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RIBONUCLEOTIDE REDUCTASE: ASSOCIATION OF THE REGULATORY SUBUNIT IN THE PRESENCE OF ALLOSTERIC EFFECTORS

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SUMMARY: Ribonucleotide reductase from Ehrlich tumor cells moves as a 9 S particle in sucrose gradient centrifugation. In the presence of ATP or dATP, but not dGTP, there is a loss of enzyme activity in the 9 S region. When a fraction from the 6 S region of the gradient or the reductase component not bound by blue-dextran Sepharose or ATP-agarose columns, is added to each gradient fraction, essentially full activity can be recovered, the major portion of which is in the 16-18 S region. The reductase subunit which is bound by blue-dextran Sepharose moves as a 6.5 S particle but ATP shifts this component to 16-18 S. These results indicate that ATP or dATP causes association of the nucleoside triphosphate-binding subunit and dissociation of the remainder of the enzyme from this aggregate.

INTRODUCTION: Ribonucleotide reductase (EC 1.17.4.1) which catalyzes the conversion of ribonucleoside diphosphates to 2'-deoxyribonucleoside diphosphates, is allosterically regulated by nucleoside triphosphate effectors (1). These nucleoside triphosphates have also been found to cause changes in the sedimentation behavior of the reductase from E. coli (2), calf thymus (3), and human cells (4). The presence of 0.1 mM dATP caused a change from 9.7 S to 15.5 S in the E. coli enzyme which was reversed by 2 mM ATP. The 15.5 S complex contained both B1 and B2 subunits but was reported to be inactive. Calf thymus ribonucleotide reductase was also reported to undergo a shift from 11 S to 19 S in 1 mM ATP. In this case, the enzyme apparently retained activity but no mention was made of enzyme activity recovery (3). The reductase from human cells increased in sedimentation rate in the presence of ATP and dGTP (4).

We have found that the sedimentation behavior of ribonucleotide reductase

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from Ehrlich tumor cells changes in the presence of ATP and dATP, but that only the nucleoside triphosphate-binding regulatory subunit is contained in the aggregate.

EXPERIMENTAL: Ehrlich tumor cells were grown in ICR/Dub mice and removed 7 days after transplantation. Preparations of crude extracts and the partial purification of ribonucleotide reductase through the ammonium sulfate step were done as described previously (5).

Twelve- or 36-ml sucrose gradients (5-20%) were prepared in 0.02 M Tris-HCl buffer, pH 7.0, 0.1 mM in dithioerythritol. Gradients also contained ATP, dATP or dGTP (0-5 mM) and magnesium acetate (0-16 mM). Samples applied to gradients were made identical in nucleotide and in Mg^{++} concentrations with the gradients. Runs were on a Beckman SW 41-Ti, rotor for 18 hr at 39,000 rpm (12 ml tubes) or a SW 27 rotor for 24 h at 26,000 rpm (36 ml tubes), all at 2°. Sedimentation coefficients were estimated from a standard curve expressing the sedimentation behavior of a series of proteins of known S value.

Affinity chromatography of ribonucleotide reductase on blue-dextran Sepharose was done as described previously (6). The fraction not bound to the affinity column is the "Tris fraction" while that bound, but eluted by Cibacron blue 3G-A dye is referred to as "Dye fraction". A similar separation of reductase subunits was effected using ATP-agarose (Sigma Chemical Co.) with ATP attached to beaded agarose through the N^6-amino with an 8-carbon spacer. The fraction not bound was eluted with 0.1 M potassium phosphate buffer, pH 7.0, 0.1 mM in dithioerythritol and the fraction bound was removed using 5 mM ATP in the same phosphate buffer.

The enzyme was assayed for CDP reductase and ADP reductase activities by the methods of Steeper and Steuart (7), and Cory, et al. (8), respectively, using [^{3}H]CDP and [^{3}H]ADP as substrates. Assays were done in duplicate. Activities are reported as moles product formed in 30 min by a 100 µl sample in a final assay volume of 150 µl. In assaying the dATP gradient, 3-tube pools were concentrated in an Amicon Minicon B-15 concentration cell before assay in order to remove most of the inhibitory dATP. In addition, the final ATP concentration in these assays was made 6 mM.

RESULTS: Ribonucleotide reductase activity moves in sucrose gradients with a sedimentation coefficient of 9 S in the absence of added nucleoside triphosphate (6) (Fig. 1a). No change occurs upon addition of 4 mM or 16 mM Mg^{++}. When 1 mM ATP is added to the gradient, the 9 S peak shifts to near 11 S and a new peak of very low activity appears in the 16-18 S region. Addition of gradient fractions 5-7, which are inactive alone, to all the other fractions of the gradient increases the activity of the 16-18 S peak more than 2-fold. Addition of a fraction obtained by affinity chromatography on an ATP-agarose column, a fraction which was not bound to the column, increased the size of the 16-18 S peak 3-fold. These data are shown in Fig. 1a. Similar results were obtained in gradients containing 0.5 mM and 2 mM ATP.

In 5 mM ATP, little activity was found anywhere in the gradient but addition of "Tris fraction" (the reductase subunit not bound by blue-dextran

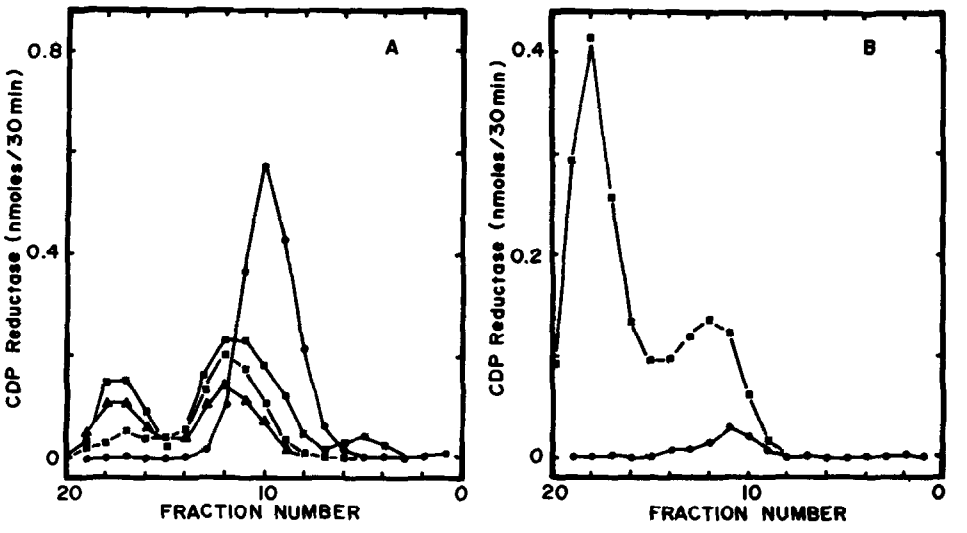

FIGURE 1. Sucrose gradient centrifugation of ribonucleotide reductase. Each gradient was 4 mM in magnesium acetate.

A. 0.6 ml of enzyme solution (5.1 mg protein, 28.7 nmoles/30 min CDP reductase activity) on each 12 ml gradient: ●—●, control; □—□, centrifuged in the presence of 1 mM ATP; Δ—Δ, centrifuged in the presence of 1 mM ATP and assayed by adding an aliquot of gradient fractions 5-7 to each assay; ■—■, centrifuged in the presence of 1 mM ATP and assayed by adding an aliquot of the first fraction from ATP-agarose chromatography to each assay.

B. 0.5 ml of enzyme solution (9.9 mg protein, 22.9 nmoles/30 min CDP reductase) on each 12 ml gradient; ●—●, centrifuged in the presence of 5 mM ATP; ■—■, centrifuged in the presence of 5 mM ATP and assayed by adding an aliquot of "Tris fraction" to each assay.

Sepharose) produced a large peak of reductase activity at 16-18 S and a smaller one (about 30% of the activity) in the 11 S region (Fig. 1b).

dGTP (1 mM) caused, at most, a slight shift in the position of the ADP reductase peak (Fig. 2a). Addition of "Tris fraction" did not reveal any other peaks, indicating that ATP and dGTP are different in their effect on the sedimentation of the enzyme.

The effect of 1 mM dATP (Fig. 2b) was similar to that of 5 mM ATP with activity moving much faster than in the control and with a 7-fold increase in activity being effected by the addition of "Tris fraction".

These data indicated that the nucleoside triphosphate-binding subunit of the enzyme was associating in the presence of ATP or dATP causing dissociation of the remainder of the molecule. To test this hypothesis, "Dye frac-

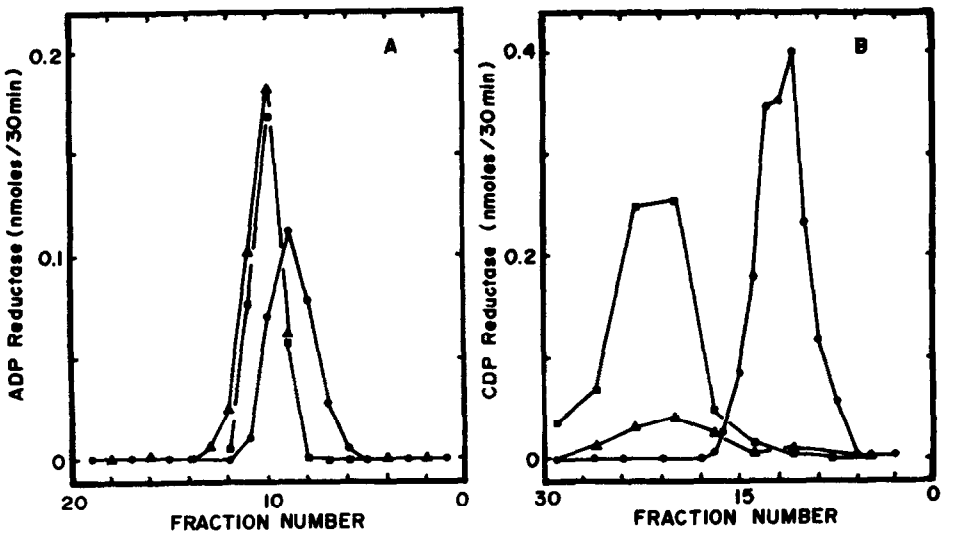

FIGURE 2. Sucrose gradient centrifugation of ribonucleotide reductase. Each gradient was 4 mM in magnesium acetate.

A. 0.5 ml of enzyme solution (7.0 mg protein, 25.0 nmoles/30 min ADP reductase) on each 12 ml gradient: ●—●, control; ■—■, centrifuged in the presence of 1 mM dGTP; ▲—▲, centrifuged in the presence of 1 mM dGTP and assayed by adding an aliquot of "Tris fraction" to each assay.

B. 2.0 ml of enzyme solution (48.6 mg protein, 68.8 nmoles/30 min CDP reductase) on each 36 ml gradient: ●—●, control; ▲—▲, centrifuged in the presence of 1 mM dATP; ■—■, centrifuged in the presence of 1 mM dATP and assayed by adding an aliquot of "Tris fraction" to each assay.

tion" from a blue-dextran Sepharose column was centrifuged with and without 1 mM ATP and assayed by the addition of aliquots of "Tris fraction". The results (Fig. 3) indicate that, in the absence of ATP, this enzyme subunit sediments near 6 S but that ATP shifts the activity peak to the 16-18 S region.

To determine the sedimentation behavior of the remainder of the enzyme (the component analogous to the "Tris fraction") each fraction of a 5 mM ATP-containing gradient was assayed in the presence of exogenous "Dye fraction" (Fig. 3). This subunit peaks near 6 S but trails off toward higher sedimentation rates. None of this subunit remains in the 16-18 S region, indicating that the aggregate of nucleoside triphosphate-binding subunits was devoid of this complementary ("Tris fraction") enzyme subunit.

DISCUSSION: In a previous study (6), it was demonstrated that both the "Tris

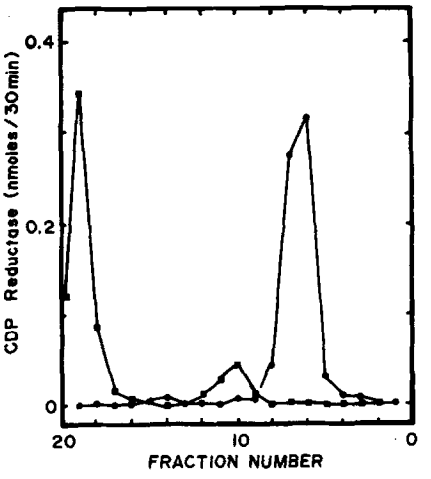

FIGURE 3. Sucrose gradient centrifugation of "Dye fraction" of ribonucleotide reductase. Each gradient was 4 mM magnesium acetate. Assays were done by adding an aliquot of "Tris fraction" to each assay: ●—●, "Dye fraction"; ■—■, "Dye fraction" centrifuged in the presence of 1 mM ATP.

fraction" (5.7 S) and "Dye fraction" (6.5 S) are required for reductase activity. The "Dye fraction" was shown to contain the ATP-binding site while the "Tris fraction" was inactivated by hydroxylamine. We now show that ATP and dATP cause the nucleoside triphosphate-binding subunit ("Dye fraction") of ribonucleotide reductase to increase in sedimentation rate, most likely as a result of aggregation. dGTP does not have this effect at a concentration of 1 mM. The complementary enzyme subunit (presumably the "Tris fraction") does not aggregate but progressively dissociates from the aggregating subunits leaving a trail of these subunits through the middle portion of the gradient.

The fact that dGTP acts differently than ATP or dATP is consistent with the possibility that two distinct classes of nucleoside triphosphate-binding site exist in the mammalian enzyme as in the E. coli reductase (9,10). In that enzyme, one class (l-site) binds dTTP, dATP, dGTP, and ATP while the second (h-site) binds only ATP and dATP. The h-site is thought to control overall activity while the l-site controls specificity. In the mammalian enzyme, the aggregation of the regulatory subunit and the accompanying dis-

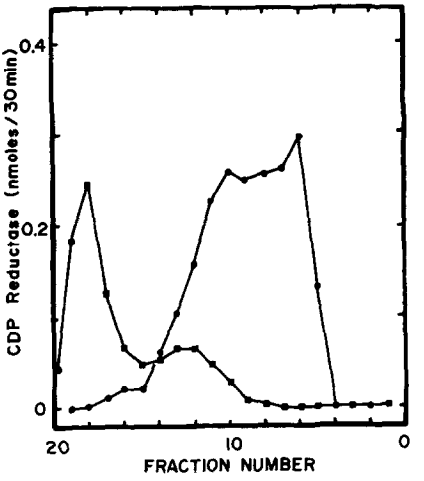

FIGURE 4. Sucrose gradient centrifugation of ribonucleotide reductase. Each gradient was 16 mM in magnesium acetate. 0.5 ml of enzyme solution (7.8 mg protein, 13.1 nmoles/30 min CDP reductase) on each 12 ml gradient: ■—■, centrifuged in the presence of 5 mM ATP and assayed by adding an aliquot of "Tris fraction" to each assay; ●—●, centrifuged in the presence of 5 mM ATP and assayed by adding an aliquot of "Dye fraction" to each assay.

sociation of the remainder of the enzyme in the presence of h-site ligands may well be a major event in the inhibition of enzyme activity. Both subunits are required in the fully active state.

The apparent aggregation of intact enzyme from other mammalian sources in the presence of nucleoside triphosphates may be a result of incomplete dissociation of the complementary subunits from the regulatory subunit aggregate. We observed a little activity in the 16-18 S region in 1 mM ATP or dATP even in the absence of added "Tris fraction".

The association phenomenon may be different in the E. coli system, since the enzyme aggregate formed in the presence of dATP, though assumed to be inactive, contained both B1 and B2 subunits. However, Brown et al. (2) did observe a tendency for the aggregate to dissociate and centrifugation was therefore done with an excess of one of the subunits present in the gradient to prevent this dissociation. Furthermore, sedimentation rates were dependent on enzyme concentration and the concentrations used in their experiments were far higher than we have used. In addition, subsequent experiments have re-

vealed that the sedimentation behavior of the E. coli enzyme was strongly affected by sucrose (12). It seems likely, therefore, that the pattern we have observed is applicable to both the E. coli system and the mammalian ribonucleotide reductases.

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