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PURIFICATION OF THE TWO COMPLEMENTARY SUBUNITS OF RIBONUCLEOTIDE REDUCTASE FROM CALF THYMUS

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SUMMARY: Subunits M1 and M2 of calf thymus ribonucleotide reductase have been purified to homogeneity. Each runs as a single band in SDS-polyacrylamide gel electrophoresis with polypeptide molecular weights of 84,000 for M1 and 58,000 for M2. Additional evidence for homogeneity was obtained using high performance liquid gel chromatography. The native M1 subunit is predominantly monomeric under experimental conditions while the M2 subunit is oligomeric. The M2 subunit has a uv-visible absorption spectrum with a major peak at 404 nm but this spectrum is different from that of the B2 subunit of E. coli ribonucleotide reductase.

INTRODUCTION

Ribonucleotide reductase (2'-Deoxyribonucleoside-diphosphate:oxidized thioredoxin 2'-oxidoreductase, EC 1.17.4.1) catalyzes the conversion of ribonucleoside diphosphates to 2'-deoxyribonucleoside diphosphates. This reaction is one of the rate-controlling steps in DNA synthesis and cell division (1-3). The enzyme from E. coli has been purified (4) and studied extensively (3). The mammalian ribonucleotide reductases have been more difficult to purify but several, including those from rabbit bone marrow (5), Ehrlich tumor cells (6), regenerating rat liver (7), and calf thymus (8,9) have been highly purified. Intact calf thymus enzyme was shown to yield

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several bands in SDS-polyacrylamide gel electrophoresis, two of which were the complementary subunits M1 and M2 (8). The M2 subunit was found in small, non-stoichiometric amounts with respect to M1. Subunit M1 was subsequently purified to homogeneity (9), but M2 was not obtained in pure form. We report here a method by which both the M1 and M2 subunits can be isolated in homogeneous form and describe some properties of these subunits.

METHODS

Assay of ribonucleotide reductase activity. CDP reductase activity was assayed in a final volume of 120 µl containing 7.7 mM potassium phosphate buffer, pH 7.0 which was 0.10 mM in CDP, 6.2 mM in dithioerythritol, 3.3 mM in ATP, 2.0 mM in magnesium acetate, 8.3 mM in sodium fluoride, 0.06 mM in ferric chloride, and contained 0.5 µCi of [^{3}H]CDP. Assays were at 37° for 20 min using the method of Steeper and Steuart (10). A unit of enzyme activity converts 1.0 nmole of CDP to dCDP per minute at 37° under the conditions described.

Purification of intact ribonucleotide reductase. The method used was patterned after that of Engstrom et al. (8) but was simplified by eluting the DEAE-cellulose column in a stepwise manner, deleting the hydroxylapatite column step and replacing the gel chromatography desalting step with dialysis. Details of this procedure will be published elsewhere.

Separation of subunits M1 and M2. A 0.9 x 2.3 cm DEAE-cellulose column (Whatman DE-52) was equilibrated at 0-4° with 0.05 M Tris hydrochloride buffer, pH 7.6, which was 0.10 mM in dithioerythritol, 4 mM in ATP, and 4 mM in magnesium acetate (buffer A). Purified intact ribonucleotide reductase (25-60 units) in buffer A was made 0.05 M in KCl and allowed to stand at 0° for 1 h. The sample was applied to the column at 6 ml/h and eluted first with about 10 ml of buffer A containing 0.1 M KCl. When the absorbance of fractions (1.5 ml) at 295 nm had returned to 0, 10 ml of buffer A containing 0.25 M KCl was applied to the column. Subunit M2 elutes in the 0.1 M KCl-containing buffer while M1 is found in the 0.25 M KCl fractions. The purified subunits were concentrated by precipitation with 80% saturated ammonium sulfate followed by centrifugation (13,200 x g, 40 min) and were then dissolved in 0.5-1.0 ml of 0.05 M Tris hydrochloride buffer, pH 7.6, 0.1 mM in dithioerythritol and 0.1 M in KCl and dialyzed against two, 4 l portions of the same buffer for 4 h. Fractions were assayed for enzyme activity and protein (11) and then frozen in dry ice/acetone and stored at -85°.

Electrophoresis. SDS-polyacrylamide gel electrophoresis was done using a 11-14% exponential gradient slab gel as described by O'Farrell (12) or in tubes using the Weber and Osborn method (13). Staining was with 0.3% Coomassie brilliant blue G-250.

Gel Chromatography. Analytical gel chromatography was performed using a Beckman model 332 HPLC equipped with an Altex Spherogel TSK-3000SW (7.5 x 600 mm) column. The column was equilibrated with 0.25 M KCl, 50 mM Tris hydrochloride buffer, pH 7.6. Chromatography was performed at 0° and at a flow rate of 30 ml/hr. The effluent was monitored at 280 nm.

Table 1. Purification of M1 and M2 Subunits of Ribonucleotide Reductase from Calf Thymus

	Activity (units)[a]					
	Intact	M1	M2	Protein (mg)	Specific Activity	Yield (%)
Intact ribonucleotide reductase	59.5	0		6.81	8.73	
DEAE-cellulose column (4 mM in ATP and Mg^{++})						
0.1 M KCl eluate	0		26.8	0.57	47.0	45
0.25 M KCl eluate	3.14	10.2		3.86	2.64	22

a. M1 and M2 activity determined in the presence of an excess of the complementary subunit.

Spectra. UV-visible spectra were obtained using a Beckman DU-8 spectrophotometer and 1 cm pathlength cuvettes. Molar extinction coefficients were calculated using a molecular weight of 84,000 for M1 and 58,000 for M2.

RESULTS

The separation of the M1 and M2 subunits of calf thymus ribonucleotide reductase is summarized in Table 1. Chromatography of the enzyme on a DEAE-cellulose column in the presence of 4 mM ATP and 4 mM Mg^{++} results in the dissociation of the two complementary subunits. Their separation is a result of binding of the allosteric effector, ATP, to the M1 subunit so that a higher concentration of KCl (0.25 M) is required for its elution than that needed in the absence of ATP or than that needed for M2 elution (0.1 M KCl). The weight of M1 subunit recovered is always considerably larger than that of M2. This finding is consistent with the previously reported occurrence of small non-stoichiometric quantities of M2 in the intact enzyme (8). The specific activity of M1, however, does differ from preparation to preparation. This variation may be related, in part, to the inhibition of M1 by excess M2, when the complementary subunits are re-mixed for assay (9).

The purified M1 and M2 subunits run as single bands in SDS-polyacrylamide gel electrophoresis (Fig. 1). Molecular weights for the polypeptides are

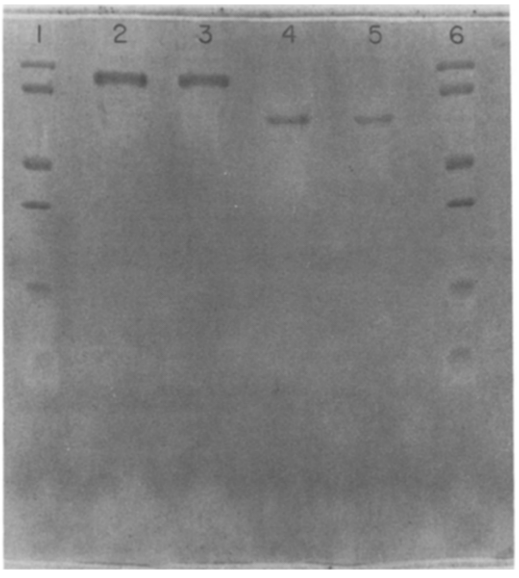


Figure 1: SDS-polyacrylamide gel electrophoresis of the complementary subunits of calf thymus ribonucleotide reductase: Standards (1 and 6) are, from top; phosphorylase b (M_r = 94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400). Subunit M1 (2 and 3), and M2 (4 and 5).

84,000 for M1 and 58,000 for M2. Further evidence for homogeneity was obtained by examining the elution profiles of proteins M1 and M2 using analytical gel chromatography (Fig. 2). Both M1 and M2 ran as symmetrical peaks with Kd's of 0.43 and 0.28 corresponding to Stokes radii of 37 Å and 53 Å, respectively. A molecular weight plot (Fig. 2) yields M_r = 70,000 and 185,000, respectively. A small peak with a Stokes radius of 54.5 Å was also observed in the M1 chromatogram. Both the M1 and M2 profiles contained a minor component eluting in the void volume (M_r > 500,000) which are probably aggregates formed during freezing and thawing.

The M2 subunit has a uv-visible absorption spectrum (Fig. 3) which includes an intense absorption maximum at 404 nm with a molar extinction coefficient near 1.5×10^4 1/mole cm. The M1 subunit does not have this feature but it is seen in the intact enzyme (data not shown). The intensity of this absorption peak is dramatically reduced by the presence of 6 M

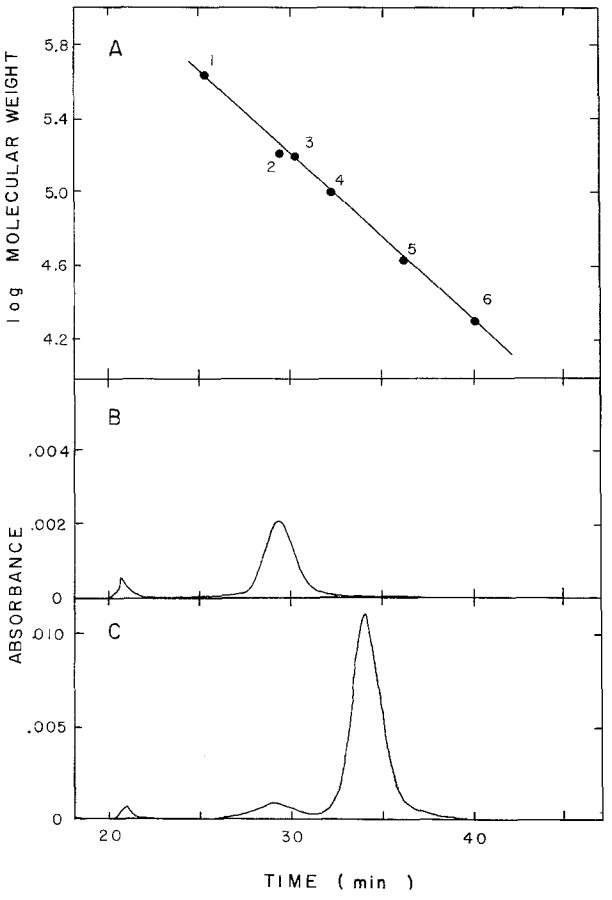
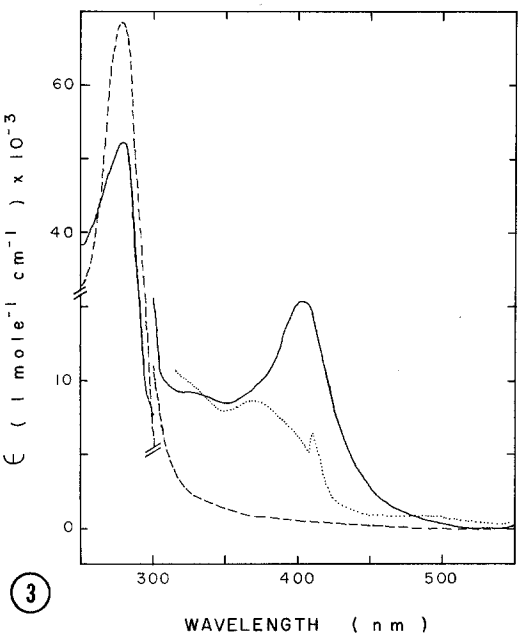


Figure 2: High performance liquid gel chromatography of native M1 and M2 subunits: A. Standard curve of ferritin (1), γ-globulin (2), aldolase (3), hexokinase (4), ovalbumin (5), and soybean trypsin inhibitor (6). B. M2 subunit. C. M1 subunit.

guanidinium hydrochloride (Fig. 4) though it does not entirely disappear even under these conditions.

DISCUSSION

The preparation of highly purified ribonucleotide reductase subunit M1 from calf thymus has been previously described (9). In this communication we report a method for the preparation of homogenous M2 as well as M1 from purified calf thymus ribonucleotide reductase. Our procedure takes advantage of enhanced binding of M1 to DEAE-cellulose in the presence of ATP under which conditions the enzyme subunits are dissociated (14).



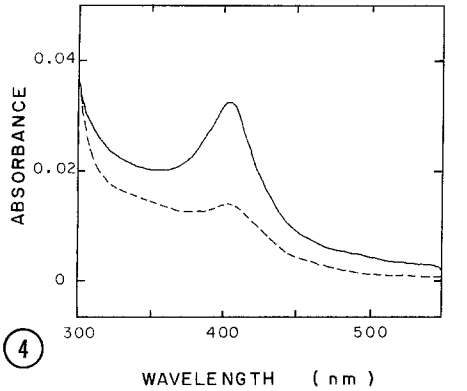


Figure 3: Absorption spectra of the subunits of ribonucleotide reductase: Subunit M2 (———); subunit M1 (-----); E. coli ribonucleotide reductase subunit B2 (.....) (from reference 15).

Figure 4: Effect of 6 M guanidinium hydrochloride on the absorption spectrum of subunit M2: Native M2 (188 µg/ml) (———); M2 (188 µg/ml) in 6 M guanidinium hydrochloride (-----).

The M1 polypeptide molecular weight of 84,000 from SDS-polyacrylamide gel electrophoresis and a slightly lower value from analytical gel chromatography indicate that the native form occurs primarily as a monomer. These results are in agreement with previous work (9). A small fraction of native M1 moves in gel chromatography as a dimer.

A polypeptide molecular weight of 58,000 has been determined for our M2 preparation. Analytical gel chromatography of native M2 gave a Stokes radius of 53 Å. This is in reasonable agreement with the 48 Å value attributed to a 110,000 molecular weight dimer (9). However, when our results are computed with respect to molecular weight, a value more consistent with a trimer is obtained. But whether this value is attributable to a trimeric quaternary structure, an equilibrium between oligomeric forms, or to a non-compact shape of native M2 is not known at this time.

A chromophore at 404 nm is observed in both the M2 subunit and intact ribonucleotide reductase. An absorption peak at 370 nm is seen in the B2 subunit from E. coli ribonucleotide reductase, but with a much lower extinction coefficient. The M2 and B2 subunits each absorb at 325 nm but the extinction coefficients at this wavelength differ considerably as well (it should be noted that molar extinction coefficients for E. coli B2 are based on a 78,000 molecular weight dimer while those for thymus M2 are based on a 58,000 molecular weight monomer). The E. coli enzyme spectrum also contains a sharp peak at 410 nm, attributed to a tyrosyl free-radical (15). We do not detect this feature but it well could be hidden under the broad 404 nm transition. Alternatively, since the calf thymus enzyme behaves differently toward inhibitors like hydroxyurea and 2'-deoxy-2'-azidocytidine diphosphate than does the E. coli enzyme, it has been suggested that the mammalian enzyme may not contain a permanent free radical (9). The dependence of the 404 nm chromophore on the native conformation of protein M2 is established by a large decrease in spectral intensity in the presence of a protein denaturant.

It is now clear from spectral evidence that substantial differences exist between active centers in the E. coli B2 and the calf thymus M2 subunit. The procedure for the purification of calf thymus ribonucleotide reductase subunit M2 presented herein and thus the availability of homogenous M2 provides opportunity for spectral and structural studies of greater detail. Investigations of this nature are now under way.

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