The carboxyl terminus heptapeptide of the R2 subunit of mammalian ribonucleotide reductase inhibits enzyme activity and can be used to purify the R1 subunit

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The heptapeptide, FTLDADF, identical in sequence to the last seven amino acid residues of the carboxyl terminus of the R2 subunit of mouse ribonucleotide reductase (RR), and its N^{α}-acetyl derivative both inhibit calf thymus RR. The N^{α}-acetyl derivative is considerably more potent, displaying a K_i of 20 μM. The same K_i was found for N-AcFTLDADF inhibition of a reconstituted ribonucleotide reductase from calf thymus R1 and mouse R2, indicating that the C-termini of calf R2 and mouse R2 might be identical. Our results, taken together with previous results of others on inhibition of viral RR, suggest that inhibition of RRs by peptides mimicking the C-terminus of R2 may be a general phenomenon. In addition, we have shown that an affinity column, FTLDADF-Sepharose 4B, can be used to prepare ~95% pure calf thymus R1, devoid of contamination with R2, in a very simple procedure that should be generally applicable to R1 purification from many sources.

Ribonucleotide reductase; Peptide inhibition; Affinity chromatography

1. INTRODUCTION

Ribonucleotide diphosphate reductase (RR) is the enzyme responsible for nucleoside reduction in *Escherichia coli*, eucaryotic organisms, and in viral particles [1–3] and is thus a vital enzyme for DNA synthesis. RR activity depends upon the association of two unlike subunits, R1 and R2. Recently, a nonapeptide, YAGAVVDNL, corresponding to the C-terminus of the R2 subunit of herpes simplex virus RR (HSV RR) has been shown to inhibit enzymatic activity by competing with R2 for association with R1 [4–7]. Extensive structure-function studies have shown that even modest changes in the parent peptide can lead to large decreases in inhibitory potency [8,9].

The C-terminal heptapeptide of R2 from mouse RR, FTLDADF, is identical or similar to that of other eucaryotic RRs (e.g. clam, yeast) but quite different from that of HSV RR [10]. Here we demonstrate that the N^{α}-acetyl derivative of this peptide is a potent inhibitor of calf thymus RR and of the hybrid RR derived from the R1 subunit of calf thymus and the mouse R2 subunit and that an affinity column, FTLDADF-Sepharose 4B, can be used to prepare R1 that is nearly homogeneous and devoid of R2 content.

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Abbreviations: PAGE, polyacrylamide gel electrophoresis; RR, ribonucleotide reductase

2. EXPERIMENTAL

A Beckman Protein-Synthesizer model 900 was used for the automated synthesis of FTLDADF by standard stepwise solid-phase Merrifield techniques. N^{α}-*t*-Butyloxycarbonyl (Boc) protected amino acids were used throughout the synthesis. The N^{α} and side-chain protected amino acids, N^{α}-*t*-Boc-β-cyclohexyl-L-aspartic acid and N^{α}-*t*-Boc-O-Bzl (benzyl)-L-threonine, were all in the L configuration. Peptide cleavage from the resin was achieved using the low-high HF procedure [11]. Peptide was solubilized by urea buffer sonication and purified by reverse-phase HPLC, using an ISCO chromatograph connected with a Hewlett-Packard Model 3390A integrator, on a Partisil 10 ODS-3 Magnum 9 column (9.4 × 250 mm) with monitoring at 214 nm. Solvent A: 0.1% TFA/H_2O; solvent B: 0.1% TFA/CH_3CN. Gradient: 5% B to 40% B, 0–40 min. The flow rate was 3 ml/min. Acetonitrile and water were HPLC grade solvent from Fisher Scientific. All other solvents and reagents were of analytical grade. Acetylation of FTLDADF by acetic anhydride to give N^{α}-acetylFTLDADF was performed in dimethylformamide prior to HF release of the peptide from the solid support [12]. ^{1}H NMR spectra, recorded on a Bruker AM-500 instrument, were consistent with the structure of both the parent peptide and the N^{α}-acetyl derivative. High resolution mass spectra, recorded on a VG ZAB E instrument under FAB conditions, gave $[M+H]^+$ molecular ions and fragmentation patterns in agreement with the amino acid sequence and compositions. The peptides were hydrolyzed for 22 h at 110°C with 6 N HCl giving the expected molar ratio of amino acids (± 3%).

FTLDADF-Sepharose 4B was prepared by suspending activated CH-Sepharose 4B (1.0 g) in 5 ml of 1 mM HCl and washing with 200 ml of 1 mM HCl in a Buchner funnel. After drying the gel briefly under suction the slightly moist cake was added with stirring to 5 ml of 0.1 M $NaHCO_3$ buffer (pH 8.4) containing 1.5 mg of the FTLDADF [13]. The reaction was allowed to proceed for 1 h at 4°C. Glycine (2 M, 5 ml) was then added to the reaction mixture at the same temperature to destroy the excess active groups on the gel [13] and stirring was continued for 40 min. The gel was then filtered, washed with 50 ml of 50 mM Tris-Cl (pH 7.6) buffer, suspended in 10 ml of distilled water and packed into a 1.5 × 8 cm column. Prior

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to use the column was washed with 40 ml of 6 M guanidine hydrochloride and 20 ml of distilled water. It was stored at 4°C.

Calf thymus RR was prepared either as described by Engström et al. [14,15] and revised by Mattaliono [16] or in a new procedure involving the use of FTLDADF-Sepharose 4B. Briefly, the first procedure involves homogenizing calf thymus, precipitating DNA from the supernatant by addition of streptomycin sulfate, ammonium sulfate fractionation, DEAE-cellulose (DE-52) chromatography of the 40% ammonium sulfate pellet, and a final chromatography on a dATP-Sepharose 4B column, prepared according to Berglund and Eckstein [17]. Calf thymus RR was eluted from this column by 50 mM ATP, giving a preparation that was 70–80% R1 as judged by SDS-PAGE analysis (see below). In the second procedure, partially purified RR obtained by DE52 column chromatography as described above (about 50 ml, 15 mg protein/ml) was loaded in portions onto the FTLDADF-Sepharose 4B column described above, which had been pre-equilibrated with 20 ml of buffer A (50 mM Tris-Cl, 0.1 mM DTT, pH 7.6) at room temperature. The column was then washed with buffer A (50 ml) and buffer A containing 0.1 M KCl (100 ml) until no further protein eluted. R1 was eluted with buffer A containing 0.5 M KCl (10 ml). Both procedures yielded about 1 mg of purified R1 from 1.5 kg of calf thymus.

Crude mouse RR was prepared from the SC2 mouse cell line derived from hydroxyurea-resistant mouse L cells, obtained as a gift from Dr J.A. Wright, essentially as described by McClarty et al. [18]. Briefly, the cells were grown in 100 mm culture dishes containing Dulbecco's modified Eagle medium (D-MEM) plus 10% fetal calf serum, 1% 2 mM glutamine, and 1% penicillin-streptomycin in the absence of hydroxyurea. Hydroxyurea (5 mM) was added 3 days before harvesting. The cells were removed from the plates by trypsinization and washed with ice-cold phosphate-buffered saline (PBS), pH 7.5, three times and the pellet was resuspended in 50 mM potassium phosphate buffer, pH 7.6, containing 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 1 mM PMSF to a density of about 2.5×10^7 cells per ml. The cells were disrupted by sonication and the suspension was cleared from cell debris by centrifugation at 15000 × *g* for 15 min at 4°C. The resulting cell extract was 1.5 mg/ml in protein and kept frozen at −70°C. Approximately 13.5 ml of crude extract was obtained from cells grown in seventy 100 mm culture dishes.

RR activities were assayed at 35°C following the method of Steeper and Steuart [19]. The reaction mixture, made up in a final volume of 100 μl of 60 mM Hepes, pH 7.6 buffer, contained 2.7 mM $Mg(OAc)_2$, 8.75 mM NaF, 0.05 mM $FeCl_3$, 25 mM DTT, 4 mM ATP, 0.1 mM [5-^{3}H]CDP (12 Ci/mol), 2 mM phenylmethane sulfonyl fluoride, RR, and varying amounts of peptide inhibitor. Peptide concentrations in stock solutions were determined spectrophotometrically, exploiting the electronic absorption of the phenyl side chains. In all assays inhibitors were added prior to enzyme. One RR unit is defined as the amount of enzyme generating 1 nmol of dCDP/h under standard conditions.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12% acrylamide as described by Laemmli [20]. Western blots were performed as described [21], using monoclonal antibody AC1 to R1 obtained from In Ro BioMedTek (Umeå, Sweden). Protein amount was determined according to Bradford [22].

[5-^{3}H]CDP was purchased from Amersham. CDP, ATP, dCDP, DE52, and Sepharose-CL-4B were from Sigma. CH-Sepharose 4B was from Pharmacia P-L Biochemicals. Dowex-1-Chloride was from Aldrich. Protein molecular weight standards were from Biorad.

3. RESULTS AND DISCUSSION

RR in crude extracts of calf thymus, though deficient in R2, retains enough of this subunit to permit measurement of enzymatic activity [14,15]. In contrast, the extract of hydroxyurea-resistant mouse cells has an excess of R2 over R1 [18]. As purification of calf thymus RR proceeds, the ratio of R2/R1 decreases, as does the yield of recovered enzymatic activity [14,15]. Accordingly, in the work reported here we used the relatively crude DE-52 eluate as the source of calf thymus RR, and the much purer dATP-Sepharose 4B eluate in combination with crude mouse extract, as the source of the hybrid calf thymus: mouse RR, following McCarty et al. [18]. The specific activities of crude DE-52 eluate and of dATP-Sepharose 4B eluate were typically 4.5 U/mg protein and 27 U/mg protein, respectively. Combination of 7 μg of dATP-Sepharose 4B eluate with 7.5 μg of crude mouse cell extract protein, gave a specific activity of 520 U/mg of purified calf thymus RR. The activity of the crude mouse extract in the absence of calf thymus RR was very low.

Both the heptapeptide FTLDADF and its N^{α}-acetyl derivative inhibit calf thymus RR (Fig. 1). The apparent K_i for the acetyl derivative (20 μM) is approximately 10 times lower than that for the underivatized peptide. This K_i value corresponds quite closely to that measured by Gaudreau et al. [8,9] for inhibition of HSV-RR by the N^{α}-acetyl derivative of YAGAVVNDL, the nonapeptide that corresponds to the C-terminus of HSV-R2 (the K_i for the corresponding underivatized nonapeptide is 3 times higher), leading to the suggestion that inhibition of RRs by the peptides mimicking the C-terminus of R2 may be a general phenomenon.

The apparent K_i for inhibition of hybrid calf thymus: mouse RR by N^{α}-acetylFTLDADF, which is a measure of the ability of the peptide derivative to compete with R2 binding to R1, is exactly the same as that for inhibition of the calf thymus enzyme (Fig. 1). Since Gaudreau et al. have shown that even small changes in the structure of the nonapeptide YAGAVVNDL can drastically reduce the potency of the resulting peptide as an HSV-RR inhibitor, our results suggest that the C-termini of calf thymus and mouse R2 are either identical or very similar. Viewed from a different vantage

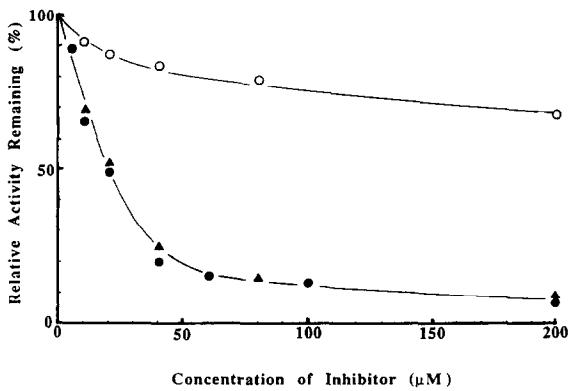

Fig. 1. Inhibition of calf thymus RR activity by FTLDADF (○—○) and by N^{α}-acetylFTLDADF (●—●) and of hybrid calf thymus-mouse RR activity by N^{α}-acetylFTLDADF (△—△). 1.0–1.5 U of RR activity was used in the assays.

point, the results of Gaudreau et al. [8,9] and ourselves suggest that even small differences in such C-terminal sequences could be exploited in designing peptide inhibitors having pronounced specificities as inhibitors of RRs from different species. Efforts in this direction are underway.

The high activity of N^{α}-acetylFTLDADF as an inhibitor of RR activity suggested that FTLDADF could be used as an affinity chromatography ligand for the purification of R1. Accordingly, an FTLDADF-Sepharose 4B column was prepared as described in section 2, the crude DE-52 eluate was applied, and, following extensive washing at low salt, R1 was eluted at high salt. It is important to emphasize that prewashing of the column with 6 M guanidine hydrochloride was essential for its proper functioning.

The crude DE52 eluate, the low and high salt washes of the FTLDADF-Sepharose 4B, and R1 prepared by dATP-Sepharose 4B chromatography were analyzed by SDS-PAGE, as shown in Fig. 2. From the Western blot results it is apparent that virtually all of the R1 (molecular mass 85 kDa [14,15]) in the crude DE52 eluate is retained on the FTLDADF-Sepharose 4B column, so that this step results in a 750-fold purification with near quantitative yield. From the Coomassie blue staining it is clear that chromatography of the crude DE52 eluate on FTLDADF-Sepharose 4B yields a somewhat purer protein (we estimate ~95%) than chromatography on dATP-Sepharose 4B. Consistent with this result is the finding that FTLDADF-Sepharose 4B-purified R1 had essentially no RR activity when assayed alone (it did, however, have a specific activity of 700 U/mg R1 when assayed in combination with 7.5 μg of crude mouse cell extract protein) whereas, as described above, dATP-Sepharose 4B-purified R1 retained RR activity, indicating the presence of contaminating R2. From these results, we consider FTLDADF-Sepharose 4B chromatography to be the method of choice for the preparation of R1. Furthermore, given the apparent generality of R2 C-terminal peptide inhibition of RR activity, it appears likely that analogous columns, prepared from the appropriate R2 C-terminal sequence, will be suitable for the purification of R1 subunits from a large variety of cells.

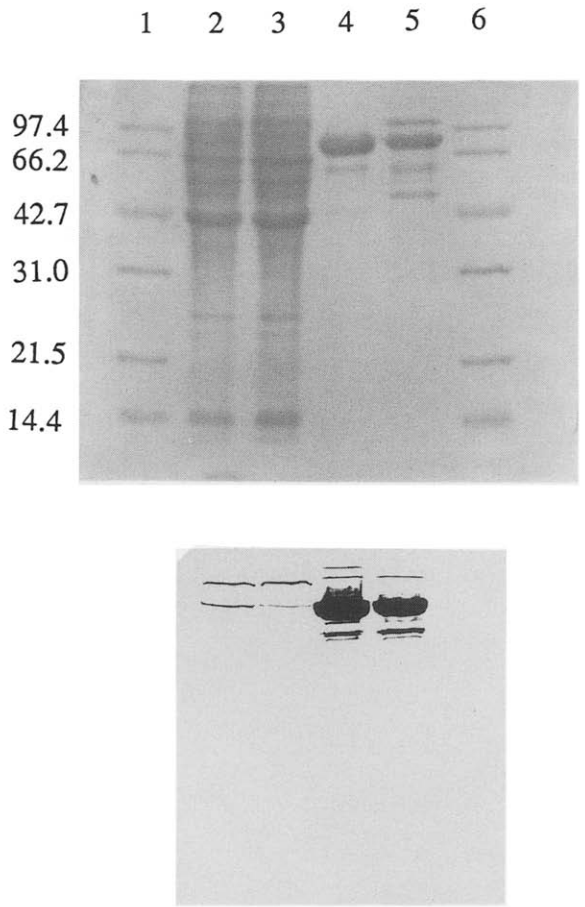

Fig. 2. SDS-PAGE analysis of RR preparations. Top: Coomassie blue staining. Bottom: Western blot. (Lanes 1 and 6) Protein molecular weight standards; 97.4 K, phosphorylase *b*; 66.2 K, bovine serum albumin;42.7 K, ovalbumin; 31.0 K, carbonic anhydrase; 21.5 K, soybean trypsin inhibitor; 14.4 K, lysozyme; (lane 2) DE52 eluate, 5 μg protein; (lane 3) buffer A eluate of FTLDADF-Sepharose 4B, 5 μg protein; (lane 4) 0.5 M KCl wash of FTLDADF-Sepharose 4B, 5 μg protein; (lane 5) R1 purified on dATP-Sepharose 4B, 5 μg protein.

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