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Properties of a DNA-dependent ATPase from rat mitochondria

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ABSTRACT

A DNA-dependent ATPase has been highly purified from rat liver mitochondria and characterized. The enzyme catalyzes the hydrolysis of ATP or dATP in the presence of single-stranded DNA cofactor and a divalent cation. The Km values for ATP and dATP are 0.15 mM and 0.35 mM, respectively. The enzyme activity is highly sensitive to N-ethylmaleimide. The sedimentation coefficient of the enzyme is 8.3 S in a glycerol gradient. From this and data on Sephadex G-200 gel filtration, the molecular weight of the native enzyme was calculated to be about 190,000. All the natural single-stranded DNAs tested were equally effective for the ATPase activity, but synthetic deoxyhomopolymer poly(dC) was found to be more effective than natural single-stranded DNAs. Synthetic and natural RNAs had no effect on the activity.

INTRODUCTION

Mitochondria have their own DNA and DNA polymerase and are able to synthesize their DNA *in situ*. In vertebrates, mitochondrial DNA is a closed-circular molecule of about 16 kilobase pairs. The mechanism of mitochondrial DNA synthesis has been shown to have very distinctive features. Recent studies have revealed that the mitochondrial DNA polymerase examined is identical to DNA polymerase γ (1,2). However, DNA polymerase γ itself cannot replicate closed-circular DNA (3), and other protein factors are expected to be involved in its replication. Although a nicking-closing enzyme has been identified in mitochondria (4), no information is available on the nature of the other protein factors involved.

Recent studies on the replication systems of bacteria and phages have revealed that many enzymes or protein factors besides DNA polymerase III holoenzyme are required for DNA replication (5). Interestingly, several of these proteins have been found to exibit DNA-dependent ATPase activity; for instance the *E.coli* dnaB gene product, which seems to act as a mobile promotor (6), the *E. coli* rep protein and DNA helicases I, II and III, which unwind double-stranded DNA (7-9), the T7 gene 4 product, which is the primase for T7 DNA

replication (10), the T4 gene 44-62 and gene 45 products, which stimulate T4 DNA polymerase (11), and E. coli DNA gyrase (12). The involvement of DNA-dependent ATPase in prokaryotic DNA replication prompted us to investigate an analogous protein in mitochondria, because ATP has been found to be essential for DNA synthesis in isolated mitochondria (13).

In this work, we purified a DNA-dependent ATPase from rat mitochondria and examined the details of its unique property of hydrolyzing ATP or dATP in the presence of natural single-stranded DNA or synthetic poly(dC). A possible role of this enzyme in mitochondrial DNA replication is discussed.

EXPERIMENTAL PROCEDURES

Preparation of mitochondria

Mitochondria were prepared from the liver of adult rats as described by Fujisawa et al. (14), except that the isolation medium was 0.25 M sucrose, pH 7.4, containing 1 mM Na_3EDTA.* Livers were homogenized with 10 volumes of the isolation medium in a Potter-Elvehjem homogenizer with a loosely fitting teflon pestle operating at less than 1,000 rpm. Nuclei, red cells, unbroken cells and cell debris were removed by three successive low-speed centrifugations at 870 x g for 15 min, and then mitochondria were obtained from the supernatant by centrifugation at 10,000 x g for 15 min. The mitochondrial pellet was washed four times with buffer A (0.3 M sucrose, 10 mM Tris-HCl, pH 7.4, and 10 mM Na_3EDTA*) by suspension and centrifugation at 10,000 x g for 15 min, and then stored at -20°C until use.

DNA-dependent ATPase assay

The standard reaction mixture in 40 µl contained 25 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 1 mM dithiothreitol, 5 % sucrose, 4 µg bovine serum albumin, 0.8 mM [γ-^{32}P]ATP (10-15 cpm/pmole), 0.5 µg denatured calf thymus DNA and 5 µl of the enzyme fraction. Incubation was carried out at 37°C for 60 min. The reaction was terminated by adding 100 µl each of ice-cold 7 % perchloric acid and 20 % (w/v) Norit. The mixture was stirred in a Vortex mixer and centrifuged for 5 min at 15,000 rpm and the radioactivity of ^{32}P inorganic phosphate was measured in a 50-µl aliquot of the supernatant. The DNA-dependent ATPase activity was determined by measuring the difference in ATP hydrolysis in the presence and absence of DNA. One unit of enzyme activity is defined as the amount catalyzing the release of 1 nmol of inorganic phosphate for 60 min at 37°C. Substrate [γ-^{32}P]ATP was prepared by the method of Glynn and Chappel (15).

Endo- and exo-nuclease assays

Endonucleolytic activity was assayed using fd phage RFI*DNA and omitting denatured calf thymus DNA from the standard reaction mixture for ATPase. In this case, [γ-^{32}P]ATP was replaced by non-radioactive ATP. After incubation for 60 min at 37°C, the reaction was terminated with 1 % SDS*, and the mixture was directly subjected to electrophoresis in 1 % agarose gel, prepared by the procedure of Sharp et al. (16). Electrophoresis was carried out at 60 V in electrophoresis buffer (20 mM Tris-HCl, pH 7.7, 20 mM sodium acetate and 2 mM Na_3EDTA*) for 2-3 h, and then the extent of conversion of RFI* to RFII*DNA was determined.

The exonucleolytic reaction was monitored using ^{32}P-labeled RSV*cDNA* under the same assay conditions as those described above. Incubation was carried out at 37°C for 60 min and the amount of ^{32}P-labeled nucleotide released into the acid-soluble fraction was measured. RSV*cDNA*was prepared by the procedure of Vogt (17).

Glycerol gradient sedimentation

Sedimentation studies were performed by glycerol gradient centrifugation. The enzyme solution (Fraction VII) eluted from an ATP-agarose column was loaded onto a 15-30 % (v/v) linear glycerol gradient prepared in 50 mM Tris-HCl, pH 8.0, 1 mM Na_3EDTA*, 1 mM dithiothreitol and 0.35 M KCl, and centrifuged in a Beckman SW50.1 rotor at 45,000 rpm for 16 h at 4°C. After centrifugation, fractions of six drops were collected from the bottom of the tube, and a portion of each fraction was used for assay for ATPase activity. The sedimentation coefficient was estimated from the position relative to those of catalase (11.3 S), bovine serum albumin (4.7 S) and cytochrome c (1.9 S), used as internal markers. Bovine serum albumin was located by the absorbance at 280 nm and catalase and cytochrome c by that at 410 nm.

Sephadex G-200 gel filtration

A column (2.0 x 38 cm) of Sephadex G-200 was equilibrated with buffer C (50 mM Tris-HCl, pH 8.0, 1 mM Na_3EDTA*, 1 mM dithiothreitol and 10 % glycerol) containing 0.35 M KCl, and then 0.5 ml of ATPase solution was applied to the column. Elution was performed with the same buffer C containing 0.35 M KCl, and enzyme activity in the eluate was measured. The column was calibrated with the following marker proteins: ferritin [Stokes radius (R)=79Å], catalase [(R)=52 Å], yeast alcohol dehydrogenase [(R)=46 Å] and cytochrome c [(R)=17 Å]. The volid volume (Vo) was determined with blue dextran.

Materials

Fd phage DNA and Qβ phage RNA were kindly provided by Dr. Mitsuru Taka-

nami (Kyoto Univ.) and Dr. Akikazu Hirashima (Keio Univ.), respectively. Deoxyribonucleotide triphosphates, ribonucleotide triphosphates, nalidixic acid, rifampicin, ferritin, catalase, yeast alcohol dehydrogenase and cytochrome c were purchased from Boehringer Mannheim GmbH. Calf thymus DNA, N-ethylmaleimide, ethidium bromide, actinomycin D, oligomycin and novobiocin were from Sigma. Agarose-ATP and synthetic polynucleotides were from P-L Biochemicals. Bio-gel P-300 was purchased from Bio-Rad, and DEAE-cellulose and phosphocellulose were from Whatman. Sephadex G-200 was from Pharmacia. Bovine serum ablumin was from Calbiochem.

RESULTS

Purification of DNA-dependent ATPase

All steps of purification were carried out 0-4°C, unless otherwise stated. The seven steps of purification are summarized in Table I.

Frozen mitochondria (wet weight 15 g, equivalent to about 100 g of liver) were thawed, ground with alumina (30 g) in 150 ml of buffer B (50 mM Tris-HCl, pH 8.0, 1 mM Na_3EDTA*, 5 mM β-mercaptoethanol, 50 mM magnesium acetate, 1 M NaCl and 0.1 % Brij 58) and extracted for 30 min by gentle mixing. The mixture was centrifuged at 180,000 x g for 60 min in a Beckman 50Ti rotor and the soluble fraction was designated as Fraction I.

Saturated ammonium sulfate solution was added to Fraction I to give 25 %

Table I. Purification of DNA-dependent ATPase from rat mitochondria

	Total protein	Total activity	Specific activity
	mg	units	
I. Crude extract	1480	(<200)*)	< 0.2
II. Ammonium sulfate	400	1120	2.8
III. Bio-Gel P-300	294	1420	4.8
IV. DEAE-cellulose	84	990	11.8
V. Phosphocellulose	9.6	450	46.9
VI. DNA-cellulose	0.40	102	255.0
VII. ATP-agarose	0.08	34	425.0

*) Total activity in the crude extract could not be determined accurately (see DISCUSSION).

saturation with constant stirring for 20 min. The mixture was centrifuged at 15,000 x g for 15 min and further saturated ammonium sulfate solution was added to the supernatant to give 50 % saturation. The mixture was centrifuged at 15,000 x g for 15 min and the precipitate was washed with buffer C containing ammonium sulfate at 45 % saturation with centrifugation at 15,000 x g for 15 min. The precipitate was dissolved in a minimal volume of buffer Co (10 mM Tris-HCl, pH 8.0, 1 mM Na_3EDTA^*, 1 mM dithiothreitol, 10 % glycerol) and designated as Fraction II.

Fraction II was loaded onto a Bio-gel P-300 column (3.5 x 35 cm) previously equilibrated with buffer Co, and material was eluted with the same buffer at a flow rate of 30 ml/h. Under this low salt condition, all the ATPase activity was eluted in the void volume as an aggregated form (designated as Fraction III). As reported previously, mitochondrial DNA polymerase activity was also eluted in this fraction (3).

Fraction III was applied to a DEAE-cellulose column (2.2 x 20 cm), previously equilibrated with buffer C containing 0.025 M NaCl, and washed with the same buffer. The enzyme was eluted with buffer C containing 0.14 M NaCl at a flow rate of 20-30 ml/h and fractions with activity were pooled (Fraction IV). The DNA polymerase activity was again eluted together with this ATPase activity.

Fraction IV was applied to a phosphocellulose column (1.2 x 10 cm) equilibrated with buffer C containing 0.025 M NaCl. The column was washed with the same buffer and then material was eluted with 50 ml of a linear gradient of 0.025 M-1.0 M NaCl in buffer C. Fractions of 1 ml were collected at a flow rate of 15 ml/h. The DNA-dependent ATPase activity was eluted as a single peak with about 0.15 M NaCl and was almost free of DNA-independent ATPase activity (Fig. 1). In this step the DNA polymerase activity was eluted at 0.35 M NaCl and was well separated from DNA-dependent ATPase activity. Active fractions, eluted with between 0.1 M and 0.2 M NaCl, were pooled and named Fraction V. Fraction V was concentrated to one-tenth of its original volume by ammonium sulfate precipitation, dialyzed overnight against 1,000 volumes of buffer C containing 0.025 M NaCl, and then stored at -20°C until use.

Fraction V was thawed and immediately applied to a single-stranded DNA cellulose column (0.5 x 2.5 cm) prepared by the method of Takanami et al. (personal communication), which was packed in a 1 ml disposable syringe and previously equilibrated with buffer C containing 0.025 M NaCl. The column was washed five times with 0.5 ml of buffer C containing 0.06 M NaCl, and then material was eluted stepwise with buffer C containing 0.3 M NaCl. Frac-

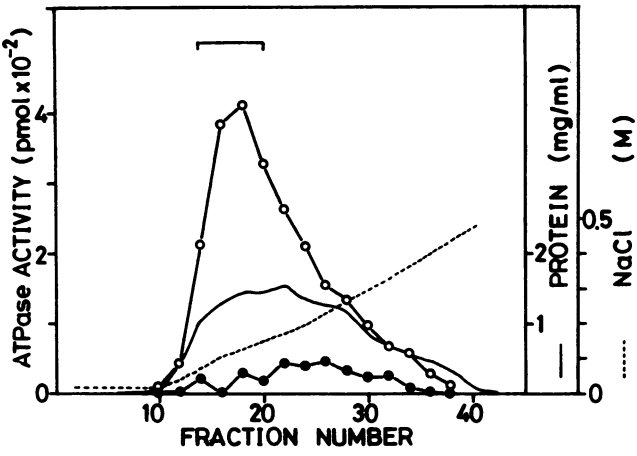

Fig. 1. Phosphocellulose chromatography of mitochondrial DNA-dependent ATPase. Chromatography was carried out as described in the text using Fraction IV. ATPase activity was assayed in the presence (o——o) and absence (●——●) of heat-denatured calf thymus DNA.

tions of 0.15 ml of eluent were collected at a flow rate of 5 ml/h. Activity fractions were combined and named Fraction VI.

Fraction VI was dialysed against buffer C containing 0.025 M NaCl and applied to an ATP-agarose column (0.5 x 2.5 cm), which had been equilibrated with buffer C containing 0.025 M NaCl. The column was first eluted with buffer C containing 0.08 M NaCl, and then with buffer C containing 0.3 M NaCl. Fractions of 0.07 ml were collected at a flow rate of 5 ml/h and a small aliquot of each fraction was assayed for enzyme activity. Peak fractions eluted with buffer C containing 0.3 M NaCl were pooled and named Fraction VII. Enzyme in Fraction VII was unstable and so was promptly used for further experiments.

Fraction VII had no detectable DNA polymerase activity, measured by the method of Tanaka et al. (2), or RNA polymerase activity, measured by the method of McMacken et al. (6). The enzyme was also free of contaminating nuclease activity. Furthermore no endonuclease activity was detectable by measuring endonucleolytic conversion of RFI* DNA to RFII* form on agarose gel, and the fraction caused no degradation of single-stranded DNA when incubated with ^{32}P-labelled RSV*cDNA* under the conditions described in EXPERIMENTAL PROCEDURES. By our procedure, the DNA-dependent ATPase of rat mitochondria was purified more than 2,000-fold from the crude extract (Fraction I). How-

ever, Fraction VII enzyme was not homogeneous as judged by polyacrylamide gel electrophoresis under nondenaturing condition (unpublished data).

Physical properties

On velocity sedimentation in a 15-30 % glycerol gradient in buffer C containing 0.35 M KCl, Fraction VII DNA-dependent ATPase sedimented at 8.3 S as a single symmetrical peak (Fig. 2A). No ATPase activity was detectable when cofactor DNA was omitted from the reaction mixture. On Sephadex G-200 gel filtration, the enzyme activity was eluted as a single peak. A plot of gel filtration data by the equation of Laurent and Killander (18), gave a satisfactory linear relationship between $(-\log Kav)^{1/2}$ and Stokes radius (Fig. 2B) and the Stokes radius of the enzyme was determined as 57 Å. From this and data on Sephadex G-200 gel filtration, the molecular weight of the native enzyme was calculated to be approximately 190,000 daltons by the procedure of Siegel and Monty (19).

Reaction requirements

As shown in Table II, the enzyme activity of ATPase depended on the presence of cofactor DNA. From a plot of the activation of ATPase as a function of the cofactor DNA concentration, the Km for heat-denatured calf thymus DNA, for instance, was calculated to be 4 μg/ml (unpublished data). The enzyme

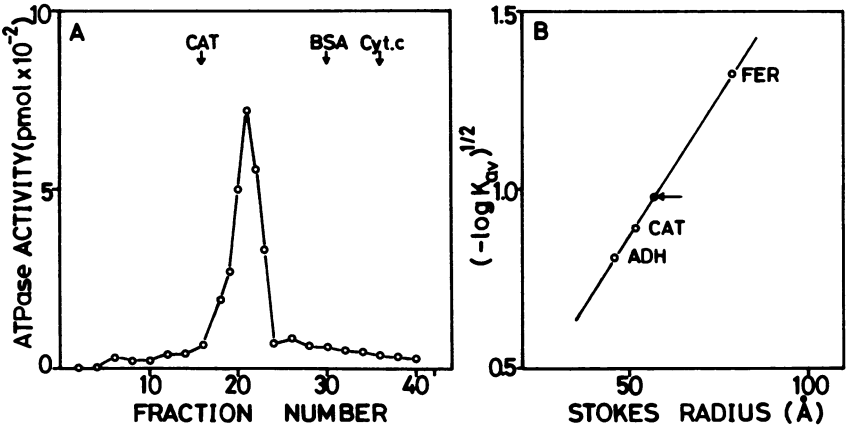

Fig. 2. A: Glycerol gradient sedimentation of mitochondrial DNA-dependent ATPase. Arrows indicate the positions of catalase (CAT), 11.3 S; bovine serum albumin (BSA), 4.7 S; cytochrome c (Cyt.c), 1.9 S.
B: Sephadex G-200 gel filtration analysis. Gel filtration was performed as described under EXPERIMENTAL PROCEDURE. Data are plotted according to the equation of Laurent and Killander (18). Marker proteins are indicated as follows: ferritin (FER), catalase (CAT), yeast alcohol dehydrogenase (ADH).

used heat-denatured DNA preferentially to native DNA. To determine the optimum assay conditions, we measured DNA-dependent ATPase activity in the presence of 12.5 µg/ml of heat-denatured calf thymus DNA and 0.8 mM ATP under various conditions. Studies on the requirement for divalent metal ion (Fig. 3) showed that activity was maximal with a concentration of about 1.5 mM $MgCl_2$, $MnCl_2$ being less effective for ATP hydrolysis and $CaCl_2$ having no effect. The enzyme activity was not influenced by NaCl concentrations of up to 80 mM, but 200 mM NaCl caused 50 % inhibition of activity. The enzyme exhibited a broad pH optimum between pH 7.0 to 9.0 when measured with 50 mM sodium phosphate buffer, 25 mM Tris-HCl buffer and 50 mM sodium glycinate buffer at pH ranges of 5.8-7.8, 7.4-9.0 and 8.6-10.6, respectively.

Substrate specificity

In the presence of heat-denatured calf thymus DNA, ATP hydrolysis was linear for up to 90 min. The products for the reaction with ATP as substrate were nucleotide diphosphate and inorganic phosphate. The enzyme activity exhibited normal Michaelis-Menten kinetics with ATP and the Km for ATP was cal-

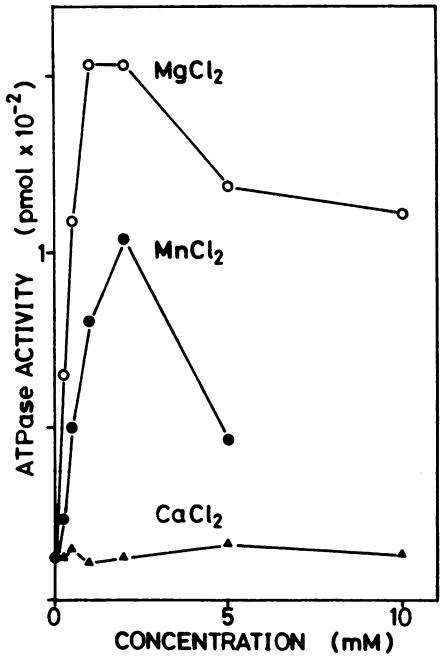

Fig. 3. Effect of divalent cations. Reactions were carried out using purified DNA-dependent ATPase (Fraction VII). $MgCl_2$(o); $MnCl_2$(•); $CaCl_2$( ).

culated as 0.15 mM from a Lineweaver-Burk plot of the activity versus ATP concentration.

Nucleoside triphosphate specificity was examined either by measuring the release of [^{32}P] inorganic phosphate from [γ-^{32}P]-labelled substrate or by testing the ability of unlabelled nucleoside triphosphate to inhibit ATP hydrolysis. Data showed that the enzyme efficiently hydrolyzed ATP and dATP, but not GTP or dGTP. The Km for dATP was calculated to be 0.35 mM. UTP and CTP were not effective as substrates or inhibitors, since these unlabelled triphosphates did not influence the DNA-dependent ATP hydrolysis when added at the same concentration as ATP (data not shown).

DNA and synthetic polynucleotide cofactor requirement

As described above, the enzyme has an absolute requirement for DNA as a cofactor for ATP hydrolysis. Because of this DNA dependency, we tested the effects of various DNAs and synthetic polynucleotides on the enzyme activity. As summarized in Table II, single-stranded DNA is a preferential cofactor for the ATPase. This preference was clearly indicated by comparison of the stimulatory effects of fd RFI* DNA and fd ssDNA*. Activated salmon sperm DNA also caused similar stimulation. None of the various double-stranded DNAs tested showed cofactor activity, except closed-circular mitochondrial DNA, probably because it has a short single-stranded region in the tertiary structure. Two different denatured restriction fragments of rat mitochondrial DNA, HpaEcoA5 containing the replication origin (20) and HpaEcoA4 adjacent to HpaEcoA5, showed only the same extent of stimulation as that of other single-stranded DNAs. The above results show that the ATPase activity has no sequence specificity for cofactor DNA.

The effects of several synthetic polynucleotides were also examined. Poly(dA) was not stimulatory, but poly(dG) and poly(dT) caused 45 % and 83 %, respectively, of the stimulation by heat-denatured calf thymus DNA. Surprisingly, poly(dC) was almost twice as stimulatory as heat-denatured calf thymus DNA. This polynucleotide preference may be due to the base specificity or to a certain secondary structure of the synthetic DNA. Oligo(dC)$_{12-18}$ stimulated the ATPase activity 53 %, but oligo(dT)$_{10}$ had essentially no effect. These data suggest that besides the base specificity, a certain chain length of polydeoxyribonucleotide molecules is required for stimulation of ATP hydrolysis. Polyribonucleotides such as Qβ phage RNA and poly(rA) were inactive as cofactors.

Inhibitors

The effects of various inhibitors on ATPase activity were tested with

Table II. Polynucleotide cofactor requirement of mitochondrial DNA-dependent ATPase. Reactions were carried out as described under EXPERIMENTAL PROCEDURES using purified DNA-dependent ATPase (Fraction VII) and various polynucleotides as cofactors. Activities are shown as percentages of that in the presence of heat-denatured calf thymus DNA.

Polynucleotide Cofactor (10 µg/ml)	Enzyme Activity (%)
None	3.6
Natural DNA	
Denatured calf thymus DNA	100
fd-ssDNA	96.2
fd-RFI DNA	13.6
Mitochondria DNA	33.6
Activated salmon sperm DNA	90.1
λ Phage DNA	11.9
HpaEcoA5 mtDNA	18.2
Denatured HpaEcoA5 mtDNA	99.9
HpaEcoA4 mtDNA	24.3
Denatured HpaEcoA4 mtDNA	96.6
Synthetic DNA	
Poly(dA)	4.1
Poly(dG)	45.4
Poly(dC)	189.9
Poly(dT)	83.0
Poly(dC)-oligo(dG)$_{12-18}$ *)	203.2
Oligo(dT)$_{10}$	2.0
Oligo(dC)$_{12-18}$	53.4
RNA	
Qβ phage RNA	9.6
Poly(rA)	7.5
Poly(rC)-oligo(dG)$_{12-18}$ *)	13.1

*) These complexes contain cytosine and guanine in a molar ratio of 20:1.

heat-denatured calf thymus DNA as cofactor and results are summarized in Table III. In contrast to their strong inhibitory effects on DNA synthesis in isolated mitochondria (13), actinomycin D had no effect and ethidium bromide only reduced the enzyme activity 43 % when added at high concentration. Nalidixic

Table III. Effects of various inhibitors. Reactions were carried out as described under EXPERIMENTAL PROCEDURES using purified DNA-dependent ATPase (Fraction VII)

Inhibitor	Enzyme Activity (%)
None	100
Actinomycin D (5 μg/ml)	99.8
Ethidium bromide (20 μg/ml)	57.0
Rifampicin (20 μg/ml)	107.6
Nalidixic acid (100 μg/ml)	106.3
Novobiocin (100 μg/ml)	108.5
Oligomycin (10 μg/ml)	101.6
Urea (2 M)	49.8
N-ethylmaleimide (1 mM)*)	22.7

*) DTT was omitted from the reaction mixture

acid and novobiocin, which are known to be specific inhibitors of DNA gyrase, did not inhibit the DNA-dependent ATP hydrolysis. Oligomycin, a specific inhibitor of mitochondrial F1-ATPase, also did not have any effect. On the other hand, the enzyme was highly sensitive to N-ethylmaleimide, suggesting that an SH-group is involved in the catalytic reaction of this DNA-dependent ATPase. Addition of 2 M urea to the reaction mixture reduced the enzyme activity by 50 %. As a high concentration of urea is known to dissociate various oligomeric enzyme proteins into monomeric species, the active form of the ATPase probably has an oligomeric structure.

DISCUSSION

In this work we purified DNA-dependent ATPase extensively from rat liver mitochondria. By our procedure, the specific activity of the enzyme was increased more than 2,000-fold from that of the crude extract (Fraction I), although we could not determine the total activity of the DNA-dependent ATPase in the crude extract accurately because there was little difference between the enzyme activities measured in the presence and absence of DNA or because of the existence of some inhibitory protein. Attempts at further purification of the enzyme to obtain a homogeneous protein have so far been unsuccessful, mainly because of considerable decrease in stability of enzyme with decrease

in the protein concentration during purification.

However, several remarkable characteristics of the enzyme were observed in the present study. The enzyme specifically hydrolyzes ATP and dATP in the presence of natural single-stranded DNA, the Km values for ATP and dATP being 0.15 mM and 0.35 mM, respectively. The reaction requires Mg^{2+} ion and is highly sensitive to N-ethylmaleimide, but completely resistant to oligomycin, which is a specific inhibitor of mitochondrial F1-ATPase, and to nalidixic acid and novobiocin, which inhibit E. coli DNA gyrase. The high sensitivity of the enzyme to N-ethylmaleimide suggests a direct role of an SH-group in the catalytic reaction. Natural single-stranded DNA is a good cofactor for ATP hydrolysis and the several species of single-stranded DNA examined showed similar extents of stimulation of ATPase activity. Another interesting feature of the enzyme is that poly(dC) is a more effective than natural single-stranded DNA. This finding suggests that some specific DNA base or secondary structure is related to cofactor function in the ATPase reaction. In this connection it is interesting that poly(dT) was found to stimulate ATPase activity associated with the large T antigen of Simian virus 40 (21).

Recently, DNA-dependent ATPase have been found in various mammalian cells, such as mouse myeloma (22), calf thymus (23), human EUE cells (24), and human KB cells (Dr. David Korn, personal communication). Like the present enzyme, these enzymes were found to exhibit ATPase activity in the presence of natural single-stranded DNA. But our mitochondrial DNA-dependent ATPase differs in two characters from other DNA-dependent ATPase: the molecular weight of the native enzyme, estimated from the sedimentation coefficient, is rather large and the enzyme shows strong preference for synthetic poly(dC). We did not obtain any information of the physiological role of mitochondrial DNA-dependent ATPase. It seems possible that the enzyme is involved in the fork movement during the process of mitochondrial DNA replication considering the properties of the enzyme shown in the present work and a previous report that highly-purified DNA polymerase γ itself cannot catalyze displacement synthesis on nicked DNA (2). In a preliminary experiment, however, we could not detect any unwinding of duplex DNA by the enzyme alone; for this, the enzyme may require the cooperation of DNA polymerase γ and/or a DNA binding protein. We are now studying the real function of DNA-dependent ATPase by constructing an in vitro replication system using the plasmid-cloned mitochondrial DNA.

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* Abbreviations used are: RSV, Rouse sarcoma virus; cDNA, complemental DNA; Na_3EDTA, ethylenediaminetetraacetic acid trisodium salt; SDS, sodium dodecyl-sulfate; RFI and RFII, closed-circualr and open-circular replicative forms, respectively; ssDNA, single-stranded DNA.

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