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RIBONUCLEOTIDE REDUCTASE ISOLATED FROM HUMAN CELLS

HETEROGENEITY AMONG THE SOURCES

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Abstract—The amount of ammonium sulfate required to precipitate the ribonucleotide reductase activities derived from histocytic lymphoma cells obtained from patients or from KB or Connaughton cultured human cells is different than for reductase activity derived from Molt-4F and HeLa-S3 cells. The reductase activity of Molt-4F cells sedimented in a sucrose density gradient at a faster rate than the enzyme activity from KB cells; CDP and ADP reductase activity from either source co-sedimented. Enzymes from both cell lines sedimented at a faster rate in the presence of CDP and ATP than in the absence of these compounds, with CDP and ADP reductase still co-sedimenting. In addition, the enzymes obtained from the two sources have different stabilities at 37°. Hydroxyurea, 1-formylisoquinoline thiosemicarbazone (IQ-1), 1,10-phenanthroline and potassium chloride inhibit the enzyme activity derived from either Molt-4F or KB cells. However, a difference in the ratio of CDP to ADP reductase activity in Molt-4F and KB cells and a difference between the sensitivities of the CDP and the ADP reductase activities from the same cell line to various concentrations of inhibitors was observed.

Ribonucleotide reductase, the enzyme responsible for the conversion of ribonucleotides to the deoxyribonucleotides, is of potential interest as a target enzyme for cancer chemotherapy. Two reductases purified from bacteria[1–4] have been investigated extensively. The enzyme from *Escherichia coli* catalyses the reduction of ribonucleoside diphosphates[1, 2], while the enzyme from *Lactobacillus leichmanii* reduces ribonucleoside triphosphates[3, 4]. Studies on partially purified enzymes isolated from animal systems have revealed that their properties are similar to those of enzymes obtained from *E. coli*[5, 6]. The possibility that different enzymes are responsible for the reduction of ADP and CDP has been proposed in the case of Chinese hamster cells[7], rat regenerating liver[8] and Ehrlich ascites cells[9]. Reductase activity was also found in human bone marrow and peripheral leukocytes of patients with acute or chronic myelocytic leukemia[10]. The inhibition of the enzyme obtained from different sources by 1-formylisoquinoline thiosemicarbazone (IQ-1)[11–13], hydroxyurea[14] and 1,10-phenanthroline[15] was also investigated.

The present study examines enzyme activities derived from several types of cultured human cells and peripheral blast cells obtained from cancer patients. The properties of CDP and ADP reductase activities and the sensitivities of the activities derived from Molt-4F and KB cells to hydroxyurea, IQ-1 and 1,10-phenanthroline inhibition are also compared.

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MATERIALS AND METHODS

Materials. The sodium salts of CDP, ADP, ATP and dGTP, dithiothreitol (DDT), Hepes (*N*-2-hydroxyethyl-piperazine-*N*′-2-ethane sulfonic acid), 1,10-phenanthroline, hydroxyurea, pyruvate kinase and lactic dehydrogenase were all purchased from the Sigma Chemical Co. St. Louis, MO. Ammonium salts of all [^{14}C]-labeled nucleotides were supplied by the Amersham Corp., Arlington Heights, IL. Dowex-1-cl was obtained from the Bio-Rad Laboratory, Richmond, VA. All materials required for cell cultures were from the Grand Island Biological Co., Grand Island, N.Y. IQ-1 was kindly given by Dr. A. C. Sartorelli. All other chemicals were of reagent grade.

Cells and enzyme preparation. HeLa-S3 cells were given to us by Dr. A. R. P. Patterson of Alberta, Canada, and KB cells by the American Type culture collection, Rockville, MD; Molt-4F (T type of human lymphoblast) and Connaughton (B type of human lymphoblast) cells were given by Dr. Minowada of this institute. They were cultured in a 1-liter spinner flask with RPMI 1640 medium containing 5% heat-inactivated fetal calf serum, penicillin (100 units/ml) and Streptomycin (50 units/ml). The cultured cells were harvested in the log phase of growth. The cells were collected by centrifugation (100 *g*) and washed six times with phosphate-buffered saline (pH 7.2). Peripheral blast cells from patients with acute lymphocytic leukemia (ALL) and histocytic lymphoma (kindly provided by the Department of Medicine A of this Institute) were collected by leukophoresis during the active phase of the disease. Red blood cell contaminants were removed by hypotonic shock using methods described previously[16].

Human cell lines growing in culture or leukemic

cells obtained from patients (about $1–2 \times 10^9$ cells in all cases) were homogenized in 2 vol. of buffer A (100 mM Hepes, pH 7.5, 1 mM $MgCl_2$ and 2 mM DTT) with a Dounce homogenizer. The homogenate was centrifuged at 100,000 *g* for 60 min. The supernatant solution was then treated with 1% streptomycin sulfate and the precipitate was removed by centrifugation (10,000 *g*). The supernatant solution was fractionated by 0–35%, 35–50%, and 50–75% ammonium sulfate saturation. The suspension, after addition of ammonium sulfate and stirring at 4° for 30 min, was centrifuged at 10,000 *g* for 20 min. The precipitate was dissolved in buffer B (50 mM Hepes, pH 7.5, 1 mM $MgCl_2$, 0.05 mM EDTA and 2 mM DTT) and the solution was dialyzed overnight against the same buffer. The dialysate was clarified by centrifugation and an aliquot (1 ml) was layered onto 11 ml of a linear 5–20% (w/v) sucrose gradient prepared in buffer B. The gradient was centrifuged at 100,000 *g* for 20 hr at 2° in a Spinco SW 41 rotor. Fractions (0.5 ml each) were collected by puncturing the bottom of the tube and were assayed for enzyme activity.

Enzyme assay. CDP reductase was assayed by the method of Steeper and Stewart[17] using Dowex-1-borate ion-exchange chromatography. The assay mixture contained, in a final volume of 0.2 ml: [^{14}C]CDP (0.1 μCi; 0.15 mM); DTT (6 mM); $MgCl_2$ (8 mM); ATP (5 mM); and a specific amount of enzyme. ADP reductase activity was determined by the method of Cory *et al.*[18]. The assay mixture contained, in a final volume of 0.2 ml: [^{14}C]ADP (0.2 μCi, 0.15 mM); DDT (6 mM); $MgCl_2$ (8 mM); dGTP (5 mM); and a specific amount of enzyme. An enzyme sample heated for 3 min in a boiling water bath prior to the addition of the labeled substrate served as the reagent blank. The incubation time for both reactions was 60 min, and the reaction was linear during this incubation period.

Protein determination. Protein was determined by the fluorometric method of Böhlen and Stein[19]; bovine serum albumin was used as the standard.

RESULTS AND DISCUSSION

The crude extract obtained from various cell lines was fractionated by streptomycin sulfate and then by ammonium sulfate. Most of the enzyme activity for CDP or ADP reduction was present in the 0–35%, the 35–50%, or both fractions of ammonium salt saturation (Table 1). The enzyme activities for both CDP and ADP reduction found in KB, Connaughton or histocytic lymphoma cells were precipitated with 0–35% ammonium sulfate saturation, whereas reductase activities in HeLa-S3 or Molt-4F cells were precipitated with 35–50% ammonium salt saturation. The enzyme activity observed in acute lymphocytic leukemia cells was present in both fractions.

Sucrose density gradient centrifugation was performed to determine the sedimentation rates of the enzymes derived from KB and Molt-4F cells and also to determine whether the ADP and CDP reductase activities would co-sediment. The enzyme obtained from KB cells after 0–35% ammonium sulfate subfractionation or the enzyme obtained from Molt-4F cells after 35–50% salt subfractionation was layered onto a sucrose gradient and centrifuged at 100,000 *g* for 20 hr; the results are shown in Fig. 1. As can be seen in the figure, both ADP and CDP reductase activities in each cell type co-sediment, but the sedimentation rate of the enzyme derived from Molt-4F cells is faster than that of the enzyme from KB Cells.

Enzyme from both types of cells after subfractionation by ammonium sulfate was incubated with substrate (3 mM CDP) and regulator (5 mM ATP) for 30 min at 4°, and 1 ml aliquots from each preparation were used for sucrose density gradient centrifugation. It was found that the total enzyme recovery (40 per cent for Molt-4F cell enzyme and 15 per cent for KB cell enzyme) did not improve dramatically by the addition of CDP and ATP, but

Table 1. Ammonium sulfate subfractionation of crude extract from various types of human cells*

	$(NH_4)_2SO_4$ subfraction (% of saturation)					
	0–35	35–50	50–75	0–35	35–50	50–75
	ADP reduction			CDP reduction		
Culture cells	Per cent of total activity					
HeLa-S3	11	89	0	15	85	0
KB	97	3	0	97	3	0
Molt-4F	3	97	0	1	99	0
Connaughton	91	9	0	82	18	0
Tumor cells from patients						
ALL	57	41	2	43	52	5
Histocytic lymphoma	85	15	0	80	17	3

*All fractions were dialyzed at 4° overnight in buffer B before assay. The specific activity of enzyme present in a crude extract of various cell lines after centrifugation at 100,000 *g* for 60 min was: 40 for HeLa-S3, 29 for KB, 33 for Molt-4F, 22 for Connaughton, 25 for ALL and 53 pmoles CDP reduced/min/mg of protein for histocytic lymphoma.

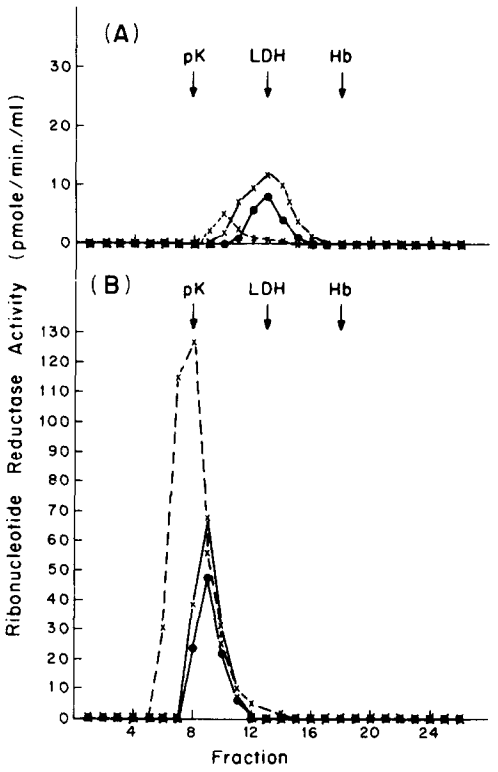

Fig. 1. Ribonucleotide reductase activity profile after sucrose density gradient centrifugation. The sucrose gradient centrifugation conditions were described in Materials and Methods. The added hemoglobin (Hb), lactate dehydrogenase (LDH) and pyruvate kinase (pK) were used as markers. The ribonucleotide reductase samples (1 ml) were layered on the top of each gradient. Panel A: CDP reduction (×——×) and ADP reduction (●——●) catalyzed by KB cell ribonucleotide reductase preparation (0–35% saturation of ammonium sulfate; the enzyme activity used was 92 pmoles/min/ml for CDP reduction with a specific activity of 25 pmoles CDP reduced/min/mg of protein). ×---× indicates CDP reduction by KB enzyme when 5 mM ATP and 3 mM CDP were included in the enzyme preparation (0–35% saturation of ammonium sulfate; the enzyme activity used was 39 pmoles/min/ml for CDP reduction). Panel B: CDP reduction (×——×) and ADP reduction (●——●) catalyzed by Molt-4F ribonucleotide reductase (35–50% saturation of ammonium sulfate; the enzyme activity used was 426 pmoles/min/ml for CDP reduction with a specific activity of 25 pmoles/CDP reduced/min/mg of protein). ×---× shows CDP reduction of Molt-4F enzyme (35–50% saturation of ammonium sulfate) when 5 mM ATP and 3 mM CDP were included. The enzyme activity used was 205 pmoles/ml/min for CDP reduction.

that the enzyme obtained from both cell types sedimented faster in the presence of these compounds than in their absence (Fig. 1). This increase in sedimentation rate may indicate that a conformational change occurs upon binding of the substrate and the regulator. ADP reductase also underwent a parallel shift (data not shown). Although an increase of sedimentation rate has been reported for CDP reductase derived from regenerating rat liver in the presence of dATP, ATP has no effect[20].

The stability of the partially purified enzymes from both cell lines was studied. They were incubated for 1 hr at 37° under the same conditions. A 50 per cent decrease in both ADP and CDP reductase activities was observed for the enzyme from the Molt-4F cells. In contrast, no loss of ADP or CDP reductase activity derived from KB cells was noted.

Potassium chloride has an inhibitory effect on enzyme derived from either source. The results of these studies are presented in Fig. 2. CDP reductase activity derived from either cell line was more resistant to potassium chloride inhibition than was ADP reductase activity. With increasing salt concentrations, there was an increase in the ratio of CDP to ADP reductase activity. The increase was more pronounced for the enzyme derived from Molt-4F than that from KB cells (Fig. 2).

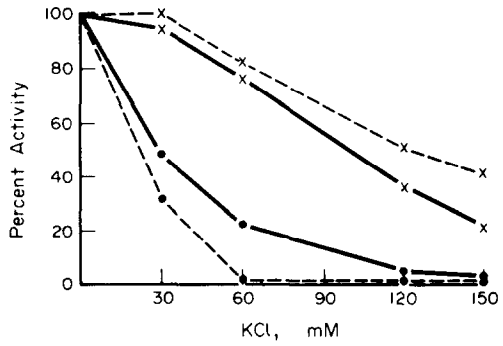

Fig. 2. Effect of KCl on ribonucleotide reductase isolated from Molt-4F and from KB cells. The enzymes from Molt-4F and KB cells used for these studies were purified by ammonium sulfate fractionation and sucrose density gradient centrifugation. The activity of Molt-4F enzyme used was 13.4 pmoles/min for ADP reduction (●——●) and 23.7 pmoles/min for CDP reduction (×——×) per assay with a specific activity of 234 pmoles CDP reduced/min/mg of protein; the activity of the KB enzyme used was 3.1 pmoles/min for ADP reduction (●---●) and 4.8 pmoles/min for CDP reduction (×---×) per assay with a specific activity of 43 pmoles CDP reduced/min/mg of protein.

Hydroxyurea, IQ-1 and 1,10-phenanthroline have been reported to be inhibitors of ribonucleotide reductase derived from a number of sources[11–13]. Their effects on the human enzymes obtained from both cell lines were studied. They all behaved as inhibitors. The results are shown in Fig. 3. IQ-1 is the most potent inhibitor among the three agents tested. CDP reductase activity from both sources seemed to be more sensitive to inhibition by these compounds than was ADP reductase activity. The ratio of CDP to ADP reductase activity derived from Molt-4F cells was decreased more by increasing the concentrations of those inhibitors, compared with that of the enzyme from KB cells (Fig. 3).

The results of this study demonstrate that the enzyme obtained from Molt-4F cells differs from that obtained from KB cells based on: (1) the amount of ammonium sulfate required to precipitate the enzyme, (2) the sedimentation rate in sucrose density gradient centrifugation, (3) the

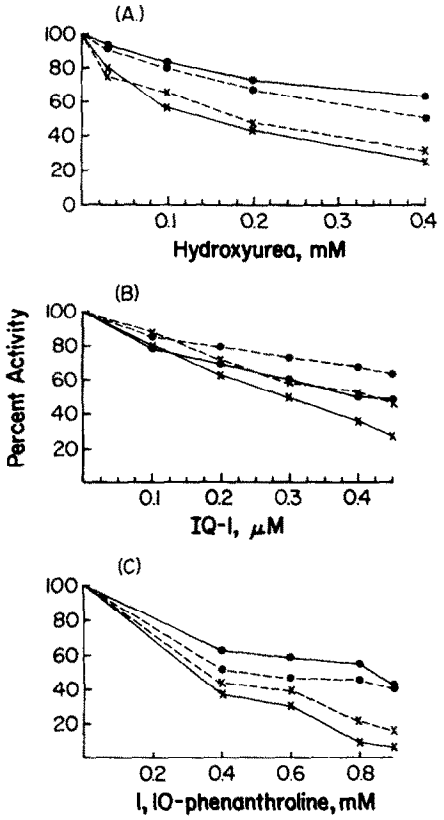

Fig. 3. Effect of hydroxyurea, IQ-1 and 1,10-phenanthroline on ribonucleotide reductase isolated from Molt-4F and KB cells. The enzyme preparations used were obtained from ammonium sulfate fractionation of Molt-4F and KB enzyme. Key: ×——× and ×---×, CDP reduction by Molt-4F and KB enzyme respectively; and ●——● and ●---●, ADP reduction by Molt-4F and Kb enzyme respectively. The enzyme activity from Molt-4F and KB cells used was 527 and 103 pmoles/hr/assay for CDP reduction, with specific activity of 117 and 25 pmoles/CDP reduced/min/mg of protein respectively. Panel A: the effect of hydroxyurea; panel B: the effect of IQ-1; and panel C: the effect of 1,10-phenanthroline.

stability at 37° and (4) the change of the ratio of CDP to ADP reductase activity in the presence of various concentrations of potassium chloride, IQ-1, hydroxyurea and 1,10-phenanthroline. Because the enzymes used for the study were not highly purified, the observed difference could be due to the association of ribonucleotide reductase with unknown factors in each of the cell lines. In order to assess this possibility, purification of the enzymes from both sources is in progress in this laboratory.

The observed difference in the sensitivity of ADP and CDP reduction catalyzed by the enzyme from either source to various agents does not necessarily suggest that different enzyme entities are involved for ADP and CDP reduction. One enzyme could have different arrangements for the reduction of ADP and CDP at their active sites. The fact that both CDP and ADP reductase activity co-sedimented in ammonium sulfate fractionation and sucrose gradient centrifugation and changed sedimentation rate in a similar pattern when regulator(ATP) and substrate (CDP) were present in the gradient seems to favor the hypothesis that there is only one enzyme entity involved in both reactions, but that the reactions have different arrangements of the active site for ADP and CDP reduction.

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