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ALLANTOINASE FROM *PSEUDOMONAS AERUGINOSA***PURIFICATION, PROPERTIES AND IMMUNOCHEMICAL CHARACTERIZATION OF ITS IN VIVO INACTIVATION**

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Key words Allantoinase; Immunochemistry; Enzyme inactivation; (Ps. aeruginosa)

The catabolic enzyme allantoinase is rapidly inactivated in cells of *Pseudomonas aeruginosa* when the stationary phase of growth is reached. This process is irreversible since the protein synthesis inhibitor chloramphenicol completely blocked the reappearance of allantoinase activity that is observed when allantoin is added to stationary cells. Purified allantoinase appeared to be a protein composed of four identical subunits with a molecular weight of 38000. With antibodies raised against purified allantoinase it was found that allantoinase inactivation is accompanied by a parallel decrease in immunologically reactive material. This suggests that allantoinase inactivation is caused or followed by rapid proteolysis.

Introduction

Allantoinase (allantoin amidohydrolase, EC 3.5.2.5) catalyzes the hydrolysis of the purine catabolism intermediate allantoin to allantoic acid. In *Pseudomonas aeruginosa*, an organism able to use allantoin as energy, carbon and nitrogen source, the formation of allantoinase is induced in the presence of allantoin, allantoate, or some of their structural analogues in the growth medium [1]. The cellular level of allantoinase is further regulated by catabolite repression and nitrogen control (Janssen et al., unpublished data) and by rapid inactivation, which occurs in induced cells upon carbon starvation [2].

Earlier results suggested the presence of a low molecular weight inhibitor as the cause of allantoinase inactivation [3]. However, the yields with which this presumptive inhibitor could be isolated were irreproducible and always far too low to account quantitatively for the inactivation observed (Janssen, unpublished data). This raised

doubt about the physiological significance of the proposed inhibitor and therefore we decided to follow an immunological approach to this problem. Our results demonstrate that the loss of enzyme activity during the in vivo inactivation is accompanied by a decrease in cross-reactive material. This suggests that allantoinase is subject to proteolytic degradation during carbon starvation [4–6].

Materials and Methods

Organism and growth conditions. *Ps. aeruginosa* strain V3003 was used in all experiments. Allantoin medium contained per l: 10 g allantoin, 4.3 g $\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$, 2.2 g KH_2PO_4 , 100 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1 ml of the chloride and sulfate solutions described previously [2]. Cells were grown in 500 ml or 8 l batch cultures at 37°C with vigorous aeration. The stationary phase of growth was reached at $A_{600 \text{ nm}}$ of 0.8, when the organism was grown on allantoin medium. Citrate/am-

monia medium contained per 10 g trisodium-citrate · 2H₂O and 2 g (NH₄)₂SO₄, instead of allantoin.

Preparation of cell-free extracts. All steps were performed at 0–4°C. Cells were harvested by centrifugation, washed once with buffer 1 (40 mM phosphate, pH 7.0, containing 0.01% sodium azide and 1 mM β-mercaptoethanol) and resuspended in buffer 1. After ultrasonic treatment and centrifugation (100000 × g for 30 min) a crude extract was obtained.

Purification of allantoinase. Allantoinase was isolated from crude extract of cells grown on allantoin medium and harvested in the exponential phase. To the crude extract 0.1 volume of a neutralized streptomycin sulfate solution (10% w/v) was added. After stirring for 30 min, the precipitate was removed by centrifugation and the supernatant was fractionated further with a saturated (NH₄)₂SO₄ solution. The precipitate obtained between 35 and 50% saturation (22°C) was collected by centrifugation, dissolved in buffer 1 and dialyzed 3 times against 20 volumes of this buffer. The dialyzed material was loaded on a Sephadex G-200 column (100 × 5 cm) previously equilibrated with buffer 1. The protein was eluted with buffer 1 and the fractions with the highest activity were pooled. This pool was loaded on a DEAE-cellulose column (Whatman DE-52, 20 × 1.8 cm) equilibrated with buffer 1. Protein was eluted with a 1200 ml linear gradient of 0–0.6 M NaCl in buffer 1. Activity was found in the fractions containing 0.15–0.3 M NaCl, which were pooled and dialyzed against 4 mM phosphate, pH 7.0, containing 1 mM β-mercaptoethanol. The dialyzed enzyme preparation was purified further on a Bio-gel-HP hydroxyapatite column (10 × 1.8 cm) equilibrated with 4 mM phosphate, pH 7.0, containing 1 mM β-mercaptoethanol. Allantoinase was eluted with a 300 ml linear gradient of phosphate buffer (4–300 mM, pH 7.0) containing 1 mM β-mercaptoethanol and the fractions with the highest activity were pooled. After concentration to 1 mg/ml protein by vacuum dialysis, solid (NH₄)₂SO₄ was added to the allantoinase solution to 75% saturation and the precipitated enzyme was collected by centrifugation. The allantoinase was dissolved in buffer 1, dialyzed against this buffer and stored at 4°C.

Molecular weight determinations. The molecular weight of purified allantoinase was determined by gel filtration on a Sephadex G-200 column (100 × 2.4 cm) according to Andrews [7], using buffer 1 for equilibration and elution. Markers used were bovine serum albumin (*M_r* 68000), alcohol dehydrogenase (*M_r* 150000), catalase (*M_r* 240000) and ferritin (*M_r* 450000). As an alternative, electrophoresis on 7.5 and 10% polyacrylamide gels was used [8] with the markers bovine serum albumin, uricase (*M_r* 100000), alcohol dehydrogenase and phosphorylase A (*M_r* 370000). The molecular weight of allantoinase subunits was estimated by polyacrylamide gelelectrophoresis (13% gels) in the presence of SDS as described by Weber and Osborn [9]. The markers were lysozyme (*M_r* 14300), trypsin inhibitor (*M_r* 21500), aldolase (*M_r* 40000), glutamate dehydrogenase (*M_r* 56000) and bovine serum albumin (*M_r* 68000).

Isoelectric point determination. An LKB model 8100 isoelectric focusing apparatus was used for the determination of the isoelectric point of allantoinase. The 110 ml column was loaded with 2 mg purified allantoinase and 1% carrier ampholines (pH 3.5–10). After focusing, 2 ml fractions were collected and the allantoinase activity was measured.

Immunological techniques. Antibodies against allantoinase were raised in two 2.5 kg New Zealand rabbits. The first injection contained 300 μg purified allantoinase in Freund complete adjuvant and four subsequent injections contained each 150 μg allantoinase in Freund incomplete adjuvant. The injections were given subcutaneously at 1 week intervals. 1 week after the last injection the animals were bled and serum was prepared.

The specificity of the serum could be increased by treatment with extract from non-induced cells. The crude serum was mixed with an equal volume of extract (10 mg/ml protein) from cells grown on citrate/ammonia medium, and after 15 min at 20°C the precipitate was removed by centrifugation. The resulting purified serum was specific for allantoinase and used in all subsequent experiments.

Immunodiffusion was carried out according to Ouchterlony [10]. Immunoelectrophoresis [11] was performed on agarose gels using a veronal buffer system (pH 8.6). After electrophoresis (1 h at 3

mA) and development with serum, the plates were washed and stained with Amido black. Rocket immunoelectrophoresis was carried out on agarose gels according to Laurell [12]. Antigen levels were calculated from the rocket heights obtained. There was a linear relationship between rocket height and the amount of purified allantoinase applied up to 0.1 μ g of protein.

Immunotitration of allantoinase activity in crude extract was performed as described by Maurizi et al. [4]. Removal of the antibody-allantoinase complex by centrifugation prior to activity determinations was necessary because the complex retained enzymatic activity.

Allantoinase determination. Allantoinase activities were determined by following the production of allantoate from allantoin in an incubation mixture consisting of 1 ml 50 mM allantoin in 150 mM triethanolamine · HCl buffer, pH 8.4, and 0.2 ml enzyme. The allantoate formation was measured with the differential glyoxylate method [13]. 1 unit of activity is defined as the amount of enzyme that produces 1 μ mol of product per min under the incubation conditions used.

Protein was determined according to Lowry et al. [14], using bovine serum albumin as the standard.

Materials. The molecular weight standards were obtained from Boehringer, F.R.G. Chloramphenicol was obtained from Gist-Brocades, Holland. CCCP (carbonylcyanide-*m*-chlorophenylhydrazine) was from Sigma, MO, U.S.A., and *p*-chloromercuribenzoate from Fluka, Switzerland. *N*-Ethylmaleimide was supplied by Koch-Light, DTNB (2,2'-dinitro-5,5'-dithiodibenzoic acid) and all other chemicals were from Merck, F.R.G.

Results

Allantoinase inactivation in vivo. When *Ps. aeruginosa* was cultured in a medium containing 1% allantoin as sole carbon and nitrogen source, growth continued until a final absorbancy of 0.8 at 600 nm was reached. During the subsequent stationary phase, allantoinase was subject to rapid inactivation and the enzyme activity dropped to about 10% of the original value within 6 h (Fig. 1) [2].

The addition of allantoin to cells containing

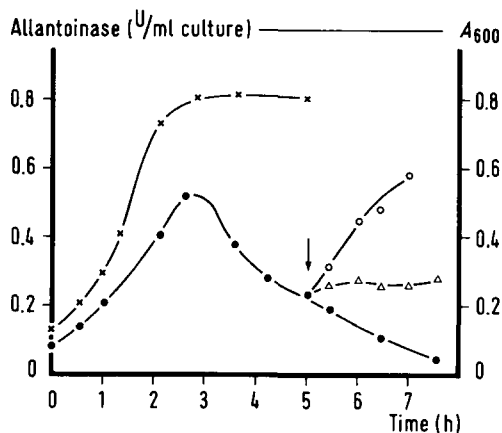


Fig. 1. Allantoinase inactivation and the effect of chloramphenicol. Cells were grown on allantoin medium and the absorbance (×) and allantoinase activities of crude extracts (●) were measured. At the time indicated by the arrow, the stationary culture was divided in three parts, and the effect of different additions on allantoinase activity was followed. ●, Control, no addition; ○, 1% allantoin added; △, 1% allantoin and 200 μ g/ml chloramphenicol added.

inactivated allantoinase caused a rapid increase in cellular levels of allantoinase (Fig. 1). This reappearance was completely blocked by the simultaneous addition of the protein synthesis inhibitor chloramphenicol. Apparently, the increase of allantoinase activity was dependent on de novo protein synthesis and reactivation of inactivated enzyme did not occur.

In order to test the energy requirement of allantoinase inactivation, the effect of uncouplers of oxidative phosphorylation was measured. Both 2,4-dinitrophenol and carbonylcyanide-*m*-chlorophenylhydrazine (CCCP) blocked allantoinase inactivation (Fig. 2).

Purification of allantoinase. For the purification of the enzyme, cells were grown on allantoin medium and harvested in the exponential growth phase. Crude extract was prepared and allantoinase was purified as described under Materials and Methods. The purification scheme is summarized in Table I. Upon polyacrylamide gelelectrophoresis, 100 μ g of the resulting preparation showed one major and two very faint additional protein bands. The major band was identified as allantoinase by slicing an unstained gel, followed

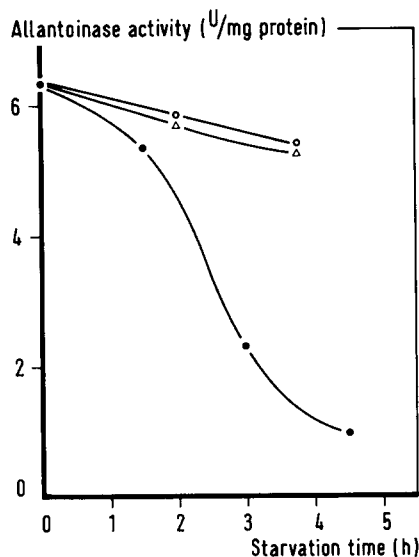


Fig. 2. Allantoinase inactivation during starvation of cells, and the effect of uncouplers. An exponential culture in allantoin medium was centrifuged, and after washing the cells were suspended in minimal medium, divided in three parts, and incubated under aeration at 37°C. ●, Control, no addition; ○, 0.01 mM CCCP added; △, 0.1 mM DNP added.

by measurement of allantoinase activity and comparison of mobilities.

The presence of 1 mM β -mercaptoethanol or 1 mM EDTA was essential during the purification and subsequent storage of the enzyme in order to prevent inactivation. The purified allantoinase did not show any loss of activity for up to 4 months when stored in buffer 1 at 4°C.

Physicochemical characterization. The molecular

weight of the purified allantoinase was estimated by means of gel filtration. By interpolation of the elution volumes obtained with known markers, a molecular weight of 140000–150000 was calculated for the native enzyme. The same value was found when polyacrylamide gelelectrophoresis on gels with different concentrations of acrylamide was used.

For the determination of the subunit molecular weight of allantoinase, electrophoresis on SDS-polyacrylamide gels was used. Only one band was observed and a value of 38000 was calculated.

The isoelectric point of allantoinase appeared to be 4.2.

Catalytic parameters. Optimal activity of the purified allantoinase was found at pH 8.4 in both triethanolamine · HCl buffer and Tris · HCl buffer.

The activity-temperature curve showed a steady increase from 20 to 45°C. At higher temperatures the activity dropped sharply and allantoinase was rapidly inactivated at temperatures above 50°C.

Under the standard assay conditions (see Materials and Methods) the enzyme followed typical Michaelis-Menten-type kinetics with respect to allantoin concentration. A K_m value of 5 mM S(+)-allantoin and a V_{max} of 1000 μ mol/mg protein per min were found.

Inhibitors. The effect of a number of potential inhibitors upon allantoinase activity was tested (Table II). It appeared that compounds, that react with sulfhydryl groups, such as *p*-chloromercuribenzoate, *N*-ethylmaleimide, DTNB and heavy metal ions caused strong inhibition. Chelators did not influence the activity. Probably, allantoinase

TABLE I
PURIFICATION OF ALLANTOINASE FROM *PS. AERUGINOSA*

Allantoinase was purified from cells from an exponential culture in allantoin medium.

Purification step	Volume (ml)	Protein (mg)	Activity (U)	Yield (%)	Spec. act. (U/mg protein)
Crude extract	276	2970	12 100	100	4.1
Streptomycin precipitation	300	2940	12 000	99	4.1
Ammonium sulfate precipitation	78	680	11 100	92	16
Gelfiltration	310	140	10 500	87	75
DEAE-cellulose chromatography	90	33	9 360	77	284
Hydroxyapatite chromatography	25	9.2	5 900	49	641
Ammonium sulfate precipitation	4	3.6	3 560	29	989

TABLE II
EFFECT OF POTENTIAL INHIBITORS ON ALLANTOINASE ACTIVITY

A volume of 0.9 ml allantoinase (0.105 unit) was preincubated for 1 h at 30°C with 0.1 ml of the inhibitor to be tested. The remaining allantoinase activity was then determined.

Potential inhibitor	Final concentration (mM)	Inhibition (%)
None	—	0
<i>p</i> -Chloromercuribenzoate	1	100
<i>N</i> -Ethylmaleimide	1	75
DTNB	1	100
Hydroxylamine	1	24
Phenylmethanesulfonylfluoride	1	4
HgCl ₂	5	100
CuSO ₄	5	100
EDTA	1	2
1,10-Phenanthroline	1	0

contains sulfhydryl groups that are essential for its enzymatic activity.

Preparation and purification of antiserum. Purified allantoinase was used for immunization of rabbits and antiserum was obtained. The serum was tested for specificity by Ouchterlony immunodiffusion. One precipitin band was formed with purified allantoinase, but two additional faint bands were produced with crude extract from allantoin-grown cells. Preimmune serum failed to produce a reaction both with purified allantoinase and with crude extract. Antiserum produced two bands with extract from non-induced cells, which were grown in medium containing citrate plus ammonia. Apparently, the serum was contaminated by antibodies that were not directed against allantoinase. Therefore, we have treated the serum with crude extract containing only a very low allantoinase activity, as described under Materials and Methods. Upon immunodiffusion, the resulting purified serum did not react with extract from non-induced cells and formed only one precipitin band with purified allantoinase or crude extract from allantoin-grown cells. This antiserum was used in all subsequent experiments.

Immunodiffusion and immunoelectrophoresis. The fate of the allantoinase protein during the course

of the inactivation process was studied with Ouchterlony immunodiffusion (Fig. 3). The antiserum was found to give the strongest reaction with crude extract from exponentially growing cells. During the stationary phase, in which allantoinase becomes inactivated, the amount of immunoprecipitable protein appeared to diminish because fainter bands were formed. Allantoinase was hardly detectable by immunodiffusion in extracts from cells that were left in the stationary phase for 10 h. Apparently, allantoinase becomes immunologically undetectable during the course of inactivation. The immunodiffusion experiments further suggested that the allantoinase protein from stationary cells is immunologically identical to allantoinase from exponential cells.

The relationship between allantoinases from different growth phases was further studied with immunoelectrophoresis. This technique should allow the detection of important structural changes in the allantoinase molecule that are not accompanied with a loss of immunological reactivity.

No immunologically detectable proteins with an electrophoretic mobility different from al-

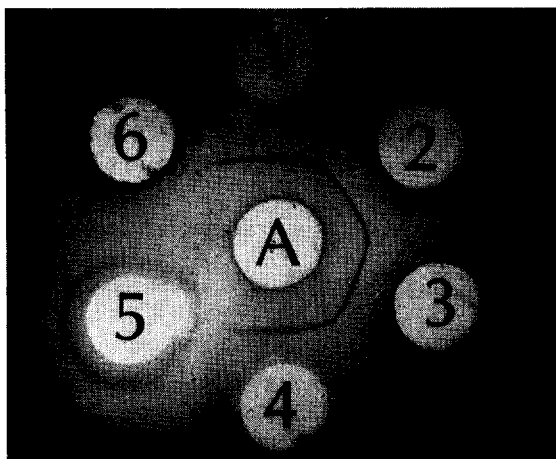


Fig. 3. Ouchterlony immunodiffusion of purified allantoinase and crude extracts from allantoin-grown cells. The wells contained: A, antiserum; 1, purified allantoinase; 2, extract from exponential cells; 3, extract from cells at the beginning of the stationary phase; 4, extract from cells left in the stationary phase for 2 h; 5, extract from cells left in the stationary phase for 10 h; 6, control extract prepared from cells grown in citrate/ammonia medium. All extracts contained 12 mg/ml protein and 25 μ l was applied per well.

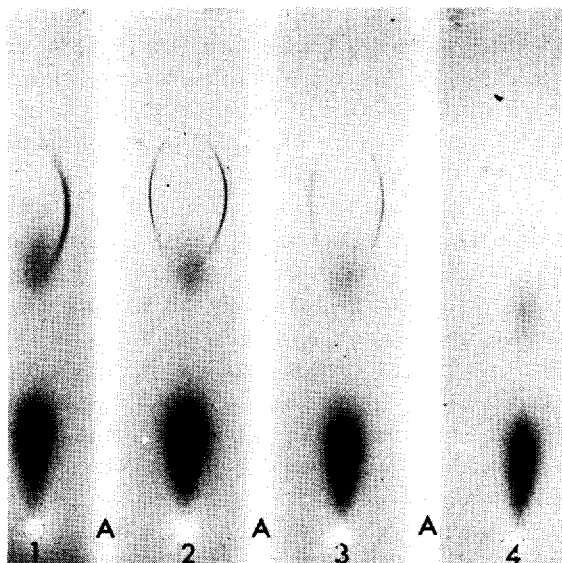


Fig. 4. Immunoelectrophoresis of crude extracts from allantoin-grown cells. The wells contained: 1, extract from exponential cells; 2, extract from cells at the beginning of the stationary phase; 3, extract from cells left in the stationary phase for 2 h; 4, extract from cells left in the stationary phase for 10 h. The protein concentrations of the extracts were 12 mg/ml and 6 μ l was applied per well.

allantoinase were found during the course of the inactivation (Fig. 4).

Quantitation of immunoreactive material. The amount of allantoinase protein present during in-

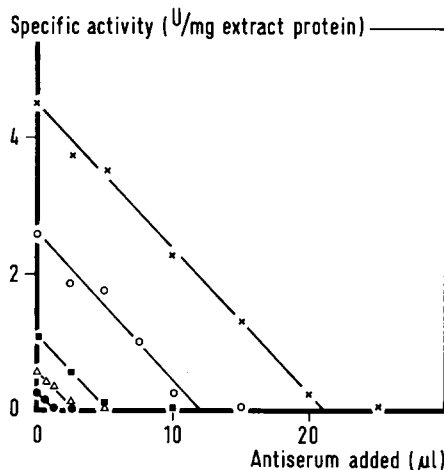


Fig. 5. Immunotitration of allantoinase activity in crude extracts from allantoin-grown cells. Samples of 50 μ l extract (12 mg/ml protein) were mixed with the amount of serum indicated and phosphate buffer to a final volume of 100 μ l. After 20 min at 20°C and centrifugation, the allantoinase activity in the supernatant was determined. Symbols: \times , extract from exponential cells; \circ , extract from cells left in the stationary phase for 2 h; \blacksquare , extract from cells left in the stationary phase for 4 h; \triangle , extract from cells left in the stationary phase for 10 h; \bullet , extract from cells left in the stationary phase for 15 h.

activation was related to the amount of active allantoinase by determination of the amount of antibody required for the inhibition of allantoinase activity in different extracts. It appeared that

TABLE III

QUANTITATION OF CROSS-REACTING MATERIAL DURING ALLANTOINASE INACTIVATION BY ROCKET IMMUNOELECTROPHORESIS.

Crude extracts were prepared from exponential and stationary cultures of *Ps. aeruginosa* in allantoin medium. Samples of 6 μ l were subjected to Laurell rocket immunoelectrophoresis. The amounts of allantoinase applied on the gel was calculated from the activities of the samples. Rocket heights were used to obtain a value for the amount of immunologically detectable antigen. A rocket height of 32 mm was obtained after electrophoresis of 0.08 μ g or 7.9 mU purified allantoinase.

Growth phase	Specific allantoinase activity (U/mg protein)	Allantoinase applied (μ g)	Rocket height (mm)	Allantoinase detected (μ g)
Exponential	4.2	0.10	40	0.10
Stationary for 0.5 h	3.7	0.09	35	0.09
Stationary for 2 h	2.4	0.06	22	0.055
Stationary for 5 h	1.3	0.03	12	0.030
Stationary for 10 h	0.6	0.015	5	0.013
Stationary for 15 h	0.4	0.01	3	0.008

no enzymatically inactive material cross-reacting with the antiserum was present in extracts from stationary cells. The amount of activity inhibited per μl of antibody was the same for extracts prepared from exponentially growing cells and for extracts that were left in the stationary phase for different periods of time (Fig. 5).

The amount of immunoreactive protein present during allantoinase inactivation was also quantitated by rocket immunoelectrophoresis. Essentially the same result was obtained. The amount of immunoprecipitated protein, as calculated from rocket heights, showed a decrease during the stationary phase that was parallel with the decrease of allantoinase activity (Table III).

Discussion

Allantoinase from *Ps. aeruginosa* is subject to rapid inactivation when cellular growth is stopped as a result of exhaustion of the carbon source in the medium [2]. This process seems to be irreversible since the increase of allantoinase activity observed on addition of allantoin to stationary cells, is dependent on de novo protein synthesis. The protein synthesis inhibitor chloramphenicol blocked the reappearance of allantoinase activity.

Although allantoinase inactivation occurs when the cells are depleted from a carbon source [2], it was found to be an energy-dependent process. The inactivation was stopped when inhibitors of oxidative phosphorylation, such as 2,4-dinitrophenol or CCCP, were added. This is probably related to the requirement for protein synthesis shown by the inactivation [2].

Allantoinase was purified from exponential cells, growing on allantoin medium. The results of molecular weight determinations suggested that the enzyme is composed of four identical subunits of molecular weight 38000. The inactivation of the purified allantoinase by compounds reacting with sulfhydryl groups shows that these groups play a role in the catalytic activity of the enzyme.

The inactivation of allantoinase in stationary cells was accompanied by a parallel decrease in the amount of material cross-reacting with antibodies against allantoinase. This could be shown with quantitative Laurell rocket immunoelectrophoresis. Also from immunotitration experiments it ap-

peared that during the course of inactivation there is no accumulation of inactive enzyme that reacted with the serum. Finally, with immunoelectrophoresis we could not detect a change in the electrophoretic mobility of immunologically detectable allantoinase during inactivation.

A parallel decrease in cross-reacting material during the inactivation of microbial enzymes is generally believed to point to a proteolytic mechanism. The catabolite inactivation of gluconeogenic enzymes of *Saccharomyces cerevisiae* was shown to be accompanied by a loss of material immunologically related to the enzymes [6,15,16], and the same was found for the inactivation of the NAD- and NADP-dependent glutamate dehydrogenases [5,17]. Definite proof for proteolysis would be obtained by the elucidation of the mechanism and by the isolation of protease mutants with altered inactivation. However, this has not yet been achieved and some mutants of *S. cerevisiae* with a defect in protease production appear to show normal inactivation of the NADP-dependent glutamate dehydrogenase [18] and gluconeogenic enzymes [15,19]. Also the inactivation of aspartate transcarbamylase in stationary *Bacillus subtilis* cells is accompanied by a disappearance of cross-reacting material, but is not caused by a known protease [4].

Our results suggest that allantoinase inactivation is caused or followed by rapid proteolysis of the enzyme, although it cannot be excluded that chemical modification alters the immunological properties. A proteolytic cleavage of the enzyme is consistent with the observed irreversibility. Allantoinase activity could not be detected in the growth medium of stationary cultures (data not shown), which argues against excretion of the enzyme from the cells.

Allantoinase inactivation occurs in stationary cells under carbon-starvation conditions [2], and also during nitrogen-starvation (Janssen et al., unpublished data). The inactivation was found to be dependent on energy and protein synthesis, since the addition of uncouplers or chloramphenicol caused rapid cessation of the decrease in allantoinase activity [2]. If the observed loss of cross-reacting material is caused by protease production, this points to a proteolytic enzyme with a high turnover rate. On the other hand, if protein

modification is the cause of inactivation, our data are consistent with a labile protein being involved in this modification.

Reconstruction of the inactivation *in vitro* and the isolation and analysis of mutants altered in allantoinase inactivation will be necessary for a better understanding of this process.

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