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PURIFICATION AND PROPERTIES OF PIG BRAIN GUANINE DEAMINASE

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Summary

Guanine deaminase (guanine aminohydrolase, EC 3.5.4.3) from pig brain was purified to homogeneity by column chromatography and ammonium sulphate fractionation. Homogeneity was established by polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulphate (SDS). The molecular weight of 110 000 was determined by gel filtration and sucrose density gradient centrifugation. SDS polyacrylamide gel electrophoresis indicated subunits of a molecular weight of 50 000. The amino acid composition, the isoelectric point and the number of -SH groups were determined. 5,5'-Dithiobis-(2-nitrobenzoic acid) reacts with about seven -SH groups in the native enzyme, but upon denaturation with SDS, 10 -SH groups react with this former reagent.

Using electrolytic reduction, 44 half-cystines were determined in accordance with the number of cysteic acid residues determined by amino acid analysis after performic acid oxidation. The K_m values determined for substrates of the enzyme were $1.1 \cdot 10^{-5}$ M for guanine in 0.1 M Tris · HCl buffer (pH 8.0) and $3.3 \cdot 10^{-4}$ M for 8-azaguanine in 0.1 M phosphate buffer, pH 6.4.

The pK_a values determined for ionizable groups of the active site of the enzyme were near pH 6.2 and pH 8.2. The chemical and kinetic evidence suggests that cysteine and histidine may be essential for the catalysis.

Introduction

Guanine deaminase (guanine aminohydrolase, EC 3.5.4.3) catalyses the transformation of free guanine to xanthine and ammonia and occupies a central position in the metabolism of guanine.

Among mammals, significant levels of activity have been found in the rat, rabbit, mouse, monkey and sheep [1—5], while current biochemical textbooks report the absence of guanine deaminase in the pig. Recently Simmonds et al. [6] observed that a large oral guanine load given to the pig is followed by a

rapid increase in urinary allantoin and xanthine with negligible alteration in guanine excretion. This suggests that this animal is able to metabolize guanine rapidly and is not deficient in guanine deaminase, although activity in blood and hepatic tissue is absent.

In the pig, the highest level of activity has been found in the brain [7]; the enzyme was seen to be localized in the supernatant (30 000 × *g*) fraction and exhibited some differences as regards molecular and kinetic properties when compared with guanine deaminase from rabbit liver, rat brain and liver.

The present paper describes purification and characterization of the enzyme. This procedure has yielded sufficient quantities of the purified enzyme to enable us to perform many quantitative studies, such as amino acid analysis and sulphydryl estimation, previously not described.

Materials and Methods

Materials. Unless otherwise stated, all chemicals were of analytical reagent grade.

Guanine and purine derivatives, phenazine methosulphate, nitro-blue-tetrazolium and amino acid standards were purchased from Sigma Chemical Co.

Protein standards and xanthine oxidase were obtained from Boehringer, DEAE-cellulose and hydroxyapatite were purchased from Bio-Rad.

Sephadex G-200 was a product of Pharmacia Fine Chemicals. 5,5′-Dithiobis-(2-nitrobenzoic acid) (Nbs_2) and 2-mercaptoethanol were obtained from Eastman Kodak. Ninhydrin and Coomassie Brilliant Blue R 250 were supplied by Merck.

Assay for guanine aminohydrolase. Activity measurements were performed at 37°C on a Zeiss PMQ III spectrophotometer equipped with a Servogor recorder. The assay mixture contained 0.2 μmol guanine in 0.1 M Tris · HCl buffer (pH 8) with a final volume of 3 ml. The reaction was initiated by addition of the enzyme (20—50 μl) and was followed by the decrease in absorbance at 245 nm [8].

A unit was the amount of enzyme which transformed 1 μmol guanine/min per mg protein at 37°C and pH 8. In some cases, enzyme activity was determined estimating ammonia by the method described by Kaplan [9].

Protein assay. The estimation of protein was based on the method of Lowry et al. [10]. Bovine serum albumin was utilized as standard.

Enzyme purification. All steps were performed at 4°C. Fresh pig brain from a commercial slaughterhouse was diced and homogenized in a Waring blendor in 2 vols. cold 2 mM 2-mercaptoethanol.

After centrifugation for 20 min at 30 000 × *g*, the pH of the supernatant was adjusted to pH 5.5 with 0.1 M HCl. The solution was stirred for 10 min and then centrifuged for 20 min at 30 000 × *g*. The precipitate was discarded. After neutralization with 0.1 M NaOH, the supernatant was taken to 40% saturation with $(NH_4)_2SO_4$ by the addition of the solid salt. After 1 h, the suspension was centrifuged for 10 min at 10 000 × *g*. The supernatant was taken to 60% saturation with $(NH_4)_2SO_4$ by the addition of the solid salt, stirred for 2 h and centrifuged for 10 min at 10 000 × *g*. The precipitate which resulted from the

40—60% $(NH_4)_2SO_4$ fractionation was dissolved in a small volume of 30 mM sodium phosphate (pH 7.5) and dialyzed overnight against the same buffer containing 2 mM 2-mercaptoethanol.

The enzyme solution was then passed through a DEAE-cellulose column (2.6 × 25 cm), previously equilibrated with 30 mM phosphate buffer (pH 7.5)/ 2 mM 2-mercaptoethanol; the gel was washed with three column volumes of the same buffer and eluted with a phosphate buffer (pH 7.5) linear gradient 30—300 mM (400 ml total volume); this operation was carried out at 10°C. The fractions were assayed for both protein concentration and enzyme activity and those of highest specific activity were pooled and taken to 75% saturation with $(NH_4)_2SO_4$. After centrifugation, the precipitate was dissolved in 40 mM phosphate buffer (pH 6.8)/2 mM mercaptoethanol and dialyzed against the same buffer.

The preparation was run through a column of hydroxyapatite (1.6 × 10 cm) at a low rate (15 ml/h); elution was carried out with 90 mM phosphate buffer, pH 6.8. Best fractions were dialyzed and again passed through hydroxyapatite, and the activity was eluted by a phosphate buffer linear gradient, 40—150 mM (140 ml total volume), pH 6.8 (Fig. 1).

Fractions containing substantial guanine deaminase activity were combined and concentrated in an Amicon Diaflo apparatus equipped with an XM-50 membrane.

Molecular weight determination. The molecular weight was determined by running the enzyme through a column of Sephadex G-200, previously equilibrated with 20 mM sodium phosphate buffer (pH 7.5) [11].

Ribonuclease (12 700), adenosine deaminase (33 000), ovalbumin (45 000), lactate dehydrogenase (140 000) and catalase (248 000) were used as standards.

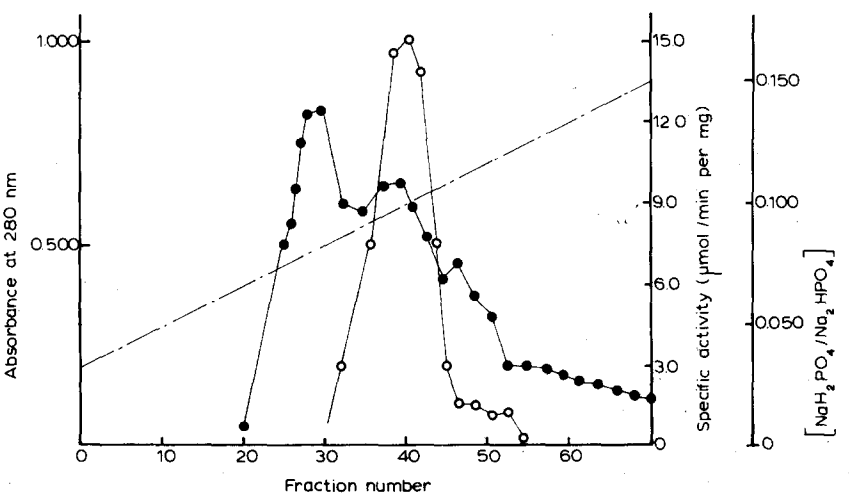

Fig. 1. Chromatography on hydroxyapatite. The column (1.6 × 10 cm) was equilibrated with 40 mM phosphate buffer, pH 6.8. Elution was performed using a phosphate buffer linear gradient 40—150 mM (140 ml total volume), pH 6.8 (— · — · —). Flow rate was 15 ml/h and 2.0-ml fractions were collected. Absorbance was measured at 280 nm (●———●). Enzyme activity was determined under standard conditions (○———○).

3.0-ml fractions were collected and assayed for enzyme activity and protein concentration.

The molecular weight was also determined by centrifugation using a sucrose density gradient of 5—20% (w/w) sucrose in 20 mM phosphate buffer (pH 7.5).

0.2 ml of guanine deaminase solution containing catalase (248 000), lactate dehydrogenase (140 000) and adenosine deaminase (33 000) as standards were loaded on the gradient (final volume 4.2 ml).

Centrifugation was carried out at 4°C and 150 000 × *g* for 16 h [12].

Cellulose acetate electrophoresis and determination of the isoelectric point. The enzyme solution (1 μl containing 1 μg) was placed on a cellulose acetate strip. A potential of 10 V/cm was applied to the strip at 4°C for 90 min. The electrode buffer used was 15 mM sodium phosphate (pH 7.5).

The enzyme activity was revealed by a substrate-specific staining method. The strip was placed on a layer of agar gel prepared from a solution containing 2 mg guanine, 5 mg phenazine methosulphate, 5 mg nitro-blue-tetrazolium and (0.08 unit) xanthine oxidase in 50 ml of 30 mM Tris · HCl buffer (pH 7.9)/ 10 mM $MgCl_2$ and 500 mg agar.

The position of the guanine deaminase activity, via the reduction of the tetrazolium reagent by xanthine oxidase, could be located by the appearance of a mauve-blue area after incubation at 50°C in the dark for 20 min.

The isoelectric point was determined from the mobility vs. pH plot, carrying out the electrophoretic runs at various pH values (4.5—7.5).

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was carried out in the absence or presence of sodium dodecyl sulphate (SDS) on 5% polyacrylamide gels according to Davis [13] and Weber and Osborn [14], respectively.

For molecular weight determination, the following marker proteins were used as standards; albumin monomer and dimer (67 000 and 134 000), aldolase and catalase subunits (40 000 and 62 000, respectively). Protein bands were stained with Coomassie Brilliant Blue R 250.

pH vs. activity. The pH vs. activity profile and the effects of pH on the saturation of the enzyme with guanine as variable substrate were determined between pH 5.5 and pH 10.0. The results were fitted to double reciprocal plots, from which the kinetic parameters K_m and V were calculated. Determination of ionizable groups at the active site of the free enzyme, and in the enzyme-substrate complex, were made from plots of pK_m vs. pH as outlined by Dixon [15].

Amino acid composition. The amino acid composition was determined in samples dialyzed exhaustively against distilled water and hydrolyzed for 24 h with constant-boiling HCl at 110°C following the method of Moore and Stein [16] or the method of Liu [17] for the quantitative estimation of tryptophan. Aliquots of the hydrolyzed fraction were subjected to chromatographic analysis in the amino acid analyzer (Beckman Multichrom B) to determine the amino acid composition. The concentration of cysteine was determined after performic acid oxidation by the method of Moore [18].

Determination of -SH groups. A slight modification of Ellman's method was used [19]. A known amount of the dialyzed enzyme (40—50 μg) was incubated in 1.0 ml of 0.1 M sodium phosphate (pH 8) with 0.2 mM Nbs_2 at room

temperature in the dark. The number of sulphydryl groups that reacted was determined by measuring the increase in absorbance at 412 nm due to the formation of 5-thio-nitrobenzoic acid (ϵ = 13 600).

Disulphide group reduction. 0.22 mg freeze-dried enzyme were dissolved in 1 ml 50 mM sodium acetate buffer (pH 4) 6 M guanidine · HCl. The solution was reduced at the cathode of an electroreduction apparatus as described by Weitzman [20].

Results

Using the purification procedure outlined in the experimental section, 1400-fold purification was achieved (Table I). The enzyme purified by the procedure described above was homogeneous by polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulphate and had the following properties.

Storage

The enzyme preparation was found to be stable at 4°C for at least 3 months when stored in 0.1 M phosphate buffer (pH 7.5)/2 mM 2-mercaptoethanol. Dilute solutions of the enzyme were irreversibly denatured by freezing.

Isoelectric point

Plotting the enzyme mobility on cellulose acetate eletrophoresis against pH, an isoelectric point near 5.0 was calculated (Fig. 2).

Substrate concentration

Values for K_m were obtained in 0.1 M Tris · HCl buffer (pH 8) for guanine, and in 0.1 M phosphate buffer (pH 6.4) for 8-azaguanine by drawing Lineweaver-Burk graphs. The K_m of the enzyme with guanine as substrate was $1.1 \cdot 10^{-5}$ M and with 8-azaguanine it was $3.3 \cdot 10^{-4}$ M. 2-Amino-6-chloropurine, 8-bromoguanine, 7-methylguanine, thioguanine, guanosine, guanosine

TABLE I

PURIFICATION OF GUANINE DEAMINASE FROM PIG BRAIN

Results are tabulated per 500 g pig brain. Enzyme activity was measured spectrally at 245 nm. A molar extinction difference of $3.15 \cdot 10^3$ was used to convert observed absorbance changes to μmol of guanine deaminated. Specific activity is based on the conversion of 1 μmol guanine/min per mg protein at pH 8 and 37°C.

Purification steps	Total volume (ml)	Total activity	Specific activity (units/mg)	Purification	Yield (%)
Crude extract	1500	245	0.01	1	100
40–60% saturated $(NH_4)_2SO_4$ fraction	32	174	0.17	17	71
DEAE-cellulose chromatography	60	116	1.16	116	46
Hydroxyapatite chromatography, 90 mM phosphate buffer, pH 6.8 elution	16	74	2.90	290	30
Hydroxyapatite chromatography gradient elution	20	62	14.00	1400	25

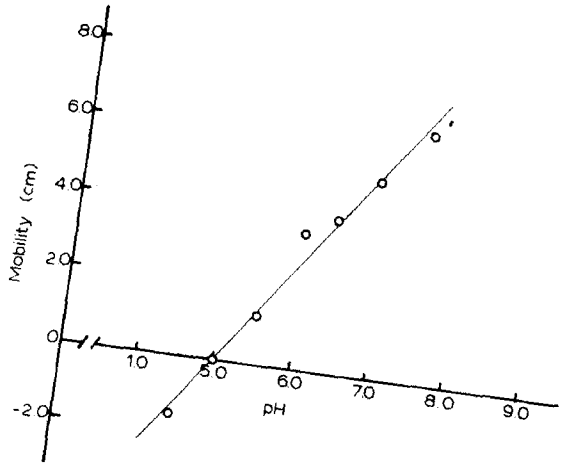

Fig. 2. Estimation of isoelectric point by cellulose acetate electrophoresis.

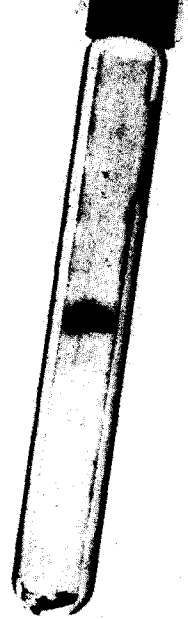

Fig. 3. Polyacrylamide gel electrophoresis. Gel electrophoresis of guanine deaminase after the final step of purification. 25 μg protein was electrophoresed in 5% running gel. The electrophoresis buffer was Tris/glycine buffer, pH 9.5. Electrophoresis was carried out at 4.0 mA per tube for 3 h. Protein was stained with 0.25% Coomassie Brilliant Blue R and destained with a mixture of methanol (5%) and acetic acid (7.5%).

5′-monophosphate, adenosine and 2-aminopurine were tested as substratres (0.2 mM in 0.1 M phosphate buffer, pH 7). Only thioguanine showed a detectable ammonia liberation.

Molecular weight and amino acid composition

The enzyme displays a single band on gel electrophoresis carried out in the presence or absence of SDS (Fig. 3). In the former case, a molecular weight of 50 000 was estimated by comparison with the protein markers listed under Materials and Methods (Fig. 4) [14].

Gel filtration on Sephadex G-200 (Fig. 5), and sucrose density gradient centrifugation experiments on the native enzyme, gave a molecular weight of 110 000 ± 10 000 (six observations).

Amino acid analysis of pig brain guanine
Table II. The values are
molecular

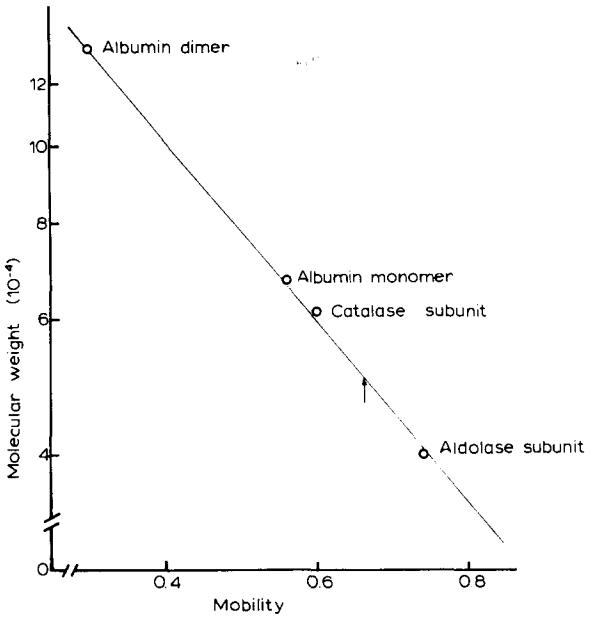

Fig. 4. Estimation of molecular weight by SDS polyacrylamide gel electrophoresis of guanine deaminase subunit. Protein samples were incubated in 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1% SDS and 0.1% 2-mercaptoethanol at 100°C for 2 min. Electrophoresis was carried out at a constant current of 8 mA per tube for 4 h using bromphenol blue as running marker. Gels were stained with 0.25% of Coomassie Brilliant Blue and destained by washing with a mixture of acetic acid/methanol/water (7.5 : 5.0 : 87.5 v/v).

benzoate outlined the essential role of -SH groups in the enzymatic activity [21].

Our recent investigations confirm these observations and suggest that non-essential sulphydryl groups are also present in guanine deaminase; in fact, the

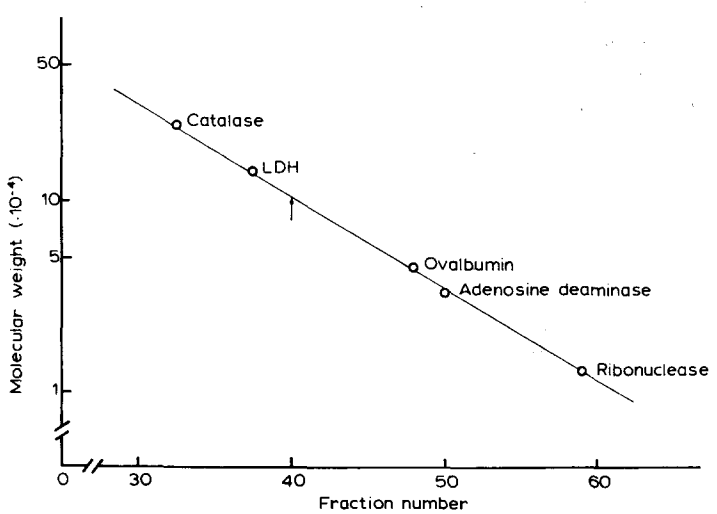

Fig. 5. Gel filtration on Sephadex G-200. The column (1.6 × 80 cm) was equilibrated with 20 mM sodium phosphate buffer, pH 7.5, containing 2 mM mercaptoethanol. Flow rate was 10 ml/h and 3-ml fractions were collected.

TABLE II

AMINO ACID COMPOSITION OF PIG BRAIN GUANINE AMINOHYDROLASE BASED ON A MOLECULAR WEIGHT OF 110 000

The results were obtained after four observations.

Amino acid	Residues per mol
Lysine	24.8
Histidine	55.3
Arginine	22.7
Half-cystine	46.3 *
Aspartic acid	86.8
Threonine	24.9 **
Serine	60.7 **
Glutamic acid	89.0
Proline	42.0
Glycine	93.8
Alanine	109.9
Valine	31.9
Isoleucine	18.4
Leucine	79.7
Tyrosine	38.2
Phenylalanine	31.3
Methionine	20.6
Tryptophan	3.8 ***

* Determined as cysteic acid. Oxidation of the protein was performed by the method of Moore [18].
** Extrapolated to $t = 0$ of hydrolysis.
*** Determined by the method of Liu [17].

enzyme is rapidly coupled to thiol-Sepharose, it retains its activity in the column and can be eluted with cysteine with a total recovery [21].

The homogenous enzyme enabled us to calculate the number of -SH groups. Fig. 6 shows the rate of reaction of sulphydryl groups of guanine deaminase with Nbs_2. It is seen from the figure that there are two sulphydryl groups which react very rapidly (2 min); after that, the rate is very slow and about five sulphydryl groups react upon further incubation for 100 min (room temperature). However, on addition of 2% SDS, a very rapid reaction occurs and about three more -SH groups react within 5 min.

The high content of half-cystines (46 residues/mol) determined by amino acid analysis suggests the existence of several disulphide bridges. The number of total -SH groups was then determined by reaction with Nbs_2 after treatment with 6 M guanidine · HCl and electroreduction. The results of this titration are reported in Table III.

From these experiments the total number of half-cystines results as 44 ± 2 (six observations) per enzyme molecule. Taking into account that 10 ± 1 (six observations) cysteine residues react with Nbs_2 prior to the reduction, it appears that the enzyme structure comprises 17 ± 1 disulphide groups.

Effect of pH on kinetic properties

K_m and V values for guanine were determined by extrapolation of the linear portions of the Lineweaver-Burk graphs to pH values from 5.5 to 10.0. The results have been plotted according to the method of Dixon [15] (Fig. 7). In

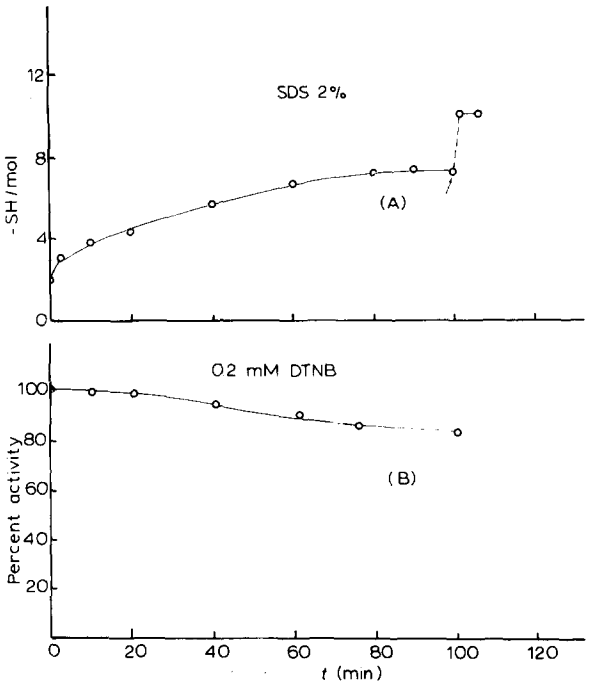

Fig. 6. Rate of reaction of sulphydryl groups with Nbs_2. (A) The incubation mixture contained in 1 ml: 0.1 M sodium phosphate, pH 8; 50 μg guanine deaminase; 0.2 mM Nbs_2. After 100 min of incubation, SDS was added, as indicated by the arrow, and a total number of 10 -SH/mol was calculated. (B) Aliquots from an experiment similar to A were withdrawn at different times and assayed for enzymic activity.

the plot of pK_m against pH, a concave downward break at about pH 6.2 is consistent with the occurrence of an ionizing group in the enzyme (histidine group). The wave at about pH 8.2 suggests than an ionizing group in the enzyme · substrate complex is affected by the substrate. It is possible that a sulphydryl group in the enzyme · substrate complex is responsible for these findings.

TABLE III

ELECTROLYTIC REDUCTION OF THE DISULPHIDE BONDS

Aliquots of guanine deaminase solution (50 μl) from the electrolytic reduction apparatus were treated with 0.550 ml of 0.2 mM Nbs_2 in 0.1 M sodium phosphate, pH 8. The data are reported for a typical experiment.

Time (min)	Absorbance at 412 nm	Total -SH per mol
10	0.055	24.1
20	0.072	31.5
30	0.088	38.5
40	0.096	42.0
60	0.102	44.7
80	0.103	45.1

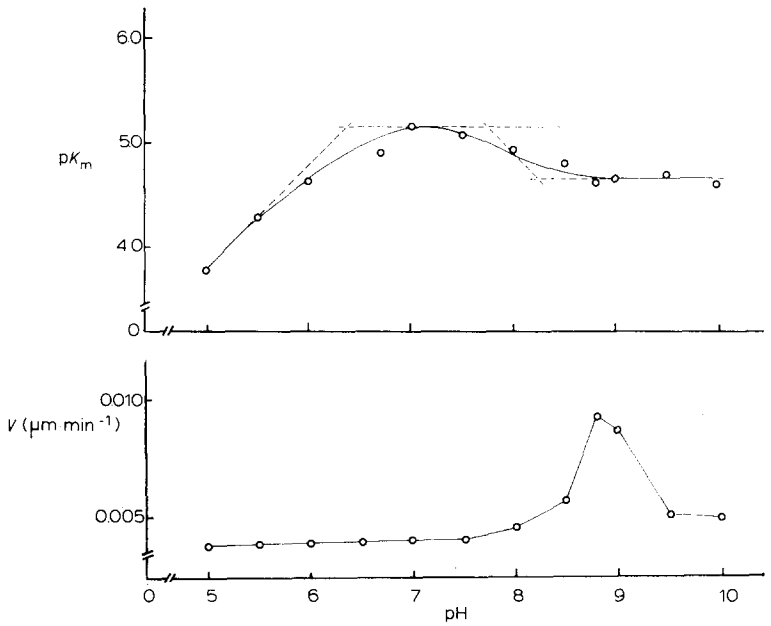

Fig. 7. The pH dependence of the Michaelis constant (K_m) and the maximal velocity (V) of guanine deaminase for guanine. K_m and V were calculated from the linear portions of the Lineweaver-Burk plots.

Discussion

Although guanine deaminase has been under investigation for the last 40 years and its presence in many tissues has been observed, relatively few purified preparations of the enzyme have been described [1,5].

Kinetic data have been reported for guanine deaminase isolated from many sources, but little other information on the nature of the enzyme is available.

Currie et al. [2] reported molecular weights of 170 000 and 525 000 for the enzyme from rabbit liver; more recently, Lewis and Glantz [5] calculated a molecular weight of 55 000, while Fogle and Bieber [1] obtained a minimum molecular weight of 100 000 for the enzyme purified from the same source. The latter investigators also observed polymeric forms, one of which had a molecular weight of 207 000, and one of the others was eluted near the void volume of Sephadex G-200 column.

By our purification method a homogeneous preparation from pig brain was obtained; homogeneity was established by gel filtration on Sephadex G-200, by polyacrylamide disc gel electrophoresis and dodecyl sulphate polyacrylamide disc gel electrophoresis.

Guanine deaminase from pig brain shows a mean molecular weight of 110 000 by gel filtration on Sephadex G-200 and sucrose density gradient centrifugation; SDS-polyacrylamide gel electrophoresis shows a single band with a molecular weight of 50 000, suggesting the possibility of two, probably identical, subunits.

Stability tests and electrophoretic experiments [21] in the presence and absence of 2-mercaptoethanol suggested the use of the thiol reagent during the

purification; this reagent possibly plays a role in the dissociation of polymeric forms obtained from other sources.

Concerning the amino acid composition of the enzyme, no comparison can be made with other preparations because this is the first report. The number of half-cystines determined by the amino acid analysis is in good agreement with the number of thiol groups determined by the spectrophotometric method on the reduced protein.

The enzyme has high specificity towards guanine among naturally occurring purines and their derivates, as no deaminase activity was detected when guanosine, guanilic acid, adenosine and 2-aminopurine were used as substrates under the conditions of the assay.

The plot of pK_m vs. pH is similar to the plot obtained by Lewis and Glantz [5] for the rabbit liver enzyme; in fact the curve has discontinuity points at about the same pH: 6.2 and 8.2. The wave at pH 8.2 is consistent with the presence of an -SH group at the active site of the enzyme, in agreement with previous experiments of inhibition and inactivation by thiol reagents [21].

As regards sulphydryl groups, it is interesting to compare the rate of reaction with Nbs_2 and the rate of inactivation of the enzyme (Fig. 6). It is possible to observe that the modification of two -SH residues per molecule does not affect the guanine deaminase activity. The observation can explain our previous experiments where the enzyme covalently bound to a Sepharose column retained all its activity [21].

The pK_a value of 6.2 calculated for ionized group at the active site of the emzyme is consistent with the ionization of histidine residues. Further experiments with imidazole group specific reagents are in progress with the aim of clarifying this problem.

Acknowledgements

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