BBA 65973

STABILIZATION OF DEOXYCYTIDYLATE DEAMINASE FROM MAMMALIAN TISSUES

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(Received May 5th, 1969)

SUMMARY

1. Preparation of stable deoxycytidylate deaminase from several mammalian tissues is described. Ethyleneglycol stabilized deoxycytidylate deaminase in both crude extract and purified preparation.

2. Under the stabilized condition, deoxycytidylate deaminase was purified 150-fold over the activity in the crude extract from regenerating rat liver. The substrate specificity, requirements, pH dependence and effects of nucleotides on the reaction were tested in the purified preparation.

INTRODUCTION

Deoxycytidylate (dCMP) deaminase (EC 3.5.4.12) is one of the regulatory enzymes[1-3] in the deoxypyrimidine nucleotide metabolism. In the rapidly growing mammalian tissue[4], in virus-infected cultured cells[5] and in bacteriophage-infected bacteria[6], there is a direct correlation between the rate of DNA synthesis and the activity of dCMP deaminase. Therefore, this enzyme has been frequently used as one of the marker enzymes which are related to the cell growth and DNA synthesis[7-11].

In the survey of the level of dCMP deaminase activity in regenerating rat liver and azo-dye-induced hepatoma tissue from rat, this enzyme was shown to be so labile, even in the crude extract, that a reproducible estimate was largely unsuccessful by using the conditions of other workers[2,12]. The lability of dCMP deaminase has been recognized in several sources[12-16], but a reliable method to stabilize this enzyme has not yet been established. It was desirable to develop an useful method to stabilize this enzyme for the general survey of dCMP deaminase in many different sources. To this end, several possible reagents were investigated for a stabilizing action for the deaminase from some mammalian tissues.

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PURIFICATION PROCEDURE

The purification procedures were summarized as follows. All procedures were carried out at 4°. Every solution and buffer contained 20% (v/v) ethyleneglycol to stabilize the enzyme. 50 g frozen regenerating rat liver[17] were homogenized with 200 ml of isotonic KCl and centrifuged at 105 000 × *g* for 120 min. The supernatant was the crude extract which was adsorbed onto calcium phosphate gel* and eluted by 0.1 M potassium phosphate (pH 7.5). This extract was fractionated by acetone at −15°. The fraction (38–44% acetone (v/v)) was collected and subjected to DEAE-cellulose column chromatography (2 cm × 40 cm) by linear gradient elution which was established by using each 600 ml of 0 and 0.2 M KCl in 0.01 M Tris–HCl buffer (pH 8.0) with 2 mM mercaptoethanol. The active peak, eluted at 0.09–0.11 M KCl, was followed by hydroxylapatite column chromatography (1.5 cm × 9 cm) after changing Tris buffer to 0.01 M phosphate buffer through Sephadex G-25. The elution of the enzyme was done by a linear gradient of each 100 ml of 0.01 and 0.2 M phosphate buffer (pH 6.8) with 2 mM mercaptoethanol. The active peak was obtained at 0.05–0.07 M phosphate.

TABLE I

STABILIZING EFFECT OF ETHYLENEGLYCOL ON dCMP DEAMINASE FROM DIFFERENT SOURCES

The crude extracts in isotonic KCl were prepared with or without ethyleneglycol (20%). dCMP deaminase activity was measured at 3.5 and 8.5 h after a storage at 0°.

*Enzyme source**	*Ethylene-glycol*	*Activity × 10² (units/0.1 ml) at*	
		3.5 h	*8.5 h*
Monkey liver	+	31.0	29.5
	−	15.5	3.0
Ascites hepatoma 7974	+	12.3	11.8
	−	10.3	6.0
Rat liver	+	1.2	1.1
	−	0	0
Rat-regenerating liver	+	22.4	24.1
	−	8.3	0
Rat thymus	+	38.4	31.2
	−	5.9	0.4
Rat spleen	+	3.8	2.8
	−	0	0
Rat testis	+	11.8	12.0
	−	1.3	0.7

* Monkey liver from African green monkey was kindly provided by Dr. Tokuda of the Institute for Virus Research of Kyoto University. Ascites hepatoma was derived from the strain supplied by the Sasaki Institute, Tokyo. Rats (Wistar–King strain, 150–200 g, male) were obtained from the hatching center of Kyushu University.

* Calcium phosphate gel (8 g dry wt.) suspended in water was packed by centrifugation (300 × *g*, 10 min) and resuspended in 0.154 M KCl containing 20% ethyleneglycol, followed by centrifugation to get the packed gel.

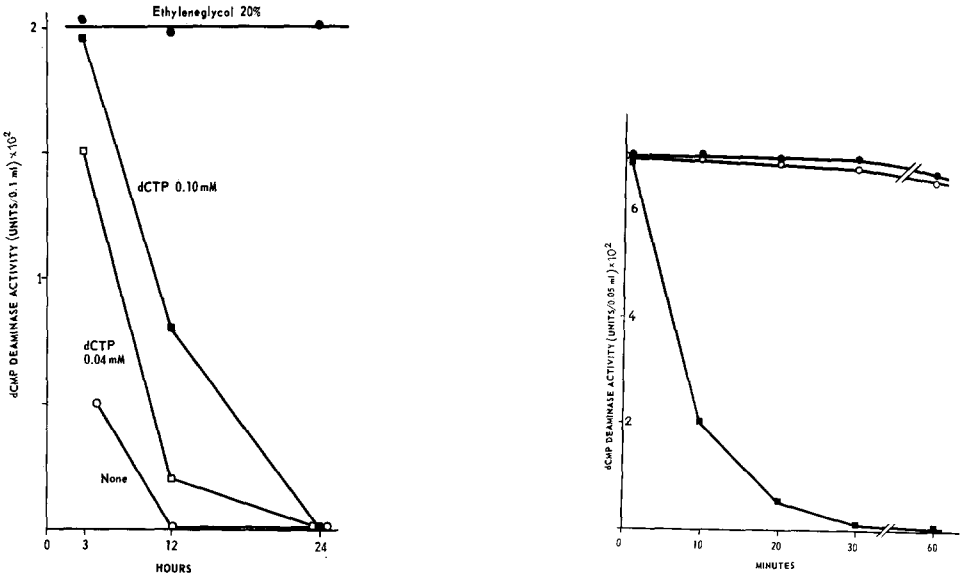

Fig. 1. Stabilizing effect of ethyleneglycol on dCMP deaminase in the crude extract. A 20% homogenate in isotonic KCl of regenerating rat liver was made with dCTP or ethyleneglycol in a final concentration indicated and centrifuged for 2 h at 105 000 × *g*. The activity of dCMP deaminase in the supernatant fluid was measured at the indicated time of storing at 0°. At 0 h, the homogenization was started. The enzyme assay[4] was modified as follows. The reaction was done for 5–20 min at 37° in the mixture (0.5 ml) of 0.2 M Tris–HCl (pH 8.0), 2 mM $MgCl_2$, 0.04 mM dCTP, 2 mM [^{3}H]dCMP and enzyme (0.05–0.5 units). The reaction was stopped by addition of 0.15 ml of 5.0 M $HClO_4$, heated at 100° for 10 min and neutralized with 0.15 ml of 5.0 M KOH. After removal of precipitate by centrifugation, a 0.5-ml aliquot was applied on Dowex-50 (H^+) (0.6 cm × 3.0 cm) and washed with 1 ml of water. Nonadsorbable fraction (1.5 ml) was counted in a liquid scintillation counter. One enzyme unit corresponds to the deamination of 1 μmole of dCMP per h at 37°. Specific activity was expressed in units per mg protein measured by the method of LOWRY *et al.*[22].

Fig. 2. Effect of ethyleneglycol and dCTP on the thermal inactivation of dCMP deaminase. The enzyme was incubated at 21° with 0.25 M Tris–HCl (pH 8.0) (■—■), Tris–HCl with 0.04 mM dCTP (●—●), and Tris–HCl with 20% ethyleneglycol (○—○). At the indicated time, 0.05 ml of incubated enzyme solution was transferred to 0.45 ml of reaction mixture containing (in μmoles) [^{3}H] dCMP 1.0, $MgCl_2$ 1.0, dCTP 0.02, Tris–HCl (pH 8) 100, and ethyleneglycol 0.1 ml, followed by the incubation at 37° for 10 min.

RESULTS AND DISCUSSION

As shown in Fig. 1, ethyleneglycol was found to be the best stabilizer for the deaminase from regenerating rat liver. The maximum stability was obtained at 20% (v/v) of this reagent. There was no effect on the enzyme assay and on the extent of deamination at the concentration of less than 5% of ethyleneglycol. Glycerol showed the same equivalent ability of stabilization as did ethyleneglycol, but the latter was preferable because of its low viscosity. Sucrose and other sugars had no effect.

The stabilizing action of ethyleneglycol was not specific for rat-liver deaminase but was common for the deaminases from other organs or species (Table I). It should be mentioned that the endogenously high level of dCMP deaminase activity in monkey

liver[12] was also increased by the addition of this reagent, and further that in spleen, testis and normal liver tissue of rat, the deaminase activity could be seen only in the extracts prepared in the presence of ethyleneglycol.

dCTP was effective as described by other authors[2,15,16] but that effect was at best short-lived, and the extent of stabilization varied in various cases (Fig. 1).

It is easy to suppose that the stabilizing ability of dCTP would depend on the level of dCTP-destroying factors, such as phosphatases or a specific dCTPase. The use of ethyleneglycol circumvented this problem and for the first time allowed the isolation of a stable deaminase preparation from regenerating rat liver (Table II).

TABLE II

PURIFICATION OF dCMP DEAMINASE FROM REGENERATING RAT LIVER

Fraction	*Vol. (ml)*	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg)*	*Recovery (%)*
Crude extract	196.0	4510.0	302	0.067	100
Calcium phosphate	245.0	1985.0	308	0.155	100
Acetone	18.0	400.0	236	0.590	78
DEAE-cellulose	9.7	30.0	100	3.380	33
Hydroxylapatite	6.4	3.2	55	17.200	17

The most purified enzyme was also unstable without ethyleneglycol. In this case, dCTP could stabilize the enzyme as well as ethyleneglycol (Fig. 2). This is a remarkable contrast to the observation in the crude enzyme which appeared to have dCTP-destroying factors as mentioned above.

It was of interest to do a comparative study on the properties of rat-liver dCMP deaminase which was induced after a partial hepatectomy, with other dCMP deaminases from chick embryo[18], donkey spleen[19,20] and bacteriophage-infected bacteria[14,21]. By using the most purified preparation, properties of rat-liver deaminase were investigated on the following criteria: (1) substrate specificity, (2) requirements of reaction, (3) pH optimum, (4) metal effects, (5) effects of nucleotides, (6) kinetic studies on the effects by substrate, dCTP activation and dTTP inhibition and (7) sedimentation properties on a sucrose gradient.

All of these properties of rat-liver dCMP deaminase were shown to be essentially similar to that of donkey spleen[19,20] which had been shown to have almost the same properties as those of other deaminase[14,18,21].

In conclusion, the application of ethyleneglycol was recognized to be the best way to stabilize dCMP deaminase in any preparation and to get a purified preparation without any detectable change of this enzyme.

ACKNOWLEDGMENT

This investigation was supported by a Grant-in-Aid for Scientific Research from Ministry of Education of Japan.

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