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SUPERHELICAL DNA-DEPENDENT ATPASE FROM CALF THYMUS

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Two physically and catalytically distinct DNA-dependent ATPases were isolated from a purified preparation of calf thymus poly(ADP-ribose) polymerase. A unique feature of these two ATPases was the high stimulation by supercoiled DNA. Other nucleic acids (including denatured DNA and ribosomal RNA) and certain polynucleotides differentially stimulated the two enzymes. We have not detected any other DNA-related activity associated with these ATPases.

In the procaryotic cell, a number of DNA-dependent ATPases are associated with DNA replication, repair and recombination functions (1). By contrast only a few of the several DNA-dependent ATPases from eukaryotic sources (2-14) have been functionally identified. Recently we observed that purified calf thymus poly(ADP-ribose) polymerase preparations (15) had an ATP hydrolyzing activity. This report is concerned with this observation and the resolution of two physically and catalytically distinguishable DNA-dependent ATPases. These two enzymes are characterized by their high activation by superhelical DNA and their physical or functional association with poly(ADP-ribose) polymerase.

MATERIALS AND METHODS

The following materials were obtained from commercial sources: [adenosine-2,8-^{3}H]-ATP, [adenosine-2,8-^{3}H]-NAD, [methyl-^{3}H]-thymidine and [γ-^{32}P]-ATP (New England Nuclear); matrex gels, red A and orange A agarose (Amicon Corp.); highly polymerized calf thymus DNA (Sigma Chemical Co.); oligo- and polynucleotides and nucleoside triphosphates (P-L Biochemicals). The following items were generous gifts: activated calf thymus DNA and Ehrlich ascites tumor DNA polymerase α (E.A. Faust, University of Western Ontario); yeast rRNA (R. Anderson, University of Western Ontario); and T4 phage topoisomerase II (B. Alberts (University California at San Francisco). [^{3}H]thymidine-labelled ColE1 plasmid DNA was prepared as previously described (16) and passed through a Sepharose CL6B column to remove RNA.

Enzyme assays. (i) DNA-dependent ATPase activity was assayed by following the formation of ADP from [adenosine-2, 8-^{3}H]-ATP in an incubation mixture (40 µl) containing 25 mM Tris-HCl (pH 8.0), 0.6 mM $MgCl_2$, 1 mM dithio-

Abbreviations. ADP-ribose, adenosine (5')diphospho(5)-β-D-ribose; SDS, sodium dodecyl sulfate.

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threitol, 0.14 mg/ml bovine serum albumin, 1.55 μM [^{3}H]-ATP (25μCi ml^{-1}), 1 μg ColE1 plasmid DNA and enzyme. After 60 min incubation at 37°C, ADP was determined by chromatography on polyethyleneimine-cellulose sheets (Brinkmann Scientific) as previously described (17). When the hydroxyapatite-purified enzyme was assayed, 1 mM EGTA was added to the incubation mixture to prevent a non-enzymic, Ca^{2+}-mediated ATP hydrolysis (18).

(ii) Other DNA-associated activities were studied under the optimal conditions described for the DNA-dependent ATPase assay. For these assays, radioactive ATP was replaced with 1 mM ATP, appropriate substrates were added and incubated at 37°C for 30 min. Commercially available and donated enzymes were used for controls.

Molecular mass determination by gel filtration. Proteins were applied to an Ultrogel AcA44 (LKB) column (0.7 x 20 cm, 30 ml) and eluted with Buffer A [50 mM Tris-HCl (pH8.0), 5mM dithiothreitol, 10% glycerol and 10 mM β-mercaptoethanol] containing 0.1M NaCl. The elution rate was 40 ml/h and 0.25 ml fractions were collected and assayed for the specific proteins. The column was calibrated using blue dextran, aldolase, bovine serum albumin, and ribonuclease A.

RESULTS

Purification of superhelical DNA-dependent ATPases I and II. We found a DNA-dependent ATP hydrolyzing activity which co-eluted with poly ADP-ribose polymerase during the final steps of purification of the polymerase [Fig. 1 and (15)]. It was also noted that topoisomerase I which co-purifies with poly(ADP-ribose) polymerase [Fig. 1A and (19, 20)] separates from the polymerase during hydroxyapatite chromatography (Fig. 1B).

The highly purified, hydroxyapatite-chromatographed poly(ADP-ribose) polymerase preparation (5 ml) [15] was desalted by passage through a BioGel P10 (BioRad) column (0.9 x 11 cm, 28 ml) which had been equilibrated with Buffer A containing 25 mM NaCl. Fractions containing poly(ADP-ribose) polymerase and DNA-dependent ATPase activities were pooled, applied to an orange A-agarose column (0.7 x 2.0 cm, 3.0 ml), and washed with 10 ml Buffer A containing 25 mM NaCl. The column was eluted with 10 ml of Buffer A containing 0.2 M NaCl followed by 10 ml Buffer A with 0.6 M NaCl.

Analysis of the column eluates indicated the presence of two separate DNA-dependent ATPase (I and II) fractions (Fig. 2). The DNA-dependent ATPase I fraction was applied to a red A-agarose column (1 ml) which retains the contaminating poly(ADP-ribose) polymerase activity, but excludes the ATPase. Table 1 provides purification data on the two DNA-dependent ATPases. Analysis of DNA-dependent ATPases I and II by SDS polyacrylamide gel electrophoresis (21) followed by silver staining (22) indicated that both preparations still contained several polypeptides. The extremely small amounts of protein available, the low enzyme activity (Table 1) and the suboptimal ATP concentrations required for the assay precluded adequate analysis of kinetic parameters.

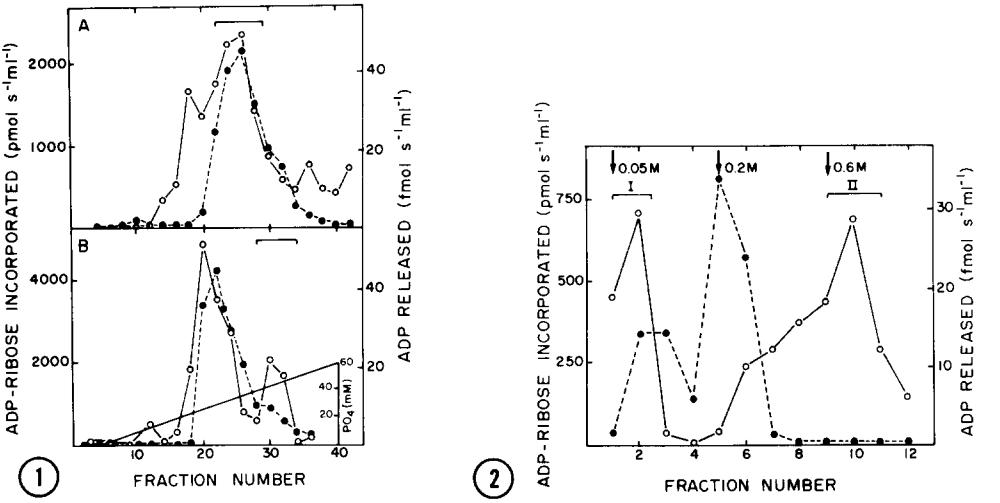

Fig. 1. Purification of DNA-dependent ATPases. Poly(ADP-ribose) polymerase (●) and DNA-dependent ATPase (o) activities were assayed under standard conditions (Materials and Methods). Topoisomerase I (┌──┐) was assayed with ColE1 DNA (11) and analyzed by electrophoresis in 0.8% agarose. A and B refer, respectively, to the enzyme activities obtained from the red A-agarose and hydroxyapatite columns. All experiments indicated in Figs. 1-2 and in the tables have been repeated on two or more occasions.

Fig. 2. Separation of DNA-dependent ATPase and poly(ADP-ribose) polymerase activities using orange A-agarose. The assay conditions and symbols are indicated in Fig. 1. NaCl concentrations used in the elution are indicated (↓).

Properties of superhelical DNA-dependent ATPases I and II. DNA-dependent ATPases I and II had molecular weights of 70,000 and 90,000, respectively, as determined by gel filtration using Ultrogel AcA44 (data not shown).

Table 1. Purification of DNA-dependent ATPases I and II

Fraction	Protein[a]	Total Activity	Yield	Specific Activity	Purification[b] A	Purification[b] B
	μg	pmol s^{-1}	%	pmol $s^{-1}mg^{-1}$	-fold	
hydroxy-apatite	35.0	0.11	100	3.1	1	1400
orange A I[c]	1.8	0.077	70	4.2	13.8	19000
orange A II[c]	0.52	0.026	23	49.0	15.9	22000

a. Protein content was determined by the method of Lowry et al. (25) as modified by Peterson (26), except for the orange A fractions which were estimated from densitometry analysis of the silver stained SDS gels. b. Purification "A" refers to purification using the purified poly(ADP-ribose) polymerase preparation ("hydroxyapatite") as a starting source; "B" approximates the total purification using the extract as a starting source. c. Orange A I and orange A II fractions refer to the purified preparations of DNA-dependent ATPases I and II, respectively. The orange A I preparation was passed through a red A-agarose chromatography step after chromatography on orange A-agarose.

The pH optima for these two enzymes ranged between pH7.8 and 8.2. Both enzymes required divalent cations, with Mg^{2+}, Mn^{2+}, Ca^{2+}, and Zn^{2+} having activity (data not shown). Salts such as KCl and NaCl at concentrations greater than 100 mM inhibited both enzymes, possibly by interfering with enzyme-DNA interactions.

Substrate and DNA requirements. The hydrolysis of ATP by DNA-dependent ATPases I and II was shown by monitoring ADP formation from [adenosine-2, 8-^{3}H]-ATP and Pi from γ-[^{32}P]-ATP (Materials and Methods). No AMP was formed (data not shown). The absence of acid-precipitable radioactivity after incubation of these enzymes with γ-[^{32}P]-ATP indicated that there was no phosphorylation of nucleic acids or polynucleotides. DNA-dependent ATPases I and II were inhibited 79% and 83%, respectively, by 0.1 mM ATPγS. While DNA-dependent ATPase I hydrolyzed dATP, DNA-dependent ATPase II was unable to do so. The other nucleoside and deoxynucleoside triphosphates were hydrolyzed by both enzymes at significant rates and GTP hydrolysis was comparable to that of ATP (data not shown).

The DNA-dependency of the ATPase reaction became evident only after red A-agarose chromatography (Fig. 1A). Both DNA-dependent ATPases showed some activity in the absence of DNA. Usually there was approximately a five-fold stimulation of the ATPase activity with the addition of DNA. A survey of different nucleic acids and polynucleotides indicated that ColE1 plasmid DNA was the most effective in stimulating the two DNA-dependent ATPases. The other nucleic acids (including denatured calf thymus DNA and ribosomal RNA) and polynucleotides differentially stimulated the two enzymes to lesser extents (Table 2). Since the ColE1 plasmid DNA was composed of 90% supercoiled circular and 10% relaxed circular DNA, this DNA was converted to the relaxed circular and linear forms and assayed in the ATPase reaction. The original, supercoiled circular form was by far the most effective (Table 3). However, the enzymes did not appear to alter supercoiled circular, relaxed circular or linear DNA, at least when tested by agarose gel electrophoresis (data not shown).

Possible Functions. We expect the DNA-dependent hydrolysis of ATP to be accompanied by some DNA-associated activity. However, no topoisomerase, endonuclease (single or double-strand), exonuclease (single- or double-strand) or DNA polymerase activities were detected with these two DNA-dependent ATPases or with poly(ADP-ribose) polymerase (data not shown). Also, experiments designed to determine whether poly(ADP-ribose) polymerase (and NAD) would affect the activity of the DNA-dependent ATPases (or the converse) have provided inconclusive results.

Table 2. Cofactor requirements

Polynucleotide	Activity (%)	
	ATPase I	ATPase II
ColE1 DNA	100	100
Calf thymus DNA	0	0
denatured calf thymus DNA	14	0
rRNA	11	0
polydA	27	0
polydT	0	21
polydC	0	37
polyrA	34	27
polyrI	6	24
polyrG.polydC	31	0

DNA-dependent ATPase activity was measured in the presence of 1 µg polynucleotide. PolyrG, polyrC, polydA.polydT, polydG.polydC, polyrA.polydT, polydA.dT_{10} and tRNA were inactive with both enzymes. The $S_{20,W}$ of the homopolymers was in the range of 4-13.

DISCUSSION

The two DNA-dependent ATPases described here are distinguished from the other mammalian DNA-dependent ATPases by their high stimulation by superhelical DNA. However in our limited tests we have not detected any alterations of supercoiled circular, relaxed circular or linear DNA.

The co-purification of poly(ADP-ribose) polymerase, DNA-dependent ATPases I and II and topoisomerase I through most of the purification steps

Table 3. The importance of DNA superhelicity for activity

Plasmid DNA	Activity (%)	
	ATPase I	ATPase II
supercoiled circular	100	100
relaxed circular	0	0
linear	5	18

Enzyme activity was measured in the presence of 1 µg ColE1 DNA. Supercoiled circular DNA refers to the original plasmid DNA preparation, relaxed circular DNA refers to supercoiled DNA treated with T4 phage topoisomerase II (27); and linear DNA refers to supercoiled DNA treated with the restriction enzyme, EcoRI as recommended by Bethesda Research Laboratories, (0.5 units).

[Results and (19, 20)] suggests a physical or functional association. A protein-protein or protein-nucleic acid complex is indicated since these enzymes became separable after exposure to the high salt conditions (2.0 M KCl) of the hydroxyapatite chromatography. Especially indicative of such an association was the finding that DNA-dependent ATPases I and II and poly(ADP-ribose) polymerase initially bound to red A agarose but after hydroxyapatite chromatography only the polymerase bound to red A-agarose. The possible existence of complexes is also suggested by the observation that poly(ADP-ribose) polymerase co-purifies with an "active" form of DNA which dissociates from the enzyme during hydroxyapatite chromatography (23). Electron microscopic analysis of this DNA indicates a branching structure (24). A special affinity for a common DNA structure is also indicated by the observations that poly(ADP-ribose) polymerase preferentially binds to superhelical DNA (16) and superhelical DNA maximally stimulates both ATPase activities as noted here.

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