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SEPARATION OF CYTIDINE DIPHOSPHATE REDUCTASE FROM RAT YOSHIDA ASCITES SARCOMA

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SUMMARY: CDP reductase was separated from the cytosol of rat Yoshida ascites sarcoma. The precipitate, which resulted from the acidification of the cytosol by acetic acid at pH 5.2, catalyzed specifically the reduction of CDP, whereas the concurrently resulted supernatant catalyzed those of UDP, ADP and GDP. The CDP reductase showed a single peak in the pattern of the enzyme activity in DEAE-cellulose and also in Sepharose 4B column chromatography with adequate recovery of the activity.

Ribonucleoside diphosphate reductase (EC 1.17.4.1) is responsible for the conversion of four different kinds of ribonucleoside diphosphates into the corresponding deoxyribonucleoside diphosphates. This enzyme from *Escherichia coli* has been studied extensively, demonstrating that a single enzyme, which consists of two subunits, catalyzes the reduction of four ribonucleotides, CDP, UDP, ADP and GDP (1). In this respect, in mammals, the reductase is proposed to resemble that of bacteria by some researchers (2), but it is supposed by others that separate enzymes are required for the reduction of the different ribonucleotides (3-5). In the present study, we can isolate the reductase fraction, which catalyzes the specific conversion of CDP into dCDP, by acid precipitation from the cytosol of rat Yoshida ascites sarcoma.

MATERIALS AND METHODS

Materials. [5-^{3}H]cytidine 5'-diphosphate (25.5 Ci/mmole), [5,6-^{3}H] uridine 5'-diphosphate (10.9 Ci/mmole), [2,8-^{3}H]adenosine 5'-diphosphate (27.5 Ci/mmole) and [8-^{3}H]guanosine 5'-diphosphate (10.2 Ci/mmole) were purchased from New England Nuclear, Mass., U.S.A. Partially purified potato apyrase was prepared by the method of Traverso-Cori *et al.* (6). Polyethyleneimine (PEI) cellulose (Polygram; CEL 300/UV254) and cellulose (Avicel SF) thin layer plates were obtained from Macherey-Nagel, Düren, Germany and Funakoshi, Tokyo, Japan, respectively.

Preparation of enzyme fractions. Male Donryu-strain rats (150-200 g) were used for serial i.p. transplantation of Yoshida ascites sarcoma as described before (7). Rats were killed 5 days after tumor inoculation and

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the collected tumor cells were homogenized with 4 volumes of 0.25 M sucrose in 50 mM Tris-HCl buffer (pH 7.5). After centrifugation of the homogenate at 105,000 x g for 60 min at 4°C, the obtained supernatant was adjusted to a pH of 5.2 with acetic acid and then centrifuged again at 10,000 x g for 10 min. Immediately after the centrifugation, both the precipitate, separated and dissolved in 50 mM Tris-HCl buffer (pH 7.5), and the concurrently separated supernatant were adjusted to a pH of 7.5 with KOH. The amount of protein was determined by the method of Lowry *et al.* (8).

Assay of ribonucleotide reductase activity. An assay was conducted separately for each ribonucleoside diphosphate by the method described before (9), except that each assay medium had a total volume of 40 µl and contained 1 µCi of the appropriate [^{3}H]ribonucleoside diphosphate and that incubation was carried out for 90 min at 37°C. After boiling of the reaction mixture for 2 min to stop the reaction, 2 µl of 20 mM appropriate ribonucleoside monophosphate and deoxyribonucleoside monophosphate was added. For assay of CDP reduction activity, this mixture was boiled for 20 min after addition of 5 µl of conc. HCl, then 15 µl of the mixture was applied to an Avicel SF thin layer plate, and developed in the solvent system of isopropanol, conc. HCl and H_2O (70/15/15; v/v/v). For the assay of reduction of the other ribonucleotides, the reaction mixture was incubated at 37°C for 30 min with 10 µl of apyrase (0.002 units) and then boiled for 2 min. Five µl of the mixture was applied to PEI cellulose thin layer chromatography, and developed in a solution of 2 M LiCl and 2% boric acid (35/65; v/v). The deoxyribonucleoside monophosphate spots were removed from the plates and their radioactivities were measured in a toluene based scintillator cocktail by a scintillation counter.

Column chromatography. DEAE-cellulose column (1 x 12 cm) was equilibrated with 50 mM Tris-HCl buffer (pH 7.5). After application of the sample, chromatography was performed with 40 ml of the same buffer and then with a linear gradient of the concentration of KCl, which was provided by 50 ml each of the same buffer and the one containing 1 M KCl, at the flow rate of 20 ml/h with fractionation of every 2 ml of the eluate. Sepharose 4B column (1.2 x 25 cm) was equilibrated with the same buffer as above containing 4 mM magnesium acetate. Chromatography was developed with this buffer at the flow rate of 20 ml/h with fractionation of every 2 ml of the eluate.

RESULTS AND DISCUSSION

All of the four ribonucleotide reduction activities that convert CDP, UDP, ADP and GDP into the corresponding deoxyribonucleoside diphosphates, were detectable in the supernatant obtained from centrifugation of the homogenate of rat Yoshida ascites sarcoma at 105,000 x g for 60 min. As shown in Table I, 80% of the total CDP reduction activity presented in the 105,000 x g supernatant of the homogenate was recovered in the precipitate fraction from acidification of 105,000 x g supernatant by acetic acid at pH 5.2, while only 8, 2 and 14% of total UDP, ADP and GDP reduction activities were recovered, respectively. In the supernatant fraction derived concurrently from the acidification, UDP, ADP and GDP reduction activities were recovered at 262, 79 and 177% of each total activity presented in the 105,000 x g supernatant of the homogenate, respectively. However, CDP

Table I. Four ribonucleotide reduction activities in the enzyme fractions prepared from rat Yoshida ascites sarcoma.

Enzyme fraction	Vol. (ml)	Prot. (mg)	Total activity (cpm × 10^{-6}/90 min)			
			CDP	UDP	ADP	GDP
105,000 x g Sup.	36.0	266	24.5 (100)*	26.5 (100)	78.9 (100)	13.5 (100)
Acid-Sup.	34.5	159	2.7 (11)	69.5 (262)	62.3 (79)	23.9 (171)
Acid-Ppt.	6.7	94	19.6 (80)	2.0 (8)	1.7 (2)	1.9 (14)

Homogenate was prepared with 9 g of the tumor as the collected cells.

*The numbers in the parentheses represent the total activity in each fraction as % of the total activity in the 105,000 x g supernatant of the homogenate.

reduction activity was recovered only at 11%. After acidification, UDP and GDP reduction activities were obviously enhanced almost 3- and 2-fold, respectively. These results clearly show that the CDP reductase can be separated from the other reductase fraction which reduces the other 3 ribonucleoside diphosphates, UDP, ADP and GDP.

This separation was also seen in another tumor, mouse S-180 ascites sarcoma, in proliferating tissue such as regenerating rat liver, and in the liver of the rat bearing Yoshida ascites sarcoma (data not shown). Higher reductase activity compared with normal rat liver has been observed in the growing tissues including tumor or in the tumor bearing animal liver. In the liver of the rat after transplantation of Yoshida ascites sarcoma, we have revealed the changes of various enzyme activities in the nucleic acid metabolism (7, 10, 11).

An aliquot of the acid-precipitate fraction was applied to DEAE-cellulose column chromatography. As shown in Fig. 1, CDP reduction activity was detected in the eluate at 0.2 M in the gradient of KCl concentration with the recovery of 70% of total activity in the applied sample. Both UDP and GDP reduction activities were not detectable, probably because of their instability during chromatography. A small yet significant amount of ADP reduction activity was seen at about 0.3 M KCl. ADP reduction activity of

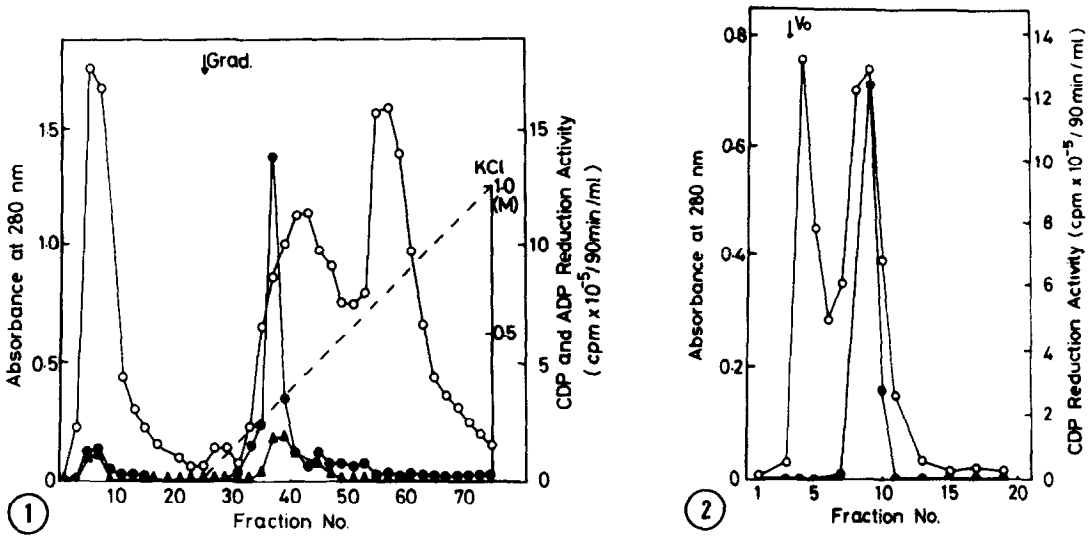

Fig. 1. DEAE-cellulose column chromatography of the acid-precipitate fraction from rat Yoshida ascites sarcoma. As described in "MATERIALS AND METHODS," 6.7 ml of the acid-precipitate fraction, prepared by acidification of the homogenate of the tumor cells, was applied to the column and chromatography was performed. (o--o) A_{280}, (•--•) CDP reduction activity, (▲--▲) ADP reduction activity and (---) concentration of KCl.

Fig. 2. Sepharose 4B column chromatography of the acid-precipitate fraction from rat Yoshida ascites sarcoma. One ml of the same sample as used in Fig. 1. was applied to the column and chromatography was performed as described in "MATERIALS AND METHODS." (o--o) A_{280} and (•--•) CDP reductase activity.

the acid-supernatant fraction was seen also at 0.3 M KCl in the same chromatography (data not shown). The identities of UDP, ADP and GDP reductase in the acid-supernatant fraction will be presented elsewhere.

As shown in Fig. 2, in the Sepharose 4B column chromatography as well, CDP reduction activity of the acid-precipitate fraction was detected as a single peak, this time in the presence of Mg^{++} in the elution buffer. Since withdrawal of Mg^{++} from the buffer diminished the recovery of activity from 80 to 20% (data not shown), the absence of Mg^{++} might induce partial dissociation of the probable subunits of the enzyme as seen in the reductase of rabbit bone marrow (12).

Youdale and MacManus isolated the subunits of the enzyme specific for the reduction of CDP from regenerating rat liver. Since the activities for the other ribonucleoside diphosphates showed different distributions in the various cytosolic fractions, a different form of enzyme for each ribo-

nucleoside·diphosphate was suggested (9). This could be the case for our reductase, since the elution pattern of the CDP reduction activity was different from that of ADP reduction activity in DEAE-cellulose column chromatography as shown in Fig. 1.

It is well known that a single reduction activity of ribonucleotide reductase, converting a certain ribonucleotide as a substrate, is subjected to negative or positive feedback regulation by its substrate-specific nucleoside triphosphates as allosteric effectors. Accordingly, another possible status of the reductase is to have a determined conformational change produced by such specific effectors, resulting in its particular behavior in the acidic condition and in the DEAE-cellulose column chromatography.

Youdale *et al.* purified the inactive subunits of the CDP reductase separated from regenerating rat liver (13). Our CDP reductase was separated from Yoshida ascites sarcoma as a whole enzyme with favorable recovery of the activity (Fig. 2), and the purification of the enzyme is currently in progress.

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