total activity (units)*	*Spec. activity*	*Recovery (%)*	*Times purified*
Crude cell-free extract	455*	1910	4.2	100	—
Acetone fractionation	51.3	1189	23.2	62	5.5
DEAE-cellulose chromatography	9.65	753	78	39	18.6
Sephadex G-200 gel filtration	2.56	536	210	28	50

* From *S. allantoicus* cells grown in 14 l allantoin–yeast extract medium[1] for 24 h at 30°.

whereas crude enzyme preparations contained more than 20%. The purified allantoate amidohydrolase preparations were found to be devoid of ureidoglycolase activity when tested with racemic ureidoglycolate as substrate.

pH-dependent activation of the enzyme

Cell-free extracts from *S. allantoicus*, *A. allantoicus*, *E. coli*, *E. coli* var. acidilactici and *E. freundii* showed only a slight allantoate amidohydrolase activity, when measured at pH 8.5, which is in the optimal pH region for enzymic activity; the specific spontaneous activities amounted to values between 0.01 and 0.1 unit per mg protein. These specific activities could be enhanced up to values between 2 and 4 by pretreatment of the enzyme preparations at pH values below 4.3 for 30 sec at 0° (refs. 1,9). The spontaneous activity was not a result of the presence of allantoicase in *S. allantoicus* as supposed by VALENTINE *et al.*[10], since our crude and purified allantoate amidohydrolase preparations incubated with allantoate yielded always 2 moles NH_3 together with 1 mole ureidoglycolate from 1 mole allantoate. NH_3 was not formed *via* urea as *S. allantoicus* was urease-negative[9].

Fig. 1 shows the effect of pretreatment of crude cell-free extract from *A. allantoicus* at pH 3.9 and pH 6.0. At both pH values the enzyme was activated and the maximal activation obtained was the same; at pH 6.0 the enhancement of activity was a slow process. Similar results were obtained with the crude enzyme from *S. allantoicus*. The purified enzyme preparations from both bacteria could also be activated at pH's 3.9 and 6.0. At pH 3.9 denaturation of the enzyme took place during activation, especially when the protein content of the sample was low. At pH 6.0 the activated enzyme was rather stable; therefore activation of purified enzyme was mostly performed at this pH.

In earlier[1] experiments no activation was found at pH values above 4.3 and, in

contrast to the results presented now, the enzyme activated at pH 2.6 was inactivated during subsequent treatment at pH 6.0. However, previously Mn^{2+} and in most cases GSH were present during pretreatment while in the present experiments both substances, which stimulate the enzymic activity measured at pH 8.5, were added together with the substrate after the acid treatment. In order to elucidate this apparent contrast we investigated the effect exhibited by Mn^{2+} added before or during the pretreatment of the enzyme at several pH values.

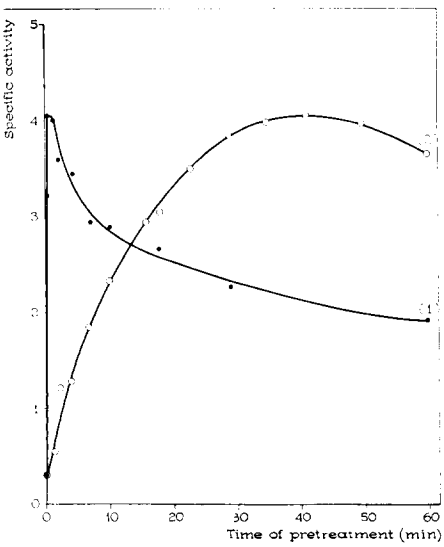

Fig. 1. Activation of allantoate amidohydrolase in cell-free extract from *A. allantoicus* by pretreatment at pH 3.9 at 0° (Curve 1) or at pH 6.0 at 30° (Curve 2). Cell-free extract (0.8 ml) containing 0.82 mg protein in 0.05 M Tris–HCl buffer (pH 7.5), which was $1.7 \cdot 10^{-4}$ M with respect to EDTA, was mixed with 2.4 ml 0.05 M sodium citrate–HCl buffer (pH 3.5) or with 2.4 ml 0.1 M KH_2PO_4–Na_2HPO_4 buffer (pH 6.0). At the indicated time intervals 0.2-ml aliquots of the mixtures were added to 2 ml 0.13 M diethanolamine–HCl buffer (pH 8.6), which contained per ml 23 μmoles sodium allantoate, 0.15 μmole $MnSO_4$ and 5.9 μmoles GSH. The resulting mixtures were incubated for 30 min at 30°. From the amounts of allantoate degraded the specific activities of the activated enzyme were calculated.

Effect of Mn^{2+} and other bivalent cations

Fig. 2 shows the effect of Mn^{2+} added during activation of the enzyme at pH 6.0. All spontaneous activity present disappeared within 10 sec at 0°, when Mn^{2+} was added at the start of the activation period. Addition of Mn^{2+} at different time intervals during the activation process resulted in a very rapid decrease in the specific activity of the activated enzyme. The effects of $2 \cdot 10^{-4}$ M Co^{2+}, Cd^{2+}, Ca^{2+} or Zn^{2+}, added during enzyme activation at pH 6.0, are presented in Fig. 3. Co^{2+}, Cd^{2+} and Zn^{2+} exhibited a similar effect to Mn^{2+}, but Ca^{2+} inhibited further activation without inactivating already activated enzyme. At pH values below 4.3, in contrast to the results obtained at pH 6.0, $2 \cdot 10^{-4}$ M Mn^{2+} did not influence the extent and rate of activation. At pH 7.5, Mn^{2+} abolished rapidly the spontaneous activity (Fig. 4a), but

at pH 8.5, the pH optimum of the enzyme, Mn^{2+} protected the enzyme against inactivation observed in the absence of Mn^{2+} (Fig. 4b).

Although Mn^{2+} inactivated the enzyme at pH values between 4 and 8, this ion was essential for the catalytic action of the enzyme at pH 8.5. Cd^{2+}, Ca^{2+} and Zn^{2+} could not replace Mn^{2+}, but Co^{2+} showed a stimulating effect on the enzymic activity, which was 16% of that exhibited by the same concentration of Mn^{2+}. The activating

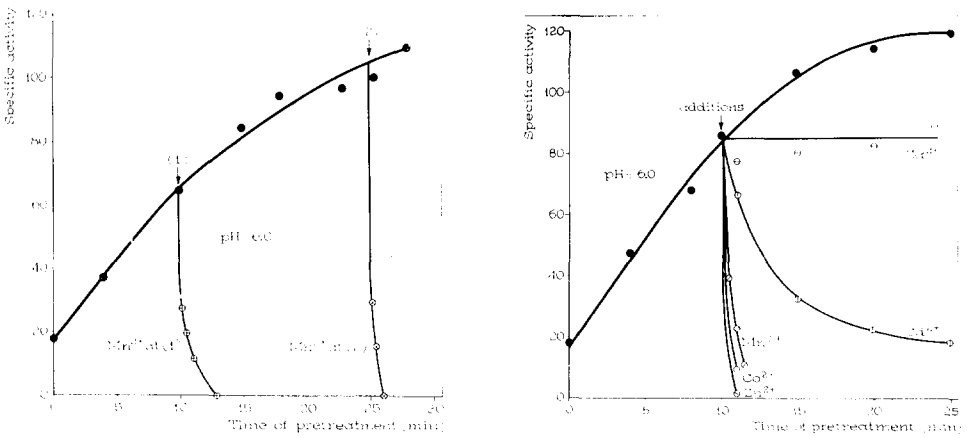

Fig. 2. Effect of Mn^{2+} on the activity of purified allantoate amidohydrolase from *S. allantoicus* activated at pH 6.0. In order to activate 0.63 ml purified enzyme containing 17 μg protein and 0.05 μmole EDTA in 0.05 M Tris–HCl buffer (pH 7.5) were mixed with 1.8 ml 0.1 M KH_2PO_4–Na_2HPO_4 buffer (pH 6.0). At the indicated time intervals the enzymic activity in 0.2-ml aliquots of the mixture was tested as given in Fig. 1. In the inactivation experiments 0.6 ml purified enzyme containing 17 μg protein and 0.05 μmole EDTA in 0.05 M Tris–HCl buffer (pH 7.5) were mixed with 1.8 ml of the 0.1 M phosphate buffer . At t = 10 min or t = 25 min of the preincubation period 0.03 ml water containing 0.46 μmole $MnSO_4$ was added. At the indicated time intervals 0.2 ml of the mixtures were tested for activity as given in Fig. 1. Mn^{2+} was added in such an amount that the final concentration in all incubation mixtures was equal.

Fig. 3. Effect of bivalent cations on the activity of allantoate amidohydrolase from *S. allantoicus* activated at pH 6.0. The same procedure was followed as described in Fig. 2. The inactivation curves, starting at t = 10 min were obtained on addition of 0.03 ml water containing 0.46 μmole of the cation tested.

effect on the enzymic activity was optimal at 10^{-4} M Mn^{2+}; the same result was found previously with crude cell-free extract[1,9].

The purified enzyme was dialyzed overnight against 0.05 M Tris–HCl buffer (pH 7.5) to remove EDTA, which was normally present at a concn. of $1.7 \cdot 10^{-4}$ M in all buffers used in the purification procedure. After dialysis the enzyme could be activated by pretreatment below pH 4.3, but not at pH 6.0. On addition of 0.17 μmole EDTA per ml of dialyzed enzyme preparation the enzyme could be activated at pH 6.0 again and to the same level as before dialysis. Apparently EDTA was important in the activating phenomenon observed at pH 6.0. This result strongly suggests that activation was due to removal of Mn^{2+} from an incorrect position in the active center of the enzyme. In purified enzyme preparations the presence of manganese could be demonstrated by the formaldoxime method[11].

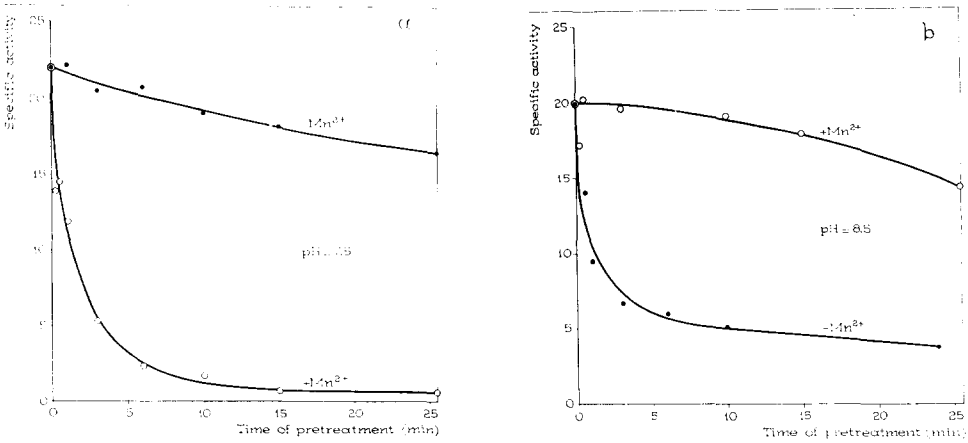

Fig. 4. Effect of pretreatment at pH 7.5 (a) and pH 8.5 (b) on the activity of allantoate amidohydrolase from *S. allantoicus* in the presence and absence of Mn^{2+}. The same procedure was followed as given in Fig. 2, except that Mn^{2+} was added at the start of the pretreatment period and that 0.1 M phosphate buffer (pH 6.0) was replaced by 0.05 M Tris–HCl buffer (pH 7.5) or 0.13 M diethanolamine–HCl buffer (pH 8.6), both without EDTA.

DISCUSSION

Allantoate amidohydrolase catalyzes the conversion of allantoate to ureidoglycine, CO_2 and NH_3. It could be activated by pretreatment at pH values below 4.3 for 30 sec at 0° (refs. 1,9). Activated enzyme was inactivated at a pH of about 6, and both activation and inactivation were reversible processes[1]. In order to study these processes in more detail, purification of the enzyme was attempted. The specific activity could be enhanced 50-fold by acetone fractionation, DEAE-cellulose chromatography and gel filtration.

Mn^{2+} has found to be essential for the catalytic activity of allantoate amidohydrolase, which was always tested at pH 8.5, the pH optimum of the enzyme. The presence of this ion in purified enzyme preparations could be demonstrated by the formaldoxime reaction.

The purified material could be activated at pH values below 4.3 at 0°, or at pH 6 in the presence of EDTA; the latter process was complete after 30 min at 30°. Since the extent of activation was the same in both cases, it seems likely that both treatments resulted in the same alteration of the enzymic configuration. The effect of EDTA suggests the involvement of a bivalent cation in the activation reaction.

Activated enzyme could be inactivated by $2 \cdot 10^{-4}$ M Mn^{2+} at pH 6 and 7.5; the enzyme was inactivated almost completely within 2 min at pH 6. It seems reasonable to assume that in this process Mn^{2+} was bound to the enzyme. Since this binding did not result in a catalytically active enzyme, Mn^{2+} was apparently bound in an incorrect position.

Three distinct pH regions for activation and inactivation of allantoate amidohydrolase can be distinguished. At pH values below 4.3 activation occurred and the presence of Mn^{2+} did not affect this process. In the pH range between about 5 and 7 the enzyme could be activated in the presence of EDTA, whereas addition of Mn^{2+} to activated enzyme resulted in a rapid disappearance of the enzymic activity. Above pH 8 the enzyme could not be activated and Mn^{2+} had no inactivating effect.

The activation and inactivation reactions observed with allantoate amidohydrolase were clearly distinguished from those observed with some proteolytic enzymes. In the latter, activation appears to consist of the cleavage of peptide links, with or without removal of free peptides. Some other enzymes can be activated at low or high pH values: 4,5-dihydropyrimidine amidohydrolase[12] (EC 3.5.2.2), latent phenolase[13] (*o*-quinol:oxygen oxidoreductase, EC 1.10.3.1), 3-hydroxyanthranilate: oxygen oxidoreductase[14] (EC 1.13.1.6), allantoin amidohydrolase[4] (EC 3.5.2.5) from higher plants and mitochondrial ATP phosphohydrolase[15] (EC 3.6.1.3). These enzymes contain bivalent cations or are strongly activated by them, and therefore the activation processes of these enzymes could be a result of an alteration in the metal–protein interaction. The phenomena observed with allantoate amidohydrolase may occur also among other enzymes, whose catalytic activity depends on the presence of bivalent cations.

ACKNOWLEDGEMENTS

We are indebted to Professor H. BLOEMENDAL and Professor S. L. BONTING for their stimulating suggestions, and helpful criticism of the manuscript.

REFERENCES

1 G. D. VOGELS, *Biochim. Biophys. Acta*, 113 (1966) 277.
2 F. TRIJBELS AND G. D. VOGELS, *Biochim. Biophys. Acta*, 113 (1966) 292.
3 F. TRIJBELS AND G. D. VOGELS, *Biochim. Biophys. Acta*, 118 (1966) 387.
4 G. D. VOGELS, F. TRIJBELS AND A. UFFINK, *Biochim. Biophys. Acta*, 122 (1966) 482.
5 G. D. VOGELS AND C. VAN DER DRIFT, *Biochim. Biophys. Acta*, 122 (1966) 497.
6 E. T. GAUDY, R. BOJANOWSKI, R. C. VALENTINE AND R. S. WOLFE, *J. Bacteriol.*, 90 (1965) 1525.
7 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
8 F. ROTHSTEIN, *J. Chromatog.*, 18 (1965) 36.
9 G. D. VOGELS, Thesis, Institute of Technology, Delft, 1963.
10 R. C. VALENTINE, R. BOJANOWSKI, E. GAUDY AND R. S. WOLFE, *J. Biol. Chem.*, 237 (1962) 2271.
11 W. BARTLEY, B. M. NORTON AND W. C. WERKHEISER, *Biochem. J.*, 67 (1957) 291.
12 D. P. WALLACH AND S. GRISOLIA, *J. Biol. Chem.*, 226 (1956) 277.
13 R. H. KENTEN, *Biochem. J.*, 67 (1957) 300.
14 N. OGASAWARA, J. E. GANDER AND L. M. HENDERSON, *J. Biol. Chem.*, 241 (1966) 613.
15 A. FONYO, L. SZENDE AND I. MEZEI, *Biochem. Biophys. Res. Commun.*, 22 (1966) 511.