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SEPARATION OF A SUBUNIT NECESSARY FOR CDP REDUCTASE FROM OTHER RIBONUCLEOTIDE REDUCTASE ACTIVITIES OF REGENERATING RAT LIVER

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SUMMARY

Purification of ribonucleotide reductase from regenerating rat liver using dATP Sepharose chromatography isolated a subunit of the enzyme which was specific for the reduction of CDP. Activities for the other ribonucleotide diphosphates showed differing distribution in the various fractions suggesting different forms of the enzyme for each ribonucleotide diphosphate.

Ribonucleotide reductase isolated from E. Coli has been shown to consist of two subunits which have been purified to homogeneity and characterised (1-5). Bacterial mutants containing up to 10% of their protein as ribonucleotide reductase have greatly facilitated these separations (6). The enzyme from mammalian cells has also been shown by some workers to consist of subunits (7-10) but their existance and properties have not been clearly established due to instability and low recoveries.

A single enzyme from E. Coli has been shown to reduce the diphosphate ribonucleotides CDP, ADP, UDP and GDP (11,12). Some researchers have shown that semi-purified enzyme from mammalian sources reduced more than one nucleotide (10,13,14), but others have suggested that separate mammalian enzymes are required for the reduction of the different ribonucleotides (15-18). Using affinity chromatography on dATP sepharose we will show that ribonucleotide reductase isolated from regenerating rat liver has

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more than one subunit necessary for activity and that one of these subunits was specific for CDP reduction.

MATERIALS

CNBr Sepharose 4B, Sephadex G25 (medium) were obtained from Pharmacia (Uppsala, Sweden); dATP, ATP (Lanthanum free), the diphosphates, deoxymonophosphates and monophosphates of adenosine, guanosine, cytosine and uridine, dithiothreitol (DTT), apyrase, and methotrexate (Amethopterin) from the Sigma Chemical Co. (St. Louis, Missouri, USA); and [5^{3}H]CDP, [2:8^{3}H]ADP, [5^{3}H]UDP, [8^{3}H]GDP (10 Ci/mMole) from Amersham Corp. (Oakville, Ontario, Canada). PEI cellulose plates Polygram CEL 300 PEI/UV$_{254}$ were obtained from Brinkmann Instruments Inc. (Westbury, NY, USA). dATP Sepharose was prepared in this laboratory by adapting the methods of Knorre et al (19) and Berglund and Eckstein (20).

METHODS

Preparation of enzyme

Ribonucleotide reductase has been isolated and measured in regenerating rat liver (21,22) but quantities were low. To increase the reductase yield advantage was taken of the reported three fold increase in enzyme yield caused by injection of methotrexate during liver regeneration (23). This was confirmed in this laboratory, and thus 20 rats were routinely injected (0.5 mg/200 g) immediately and 24 hrs after 68% partial hepatectomy (24). The rats were killed at 48 hours and liver remnants excised, homogenised in isolation medium (100 mM Tris HCl, 0.25 M sucrose, 5 mM Mg acetate, 10 mM KCl, 2 mM DTT pH 7.6) and centrifuged at 100,000 x g for one hour in a Beckmann L265B ultracentrifuge. The supernatant fluid (Fraction 1) was removed and ammonium sulphate added to 40% saturation (0.25 g/ml). The mixture was stirred for 30 min at 4^{o}C, centrifuged at 10,000 x g for 10 min in a Sorval 2B centrifuge and the precipitate dissolved in buffer (100 mM Tris HCl, 10 mM $MgCl_2$ and 2 mM DTT pH 7.6) (Fraction 2). This solution was desalted on a Sephadex G25 column (40 x 2.5 cm), the protein fraction passed through a dATP Sepharose column (2 cm x 0.2 cm), and the effluent collected and stored at -80^{o}C (Fraction 3). Fractions 1-3 were dialysed separately overnight against 3L of isolation medium before measurement.

The dATP Sepharose column was washed with buffer until the eluate was protein-free (A_{280}), then it was washed with buffer containing 0.5 mM ATP and finally with buffer contained 50 mM ATP (pH 7.6). This last eluate was concentrated (30x) using a hollow fibre concentrator (MDA Scientific Inc., Park Ridge, Ill., USA) and desalted to remove ATP on a Sephadex G25 column (30 x 0.9 cm) which had been equilibrated with isolation medium. The fractions containing protein were reconcentrated and frozen at -80^{o}C (Fraction 4).

The effluent from the dATP Sepharose column (Fraction 3) was adjusted to pH 5.2 with acetic acid, stirred for 5 min at 4^{o}C and centrifuged at 10,000 x g for 10 min. The pH of the supernatant fluid was immediately adjusted to pH 7.8 and stored at -80^{o}C.

Enzyme activity measurement

Assays were conducted for each of the ribonucleotide diphosphates. Each assay had a total volume of 60 µl and always contained 5 µCi of the appropriate [^{3}H] ribonucleotide diphosphate (Sp Act 10 Ci/mM), 10 mM DTT and 20 mM Tris HCl pH 7.6. For measurement of CDP activity it also contained 0.1 mM CDP, 3.3 mM ATP, 8.5 mM $MgCl_2$; for ADP activity 0.2 mM ADP, 0.1 mM dGTP, 8.5 mM $MgCl_2$; for UDP activity 0.2 mM UDP, 1.5 mM ATP, 4.0 mM $MgCl_2$ and for GDP activity, 0.2 mM GDP, 0.1 mM dTTP and 8.5 mM $MgCl_2$. The assay tubes were incubated for 1 hr at 31°C. Each reaction was terminated by boiling for 2 min. A mixture of appropriate nucleotide monophosphate and deoxymonophosphate (100 µg of each) was added as carrier to each sample. This mixture was incubated at 37°C for 30 min with apyrase to hydrolyse the diphosphates to monophosphates. The reaction was terminated by boiling for 2 min and the mixtures centrifuged for 5 min at 2,000 rpm in an IEC PRJ centrifuge. A sample (10 µl) of the supernatant fluid was applied to a PEI cellulose plate and developed in 35 ml 2.0 M LiCl/65 ml 2% Boric acid (25,26). Both the monophosphate and deoxymonophosphate spots were removed from the plate and extracted with 0.2 ml 1N HCl. This was followed by 10 ml scintillation cocktail ACS (Amersham Corp.) and counted in a Beckmann LS 255 scintillation counter. The total radioactivity in each sample was measured by counting 10 µl of the supernatant fluid prior to chromatography. The sum of the counts from the monophosphate and deoxymonophosphate spots was expressed as a fraction of the total radioactivity in the original sample. This fraction indicated the recovery for each reaction and the results were adjusted accordingly.

When the activity of one fraction (in this case the 50 mM ATP eluant, Fraction 4) required the presence of a second, the second fraction (in this case the dATP effluent, Fractions 3 or 5) was present in 5 fold excess to ensure the maximum activity of the first fraction was expressed. Protein was estimated by dye-binding assay (27).

RESULTS AND DISCUSSION

Passage down a dATP Sepharose column removed almost all of the CDP reductase activity from Fraction 2, leaving the effluent (Fraction 3) with no significant activity (Fig. 1). Neither the 0.5 mM ATP eluate nor the 50 mM ATP eluate (Fraction 4) showed significant CDP reductase activity. However when Fraction 4 (50 mM ATP eluant) was measured in the presence of an excess of Fraction 3 (dATP effluent) about 10% of the level of CDP enzyme activity in Fraction 2 could be recovered (Fig. 1). The specific activity of Fraction 4 had increased 250 fold (Table 1) which

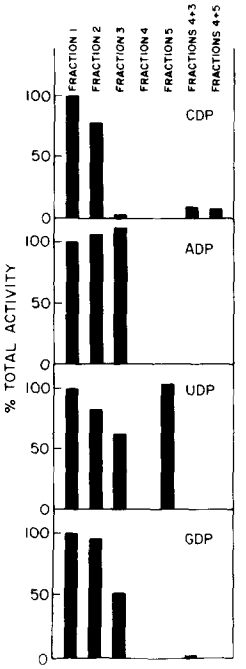

Fig. 1. Quantity of enzyme in each fraction expressed as a % of the amount in Fraction 1 for each ribonucleotide diphosphate

indicated a considerable degree of purification, however SDS gel electrophoresis showed several protein bands (not shown).

This indicates that a component necessary for CDP ribonucleotide reductase activity sticks to the dATP Sepharose column and can be eluted with ATP. Measurement of ADP, GDP, and UDP ribonucleotide reductase activity on the same fractions showed a different pattern (Table 1, Fig. 1). Components necessary for their reduction did not stick to the dATP Sepharose and activity for these three ribonucleotides could be shown to remain in the effluent from this column (Fraction 3) (Table 1, Fig. 1). It should be noted that a slight separation did occur for the GDP ribonucleotide reductase (Table 1).

When the pH of Fraction 3 was adjusted to pH 5.2 and the precipitate removed, measurements for ADP, UDP and GDP reductase

TABLE 1

Purification of Ribonucleotide Diphosphate Reductase

	Vol (ml)	Protein (mg)	CDP Total U	CDP Sp Act	UDP Total U	UDP Sp Act	ADP Total U	ADP Sp Act	GDP Total U	GDP Sp Act
Fract 1 100,000xg	60	3120	1058	0.339	735	.235	1047	0.33	852	0.273
Fract 2 AMS ppt.	23	1012	778	0.77	583	0.58	1158	1.14	830	0.820
Fract 3 dATP effluent	45	1170	23.3	.019	453	0.39	1206	1.03	464	0.396
Fract 4 50 mM ATP eluant	0.5	0.85	0	-	0	-	0	-	0	-
Fract 4+3	0.5	0.85	71.5	84.1	1.7	2.0	1.6	1.9	9.3	10.9
Fract 5 pH 5.2 effluent	45	875	0	-	763	0.872	0	-	81	0.092
Fract 4+5	0.5	0.85	68.3	-	not meas'd	-	not meas'd	-	not meas'd	-

One unit of enzyme activity was expressed as 1 nmole/hr ribonucleotide diphosphate reduced.

Specific activity was expressed as units/mg protein.

activity on the supernatant fluid (Fraction 5) again showed a different pattern (Fig. 1, Table 1). The ADP reductase activity was eliminated, the GDP reductase activity was considerably reduced while the UDP reductase activity increased. Fraction 5 was unchanged in its ability to show CDP reductase activity when measured in the presence of Fraction 4 (50 mM ATP eluate) indicating that the component in Fraction 4 required for CDP

enzyme activity was not removed by acidification (Fig. 1, Table 1). We were unable to dissolve the precipitate resulting from this acidification and so no measurements of ADP or GDP reductase activities could be made.

Ribonucleotide reductase isolated from E. Coli has subunit structure. Subunit B_1 which sticks to dATP Sepharose (2) has been shown to contain both the substrate binding sites and the effector binding sites (4). Subunit B_2 which is required to complete the catalytic site (5) does not absorb to dATP Sepharose. Moore has separated ribonucleotide reductase from Novikoff tumour cells (8) into two subunits, one of which, P_1, is absorbed to dATP Sepharose, the other of which, P_2, is not. The separation of two components of ribonucleotide reductase from rat regenerating liver having these same dATP Sepharose binding and non binding properties would suggest that these two components are indeed enzyme subunits.

Since the subunit which absorbed to dATP Sepharose was specific for CDP ribonucleotide reductase when recombined with Fraction 3 (dATP effluent), and since the activities of other ribonucleotide reductases showed variable distribution during purification, the liver enzyme was considered to have not only subunit structure, but also exist in separate forms for each ribonucleotide.

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