Studies on the Guanosine Degrading System in Bacterial Cell

Part III. Purification and Properties of Guanosine Deaminase

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A highly purified preparation of the enzyme, guanosine deaminase, has been obtained by a four-step purification procedure from the cells of *Pseudomonas convexa* No. 149. The enzyme deaminates guanosine, deoxyguanosine and 8-azaguanosine, and the deamination of guanosine is inhibited by Hg^{2+} and 4-amino-5-imidazole carboxamide ribonucleoside.

The enzyme has the optimum pH at pH range from 6.0 to 6.5 and the optimum temperature at 45°C. Metal ions are not required for the enzyme activity.

The molecular weight of the enzyme is 1×10^5 to 2×10^5 .

Adenase, adenosine deaminase, adenylic acid deaminase and ATP-deaminase deaminate adenine derivatives.^{1~4)} In the case of deamination for guanine derivatives, only guanase has been found in microorganisms and animal cells. The degradation of guanosine by bacterial enzymes was studied by some workers.^{5,6)} It was found that xanthine was formed from guanosine by a combination of nucleoside phosphorylase or nucleosidase and guanase.

In the previous papers,^{7,8)} it was shown that *Pseudomonas convexa* No. 149 produced a guanosine deaminase that deaminated guanosine to form xanthosine.

The present paper deals with characterization and substrate specificity of the partially purified guanosine deaminase.

MATERIALS AND METHODS

Preparation of crude enzyme. *Pseudomonas convexa* No. 149 was grown at 28°C for two days on a rotary shaker. The culture medium consisted of 2% of peptone, 0.5% of meat extracts and 0.5% of NaCl. After cultivation, the cells were collected, washed with 0.05 M sodium phosphate buffer (pH 7.0), and suspended in 1/4 volume of the same buffer. The suspension was treated at 10 kc for 5 min with a Kubota Sonic Oscillator. The sonicate was centrifuged at 13,000 rpm for 20 min in a Servall Refrigerated Centrifuge RC-2. The supernatant was used as a crude enzyme preparation.

Measurement of enzyme activities. The activity of guanosine deaminase was assayed by following the decrease of optical density at 260 m μ at 37°C with a Gilford Recorder Model 2000 according to a slightly modified method of Kalckar.⁹⁾ The reaction mixture (3.0 ml) consisted of 50 μ moles of sodium phosphate buffer (pH 7.0), 0.18 μ mole of guanosine and 0.1 ml

1) R. C. Hartenstein and I. Fridovich, *J. Biol. Chem.*, **242**, 740 (1967).

2) B. M. Chassy and R. J. S. Suhadolnik, *ibid.*, **242**, 3655 (1967).

3) B. Setlow and J. M. Lowenstein, *ibid.*, **242**, 607 (1967).

4) Shian-Ta Chung and K. Aida, *J. Biochem.*, **61**, 1 (1967).

5) M. Manson, G. D. Kalyankar and G. P. Talwar, *Biochim. Biophys. Acta*, **77**, 307 (1963).

6) C. Colla, R. Craveri and A. Craveri, *Microbiol. Enzymol.*, **15**, 75 (1965).

7) Y. Ishida, H. Shirafuji, M. Kida, M. Yoneda, and H. Fujitani, *Nippon Nogeikagaku Kaishi*, **43**, 1 (1969).

8) Y. Ishida *et al.*, *ibid.*, **43**, 6 (1969).

9) H. M. Kalckar, *J. Biol. Chem.*, **167**, 429 (1947).

of enzyme solution. The amounts of xanthosine formed from guanosine were calculated from the difference of the molar extinction coefficients (3.9×10^3).

Adenosine deaminase activity was assayed by almost the same procedure. The reaction mixture (3.0 ml) consisted of 150 μ moles of sodium phosphate buffer (pH 7.0), 0.195 μ mole of adenosine and 0.1 ml of enzyme solution. The quantity of inosine formed from adenosine was calculated from the change of optical density at 265 $m\mu$, based on the difference of the molar extinction coefficients (8.6×10^3).

The activity of nucleoside phosphorylase was measured in a reaction mixture (3.0 ml) consisting of 150 μ moles of sodium phosphate buffer (pH 7.0), 4.5 μ moles of inosine, 200 units of milk xanthine oxidase¹⁰⁾ and 0.1 ml of enzyme solution. The increase of optical density at 290 $m\mu$ was measured and the quantity of hypoxanthine formed was calculated from the difference of the molar extinction coefficients (10.9×10^3) between hypoxanthine and uric acid.

Guanase activity was determined in the similar manner. The reaction mixture (3.0 ml) consisted of 150 μ moles of sodium phosphate buffer (pH 7.0), 0.21 μ mole of guanine and 0.1 ml of enzyme solution. The decrease of optical density at 250 $m\mu$ was measured for the calculation of the quantity of xanthine formed from guanine. The difference of the molar extinction coefficients between guanine and xanthine was 5.3×10^3 .

In the case of adenase, the difference of the molar extinction coefficients between adenine and hypoxanthine was 5.2×10^3 at 265 $m\mu$.

One unit of enzyme was defined as the amount of enzyme which catalyzed the formation of 1 $m\mu$ mole of the product per min.

Preparation of purine nucleosides. Several purine nucleosides were synthesized by the following method.¹¹⁾ The reaction mixture consisted of 0.1 ml of 20 mM ribose-1-phosphate; 0.1 ml of 20 mM purine bases in 0.05 N NaOH; 0.1 ml of 1 M Tris-HCl buffer (pH 7.5); 0.1 ml of 0.05 N HCl; and 0.1 ml of nucleoside phosphorylase from bacteria. After incubation for 60 min at 37°C, the reaction was terminated by

heating at 100°C for 5 min. The purine base and nucleoside formed were separated by paper electrophoresis using Whatman No. 1 filter paper in 0.025 M borate buffer (pH 9.55) at 50 V/cm for 60 min. And then, the area of the corresponding purine nucleoside was eluted with distilled water. The molar extinction coefficient was calculated from the content of ribose in the eluate.

Whether the purine nucleosides were deaminated or not, was determined by the change of ultraviolet spectra of the reaction mixture before and after reaction.

The reactions were carried out under almost the similar conditions as described in "Measurement of enzyme activities." Although, under these conditions, guanosine was deaminated completely within 10 min, the reaction mixtures were incubated for an hour. In the case of inhibition studies, the compounds were added to reaction mixture at the same concentration as that of the substrate, guanosine. And, the deamination of guanosine was assayed by the decrease of optical density at 260 $m\mu$ with Gilford Recorder Model 2000.

Estimation of molecular weight. The molecular weight of guanosine deaminase was determined by the method of Andrews,¹²⁾ using a Sephadex G-100 column (2.4 $\times$ 50 cm) and several standard proteins. γ -Globulin, serum albumin, ovalbumin, pancreatic ribonuclease, T₁-ribonuclease and sucrose were used as standards. The elution pattern of these proteins was measured from the optical extinction at 280 $m\mu$, and the peak of guanosine deaminase was determined from its activity.

Others. Protein and NH₄-N were analyzed according to the methods of Lowry *et al.*¹³⁾ and Conway,¹⁴⁾ respectively.

Purine bases, purine nucleosides and the standard proteins were purchased from Calbiochem Co., in a grade A. Several purine derivatives were gifted from Dr. Y. Kuwada, Chemical Research Laboratories in this Division. Other reagents were purchased from Wako Pure Chem. Ind., Ltd. Bacterial nucleoside phosphorylase was supplied from Mr. Fujitani in Takeda Chem. Ind., Ltd.

12) P. Andrews, *Biochem. J.*, **91**, 222 (1964).

10) "Methods in Enzymology," Vol. II, ed. by S. P. Colowick and N. O. Kaplan, Academic Press Inc., New York, N. Y., 1955, p. 482.

11) A. Imada and S. Igarashi, *J. Bacteriol.*, **94**, 1551 (1967).

13) "Methods in Enzymology," Vol. III, ed. by S. P. Colowick and N. O. Kaplan, Academic Press Inc., New York, N. Y., 1957, p. 994.

14) P. D. Boyer, "The Enzymes," Vol. I, Academic Press Inc., 1959, p. 511.

RESULTS

Purification of enzyme

A saturated ammonium sulfate solution was gradually added to the crude extract with stirring and three fractions were obtained by ammonium sulfate fractionation, *i.e.*, 0~20%, 20~43% and the supernatant of 43% saturation. These fractions were tested for protein content, guanosine deaminase, guanase, adenase, adenosine deaminase and nucleoside phosphorylase activities. As shown in Table I, the great part of guanosine deaminase and about

one-half of the adenosine deaminase activity were recovered in the fraction between 20 and 43% saturation, while other enzymes were recovered in the fraction between 43 and 75% saturation. The guanosine deaminase fraction obtained by ammonium sulfate fractionation was dissolved in 8 ml of 0.05 M Na-phosphate buffer (pH 7.0) and loaded onto Sephadex G-100 (2×50 cm), equilibrated with the same buffer. Elution was carried out with the same buffer and the guanosine deaminase fractions were pooled.

The enzyme solution (20 ml) thus obtained

TABLE I. FRACTIONATION OF CRUDE EXTRACT BY AMMONIUM SULFATE

Protein	Guanosine deaminase	Guanase	Adenosine deaminase	Adenase	N. P. or nucleosidase
%	%	%	%	%	%
Crude extract	100.0	100.0	100.0	100.0	100.0
0~20%	18.0	0.2	0.0	0.0	0.0
20~43%	29.4	101.5	17.8	59.6	19.0
43% sup.	43.5	9.3	81.2	43.2	96.0
Recovery	90.9	111.0	99.0	103.0	115.0

Enzyme activities and protein were tested as described in Materials and Methods. Abbreviation; N. P., Nucleoside Phosphorylase.

FIG. 1. Column Chromatography with DEAE-cellulose.

After the treatment with Sephadex G-100, the enzyme solution was loaded on a DEAE-cellulose column and eluted stepwisely with 0.1 M and 0.2 M Na-phosphate buffer (pH 7.0) and 0.5 M K-phosphate buffer (pH 7.0) containing 0.5 M KCl. Protein (○), guanosine deaminase activity (●), and guanase activity (▲) were determined as described in the text.

was put on the top of a DEAE-cellulose column (2×20 cm) and eluted stepwisely with 0.1 M, and 0.2 M Na-phosphate buffer (pH 7.0), and 0.5 M K-phosphate buffer (pH 7.0) containing 0.5 M KCl. The equilibration of the column with 0.05 M Na-phosphate buffer yielded the best result. As shown in Fig. 1, guanosine deaminase was eluted with 0.1 M phosphate buffer at a position different from that of guanase.

The purification procedure is summarized in Table II. The recovery of the enzyme was about 45% and the specific activity was increased about 180 times over that of the crude enzyme. The activities of guanase, adenase, adenosine deaminase and nucleoside phosphorylase were not detected in the guanosine deaminase fraction. For the characterization of guanosine deaminase, 7.2 units of this enzyme fraction in reaction mixture was used unless otherwise indicated.

TABLE II. SUMMARY OF THE PURIFICATION PROCEDURE

	Volume	Total protein	Total unit	Specific activity	Recovery
	ml	mg	$\mu\text{mole/min}$	unit/mg protein	%
Crude extract	67.2	434.0	16,850	38.9	100.0
Ammonium sulfate fraction (20~43%)	8.0	88.1	13,620	154.6	88.7
Sephadex G-100 treatment	20.0	74.0	9,847	131.7	58.3
DEAE-cellulose chromatography	47.0	1.03	7,520	7300.9	44.6

One guanosine deaminase unit is defined as the quantity of the enzyme which forms one μmole of xanthosine per minute under the conditions described in Materials and Methods.

Characterization of guanosine deaminase

Effect of pH. Guanosine deaminase activity was measured at intervals of 0.5 pH unit using citrate-phosphate buffer, Tris-HCl buffer and glycine buffer from pH 3.0 to 10.5. Guanosine deaminase exhibited a relatively

high activity between pH 4.5 and pH 9.0, and showed the optimal activity at pH 6.0 to 6.5 as illustrated in Fig. 2.

Effect of temperature. The guanosine deaminase activity was measured from 20°C to 65°C at intervals of 5°C. As shown in Fig. 3, it was estimated that the optimum temperature for the enzyme reaction was 45°C, and the enzyme activity was completely inhibited at 60°C.

FIG. 2. Effect of pH.

Assay conditions are described in the text. The pH of reaction mixtures was adjusted by various buffers. Citrate phosphate buffer (○) was used in the pH range 3.0 to 6.7; Na-phosphate buffer (●), in the pH range 5.8 to 8.0; Tris-HCl buffer (□), in the pH range 7.2 to 9.9; glycine-NaOH buffer (■), in the pH range 9.7 to 10.4.

FIG. 3. Effect of Temperature on Guanosine Deaminase Activity.

The enzyme activity was determined as described in the text.

Heat stability. The residual enzyme activity was measured after incubation for 5 min at pH 5.0, pH 7.0 and pH 9.1 at each temperature. As indicated in Fig. 4, almost the

FIG. 4. Heat Stability of Guanosine Deaminase.

Enzyme solutions in pH 5.0 citrate phosphate buffer (●), pH 7.0 Na-phosphate buffer (○) and pH 9.1 glycine-NaOH buffer (□) were incubated for 5 min at each temperature. After incubation, the residual activity was determined as described in the text and was shown as the percentage against the activity of the initial enzyme activity.

same inactivation curves were obtained in the cases of citrate buffer (pH 5.0) and phosphate buffer (pH 7.0), and the activity was decreased to about 60% at 50°C and was inactivated completely at 55°C. The enzyme activity fell to about 50% at 45°C in glycine buffer (pH 9.1), and to about 10% at 50°C. Then, it appears that the enzyme is relatively unstable in the alkaline range.

Effect of metal ions. The effect of metal ions on the enzyme activity was examined in 1.7 mm metal salt solutions. As shown in Table III, it appears that the enzyme did not require metal ions for activity, and Cu^{2+} , Hg^{2+} and Co^{2+} inhibited strongly the activity, and Mn^{2+} , Cd^{2+} and Al^{3+} were also inhibitory at 1.7 mm. The inhibition by Hg^{2+} was not

TABLE III. EFFECT OF METAL IONS

Addition (1.7×10^{-3} M)	Activity m μ mole/min	Inhibition %
None	7.18	0.0
EDTA (1×10^{-3} M)	7.00	2.5
KCl	7.18	0.0
LiCl	7.02	2.1
NaCl	6.58	5.0
CuCl_2	0.0	100.0
(6.7×10^{-4} M)	4.94	31.3
CoCl_2	1.64	72.7
(6.7×10^{-4} M)	6.06	15.7
HgCl_2	0.0	100.0
(1.0×10^{-4} M)	0.0	100.0
MnCl_2	4.04	43.6
CdCl_2	5.28	26.4
NiCl_2	6.62	7.8
CaCl_2	6.66	7.1
MgCl_2	7.08	1.4
BaCl_2	7.08	1.4
AlCl_3	3.44	52.1

A reaction mixture (total 3.0 ml) consisted of 0.18 μ mole of guanosine, 50 μ moles of tris buffer (pH 7.2) and 5 μ moles of metal ions, Cu^{2+} , Cr^{2+} , Co^{2+} and Hg^{2+} were also tested at the indicated concentration.

The enzyme activity was assayed at 37°C by using a Gilford recorder model 2000.

reversed by the addition of SH-reagents, *e.g.*, glutathione, cysteine and mercaptoethanol,¹⁴ and half inhibition was achieved with 0.4 μM HgCl_2 .

Material balance. Deamination of guanosine by the enzyme was followed during incuba-

TABLE IV. MATERIAL BALANCE

Reaction time min	Guanosine μ mole	Xanthosine μ mole	$\text{NH}_4\text{-N}$ μ mole
0	4.91	0.00	0.00
40	0.00	4.85	4.60

Reaction mixture (total volume 2.2 ml) contained 5 μ moles of guanosine, 50 μ moles of Na-phosphate buffer (pH 7.0) and 26 μg of the purified enzyme. After incubation, the reaction mixture was boiled to stop the reaction and analyzed by paper electrophoresis which was carried out in 0.05 M borate buffer (pH 9.55) at 50 volts/cm for 60 min. $\text{NH}_4\text{-N}$ was determined by Conway's method.¹⁴

tion by assaying guanosine, xanthosine and $\text{NH}_4\text{-N}$, as shown in Table IV. The deamination reaction was complete in 40 min and was stoichiometric.

Substrate specificity. Twenty-two kinds of purine compounds were tested as substrates (Table V). Guanosine, 8-azaguanosine and

8-azaguanine and guanine, and is inhibited by 4-amino-5-imidazolecarboxamide.¹⁵⁻¹⁸⁾ The K_m values for guanosine, deoxyguanosine and 8-azaguanosine were 3.6×10^{-5} M, 6.2×10^{-5} M and 1.22×10^{-4} M, respectively.

Molecular weight. The elution pattern of each protein from Sephadex G-100 was obtained as described in Materials and Methods. From the plot of molecular weight with volume of eluate in Fig. 5, the molecular

TABLE V. SUBSTRATE SPECIFICITY AND INHIBITION OF GUANOSINE DEAMINASE

Additions	Substrate	Inhibitor
8-Methylguanosine	—	—
2,6-Diaminopurine ribonucleoside	—	—
8-Azaguanosine	+	—
2-Amino-6-benzylaminopurine ribonucleoside	—	—
8-Aza-2,6-diaminopurine ribonucleoside	—	—
2-Amino-6-mercaptapurine ribonucleoside	—	—
2-Aminopurine ribonucleoside	—	—
2-Amino-6-methylmercaptapurine ribonucleoside	—	—
2-Amino-6-furfurylpurine ribonucleoside	—	—
2-Ethylthioinosine	—	—
2-Methylthioinosine	—	—
2-Mercaptoinosine	—	—
2-Methylinosine	—	—
2-Aminomethylinosine	—	—
Guanine	—	—
Guanosine	+	—
5'-Guanylic acid	—	—
3'-Guanylic acid	—	—
5'-Deoxyguanylic acid	—	—
Deoxyguanosine	+	—
Adenosine	—	—
Cytidine	—	—
5-Amino-4-imidazolecarboxamide ribonucleoside	—	—

Substrate specificity was tested as described in Materials and Methods.

Symbols are used as follows:

+ available as substrate or inhibitor.

— unavailable as substrate or inhibitor.

deoxyguanosine were substrates and 4-amino-5-imidazole-carboxamide ribonucleoside was an inhibitor. There results are similar to those found for guanase, which deaminates

FIG. 5. Estimation of the Molecular Weight of Guanosine Deaminase.

According to the method of Andrews, Sephadex G-100 column chromatographies of several proteins were carried out with column equilibrated by 0.05 M Tris-HCl buffer (pH 7.5) containing 0.1 M KCl. Column size and flow rate were 2.4×50 cm and 30 ml/hr, respectively.

weight of guanosine deaminase was assumed to be 1×10^5 to 2×10^5 , because it was eluted in almost the same position with that of γ -globulin.

15) J. E. Roy and K. L. Roy, *Can. J. Biochem.*, **45**, 1263 (1967).

16) H. G. Mandel, J. L. Way and P. K. Smith, *Biochim. Biophys. Acta*, **23**, 402 (1957).

17) C. P. Rhoads, "Antimetabolites and Cancer", American Association for the Advancement of Science, Washington, 1955, p. 199.

18) H. K. Kuramitsu, S. Uda and H. S. Moyed, *J. Biol. Chem.*, **239**, 3425 (1964).

DISCUSSION

Adenosine deaminase, forming inosine from adenosine, is widely distributed and has been much studied. Guanosine deaminase has not hitherto been reported, although the degradation of guanosine by a combination of nucleoside phosphorylase and guanase is known.^{5,6)} The discovery of guanosine deaminase in *Pseudomonas convexa* and its separation from coexisting guanase suggest another pathway of guanosine degradation. It will be of interest to elucidate the biological roles of the two enzyme systems. The new enzyme has been purified 180 fold.

Guanosine deaminase is strongly inhibited by the addition of mercuric chloride, and the inhibition is not reversed by SH-reagents. It is assumed that the enzyme strictly combines with the metal ion or the enzyme activity is lost through some conformational change.

8-Azaguanine is a substrate for guanase, while 4-amino-5-imidazolecarboxamide is an inhibitor;¹⁵⁻¹⁸⁾ guanosine deaminase shows the same activities for the ribosyl derivatives. Several derivatives involving changes in the

2-amino or 6-hydroxyl groups are not active either as substrates or as inhibitors, whereas the replacement of C-8 by nitrogen has no such effect; 8-azaguanosine is deaminated. The ribosyl derivatives of 2-chloro-6-hydroxypurine and 2-hydrazino-6-hydroxypurine, both bases of which were attacked at the 2 position by guanase, have not been tested with guanosine deaminase. The ribosyl moiety is required for activity, but it is not known whether other glycosyl derivatives of guanine will also be attacked.

A higher degree of specificity for guanosine deaminase is indicated by its inability to deaminate guanylic acid or guanosine triphosphate, whereas ATP deaminase has been found to deaminate many adenine derivatives.⁴⁾

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