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Purification and Characterization of a Deoxyribonucleic Acid Dependent Adenosinetriphosphatase from Mouse FM3A Cells: Effects of Ribonucleoside Triphosphates on the Interaction of the Enzyme with Single-Stranded DNA†

Masayuki Seki,* Takemi Enomoto, Yoshinari Watanabe,‡ Yasunori Tawaragi, Katsumi Kawasaki,§ Fumio Hanaoka, and Masa-atsu Yamada

Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan

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ABSTRACT: There are at least four forms of DNA-dependent ATPase in mouse FM3A cells [Tawaragi, Y., Enomoto, T., Watanabe, Y., Hanaoka, F., & Yamada, M. (1984) *Biochemistry 23*, 529–533]. One of these, ATPase B, has been purified and characterized in detail. During the purification of the enzyme, we encountered the difficulties that the enzyme could not be recovered well from the single-stranded DNA–cellulose column and that the enzyme activity was distributed very broadly. The problems were resolved by the addition of ATP in the elution buffer. The ATPase has a sedimentation coefficient of 5.5 S in both high salt and low salt. The enzyme hydrolyzes rNTPs and dATP, but ATP and dATP are preferred substrates. Adenosine 5′-*O*-(3-thiotriphosphate) (ATP-γ-S), 5′-adenylyl methylenediphosphate (AMP-PCP), and 5′-adenylyl imidodiphosphate (AMP-PNP) inhibit the enzyme activity. The enzyme is insensitive to ouabain, oligomycin, novobiocin, and ethidium bromide. A divalent cation ($Mg^{2+} \simeq Mn^{2+} > Ca^{2+}$) as well as a nucleic acid cofactor is required for activity. Poly(dT), single-stranded circular DNA, and heat-denatured DNA were very effective. Native DNA was little effective with an efficiency of 29% of that obtained with heat-denatured DNA. In addition, the enzyme showed almost no activity with poly(dA)·poly(dT) although it showed very high activity with the noncomplementary combination of poly(dT) and poly(dC), suggesting that ATPase B requires single-stranded DNA for activity. ATP altered the affinity of ATPase B for single-stranded DNA. The interaction of the enzyme with DNA was studied by Sephadex G-200 gel filtration assay. In the presence of 0.15 M KCl, almost all of the enzyme applied to the column was coeluted with single-stranded circular *fd* DNA, while in the presence of 1 mM ATP under the same salt condition the amount of the enzyme coeluted with DNA decreased greatly. The effect of dissociation of the enzyme from the single-stranded DNA was not observed with other ribonucleoside triphosphates, CTP, GTP, and UTP.

It has been shown that ATP is required for DNA replication in eukaryotic cells as well as prokaryotes. We have demonstrated an absolute requirement for high levels of ATP for DNA synthesis in isolated nuclei, especially for the synthesis of Okazaki fragments (Enomoto et al., 1981, 1983a). However, the molecular basis of the requirement for high levels of ATP remains to be resolved. In prokaryotic systems, the role of ATP in DNA replication has been related to several proteins that have DNA-dependent ATPase activity by a combination of genetic and biochemical approaches. These proteins include the *rep* protein, which catalyzes the separation of DNA strands, the *Escherichia coli dnaB* gene product, which acts as a "mobile promoter" enabling the primase to synthesize initiator RNAs, and *E. coli* DNA gyrase, which introduces superhelical turns into closed circular DNA. Therefore, the requirement for ATP for DNA replication in eukaryotic cells could be explained by the participation of analogous proteins in the DNA replication, which perform their functions concomitantly hydrolyzing ATP in a DNA-dependent manner.

During this decade, eukaryotic DNA-dependent ATPases have been isolated from mouse myeloma (Hachmann & Lezius, 1976), bovine lymphocyte (Otto, 1977), lily (Hotta & Stern, 1978), human EUE cells (Cobianchi et al., 1979), calf thymus (Assairi & Johnston, 1979), human KB cells (Boxer & Korn, 1980; DeJong et al., 1981), yeast (Plevani et al., 1980), mouse FM3A cells (Hyodo & Suzuki, 1981), rat liver mitochondria (Yaginuma & Koike, 1981), rat hepatoma (Thomas & Meyer, 1982), and monkey CV-1 cells (Brewer et al., 1983). We have also isolated three forms of DNA-dependent ATPase from calf thymus (Watanabe et al., 1981). The lily enzyme has been shown to have helicase activity, and a limited degree of stimulation of DNA polym-

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‡Present address: Department of Biochemistry I, Fukui Medical School, Matsuoka, Fukui 910-11, Japan.
§Present address: Department of Microbiology, Institute of Physical and Chemical Research, Wako-shi, Saitama 351-01, Japan.

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erase has been reported for the KB cell enzyme (Boxer & Korn, 1980) and the rat hepatoma enzyme (Thomas & Meyer, 1982). However, the biological roles of most of the eukaryotic DNA-dependent ATPases are unknown. Therefore, a detailed analysis of the enzymological properties of DNA-dependent ATPases and the interactions of the enzymes with other replication enzymes and proteins is necessary for the understanding of the physiological roles of DNA-dependent ATPases.

In order to elucidate the molecular mechanism of DNA replication in mammalian cells, we have tried genetic and biochemical approaches using mouse FM3A cells. We have isolated several mutants related to DNA replication (Nakano et al., 1978; Nishimura et al., 1979; Tsai et al., 1979; Yasuda et al., 1981). Recently, one of the mutants, tsFT20, was found to have heat-labile DNA polymerase α activity (Murakami et al., 1985). In biochemical approaches, we have purified and characterized DNA replication enzymes and proteins such as two forms of DNA polymerase α (Enomoto et al., 1985), primase (Suzuki et al., 1985), and DNA polymerase α stimulating factors (Kawasaki et al., 1982, 1984) from mouse FM3A cells. We have also isolated four forms of DNA-dependent ATPase from the cells and initially characterized two forms of the ATPase, B and C_3 (Watanabe et al., 1982; Tawaragi et al., 1984; Enomoto et al., 1984). In this study, we have purified and further characterized one of the four DNA-dependent ATPases, ATPase B.

Materials and Methods

Materials. Nucleoside triphosphates, AMP-PCP,[1] AMP-PNP, ATP-γ-S, and oligomycin were purchased from Boehringer Mannheim. Ouabain, ethidium bromide, and novobiocin were obtained from Sigma. Actinomycin D was from P-L Biochemicals. Deoxyribohomopolymers, poly[d(A-T)], oligodeoxyribonucleotides, poly(C), and poly(A) were purchased from P-L Biochemicals. Poly(G) and poly(U) were from Boehringer Mannheim. Yeast tRNA was obtained from Sigma. Calf thymus DNA was obtained from Worthington. Heat-denatured DNA was prepared as follows. Calf thymus DNA was dissolved in the solution containing 10 mM Tris-HCl, pH 7.4, and 1 mM Na_3EDTA at a concentration of 1 mg/mL and denatured by heating for 15 min in boiling water and rapid cooling in ice. Activated DNA and S1 nuclease treated native DNA were prepared with calf thymus DNA according to the method of Aposhian and Kornberg (1962) and the method of Mace and Alberts (1984), respectively. Single-stranded circular *fd* DNA and pBR322 RFI DNA were prepared according to Herrman et al. (1980) and Maniatis et al. (1982), respectively. DEAE-cellulose was obtained from Brown. Phosphocellulose (P11) was from Whatman. Hydroxylapatite was from Bio-Rad. DNA–cellulose was prepared with heat-denatured calf thymus DNA essentially according to the method of Alberts and Herrick with a slight modification as described previously (Tanuma et al., 1980).

Buffers. Buffer 1 contained 20 mM potassium phosphate buffer, pH 7.5, 0.1 mM Na_3EDTA, 1 mM 2-mercaptoethanol, 0.25 mM PMSF, and 1% ethanol. Buffer 2 contained all of the components of buffer 1, 20% ethylene glycol, and 0.01% Triton X-100. Buffer 3 contained all of the components of buffer 1 and 50% (v/v) glycerol. Buffer 4 contained 20 mM potassium phosphate buffer, pH 7.5, 0.1 mM Na_3EDTA, 1 mM 2-mercaptoethanol, 20% ethylene glycol, and 0.2 mg/mL BSA.

Cells. FM3A cells (1×10^6) were injected into the abdominal cavity of a ddY mouse. On the fifth day after inoculation, cells were synchronized in S phase by intraperitoneal injection of 100 μg of 5-fluoro-2′-deoxyuridine and harvested as described previously (Hanaoka et al., 1981). The cells were stored at −80 °C until use.

ATPase Assay. The standard reaction mixture (50 μL) contained 50 mM Tris-HCl, pH 7.5, 20 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 5 mM ATP, and 25 μg of BSA. DNA-dependent and -independent ATPase activities were assayed in the presence or absence of 5 μg of heat-denatured calf thymus DNA. Incubations were carried out at 37 °C. The amount of ADP produced was determined by the method of Korn and Yanofsky (1976). One unit of activity is defined as the amount of enzyme that hydrolyzes 1 nmol of ATP/h at 37 °C.

DNA Polymerase α Assay. The standard reaction mixture (30 μL) contained 20 mM Tris-HCl, pH 8.0, 3.3 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 0.2 mg/mL BSA, 100 μM each of dATP, dCTP, and dGTP, 50 μM [^{3}H]dTTP (0.1 Ci/mmol), and 500 μg/mL activated calf thymus DNA. Incubations were carried out for 30 min at 37 °C in a plastic immunological microtitration tray. Each reaction mixture was spotted on a 1-in. square of DEAE paper (Whatman DE81), and the paper was washed with 5% Na_2HPO_4 3 times and then with water twice and with ethanol once. The radioactivity incorporated into acid-insoluble materials was measured with a liquid scintillation counter.

Other Enzyme Assays. DNA polymerase β ahd γ activities were assayed as described previously (Enomoto et al., 1983b). DNA topoisomerase I and II activities were assayed as described previously (Goto et al., 1984). Endonuclease activities were assayed by measuring the degree of the conversion of single-stranded circular *fd* DNA to linear DNA or pBR322 RFI DNA to open circular DNA and duplex linear DNA. Exonuclease activities were assayed by using 1.5 μg of double- or single-stranded ^{3}H-labeled calf thymus DNA labeled by nick translation with *E. coli* DNA polymerase I (6700 cpm/μg). The radioactivity remaining in high molecular weight DNA was determined by transferring the reaction mixture onto a Whatman DE81 paper disk as described in DNA polymerase α assay.

Glycerol Density Gradient Centrifugation. The sample was dialyzed against buffer 2 containing 40 mM KCl. Two hundred microliters of the dialyzate was layered onto a 4.8-mL linear gradient of glycerol from 15 to 35% (v/v) in buffer 2 containing 40 mM KCl and 0.5 mg/mL BSA. Centrifugation was performed for 20 h at 258000g in a Hitachi RPS65-T rotor at 4 °C. In the parallel run, alcohol dehydrogenase (7.4 S), alkaline phosphatase (6.1 S), BSA (4.4 S), and cytochrome *c* (1.8 S) were used as markers.

Measurement of Interaction of DNA-Dependent ATPase B with Single-Stranded DNA by Sephadex G-200 Gel Filtration. The standard binding assay mixture (151.4 μL) contained 20 mM potassium phosphate buffer, pH 7.5, 0.1 mM Na_3EDTA, 1 mM 2-mercaptoethanol, 20% (v/v) ethylene glycol, 0.238 mg/mL BSA, 50 mM KCl, 90 μg of single-stranded circular *fd* DNA, and 600 units of ATPase B. After incubation of the mixture at 0 °C for 5 min, 28.6 μL of appropriate buffers was added to make the concentration of KCl as indicated and to add the ribonucleoside triphosphate at a

[1] Abbreviations: AMP-PCP, 5′-adenylyl methylenediphosphate; AMP-PNP, 5′-adenylyl imidodiphosphate; ATP-γ-S, adenosine 5′-*O*-(3-*thiotriphosphate*); BSA, bovine serum albumin; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; RFI, replicative form I; Tris, tris(hydroxymethyl)aminomethane.

final concentration of 1 mM. The mixture was loaded onto a Sephadex G-200 column (10.5 mL; 0.62 × 35 cm) equilibrated with buffer 4 containing the indicated concentration of KCl and 1 mM of the indicated ribonucleoside triphosphate. The column was developed with 1.5 column volumes of the same buffer.

Quantitation of Protein Amount. Protein concentration was determined by the method of Bradford (1976). Bovine plasma γ-globulin was used as a standard.

Purification of DNA-Dependent ATPase B. A frozen stock of 5×10^{10} FM3A cells was thawed, suspended in 400 mL of buffer 1 containing 2 μg/mL antipain, and homogenized by sonication. The sonicate was made 0.3 M in KCl by the addition of $^1/_{10}$th volume of buffer 1 containing 3.3 M KCl. After being stirred for 30 min at 0 °C, the extract was centrifuged for 30 min at 10000*g*, and the supernatant was recentrifuged for 1 h at 125000*g*. The supernatant (fraction I) was loaded onto a DEAE-cellulose column (500 mL) equilibrated with 0.3 M KCl in buffer 1. The flow-through fractions were pooled (fraction II), and Triton X-100 was added to fraction II at a final concentration of 0.01% (v/v). Fraction II was dialyzed against buffer 2 containing 50 mM KCl. The dialyzate was loaded onto a second DEAE-cellulose column (250 mL) equilibrated with 50 mM KCl in buffer 2. The column was washed with 3 bed volumes of the equilibration buffer, and the proteins bound to the column were eluted with 3 bed volumes of 0.4 M KCl in buffer 2. Another frozen stock of 5×10^{10} FM3A cells was processed as described above. DNA-dependent ATPase activity was recovered from both flow-through and bound fractions of the second DEAE-cellulose column. The flow-through fraction contained three DNA-dependent ATPases, C_1, C_2, and C_3, as described previously (Tawaragi et al., 1984). The activity in the bound fraction was designated as ATPase B.

The two 0.4 M KCl eluates from the second DEAE-cellulose column that contained ATPase B activity were combined (fraction III) and dialyzed against buffer 2 containing 50 mM KCl. The dialyzate was loaded onto a phosphocellulose column (160 mL) equilibrated with 50 mM KCl in buffer 2. The proteins were eluted from the column with 10 bed volumes of a linear gradient of KCl from 50 mM to 0.8 M in buffer 2. The active fractions eluted at about 0.45 M KCl were pooled (fraction IV) and added with buffer 2 containing 1 M KCl to make the final concentration of KCl to 0.5 M (fraction IV′). Fraction IV′ was loaded onto a hydroxylapatite column (30 mL) equilibrated with 0.5 M KCl in buffer 2. The column was washed with 10 bed volumes of buffer 2 containing 10 mM potassium phosphate buffer, pH 7.5, and 0.5 M KCl, and the proteins bound to the column were eluted with 3 bed volumes of buffer 2 containing 130 mM potassium phosphate buffer, pH 7.5, and 0.5 M KCl. The active fractions eluted from the column were pooled (fraction V) and dialyzed against 0.1 M KCl in buffer 2. The dialyzate was loaded onto a single-stranded DNA–cellulose column (16 mL) equilibrated with 0.1 M KCl in buffer 2. The proteins bound to the column were eluted with 12 bed volumes of a linear gradient of KCl from 0.1 to 0.7 M in buffer 2 containing 1 mM each of ATP and $MgCl_2$. The active fractions eluted at about 210 mM KCl were pooled (fraction VI). Fraction VI was dialyzed against 0.1 M KCl in buffer 2, and the dialyzate was loaded onto a second phosphocellulose column (4 mL) equilibrated with 0.1 M KCl in buffer 2. The proteins were eluted from the column with 10 bed volumes of a linear gradient of KCl from 0.1 to 0.7 M in buffer 2. DNA-dependent ATPase activity was eluted at 0.45 M KCl. The active fractions were pooled (fraction VII) and dialyzed against 0.1 M KCl in buffer 3. The purification of ATPase B is summarized in Table I.

Table I: Purification of DNA-Dependent ATPase B from FM3A Cells[a]

purification step	protein (mg)	total units ($\times 10^{-3}$)	sp act. (units/mg)
crude extract (I)	12100	*b*	*b*
first DEAE–cellulose (II)	10600	*b*	*b*
second DEAE–cellulose (III)	2400	4900	2000
first phosphocellulose (IV)	150	640	4300
hydroxylapatite (V)	56	560	10000
ssDNA–cellulose (VI)	7.3	190	26000
second phosphocellulose (VII)	3.5	180	51000

[a] ATPase activity was assayed under the standard conditions in the presence or absence of heat-denatured calf thymus DNA. DNA-dependent ATPase activity was determined by subtracting the activity in the absence from that in the presence. [b] DNA-dependent ATPase activity in fractions I and II could not be determined because of the abundant presence of DNA-independent ATPases.

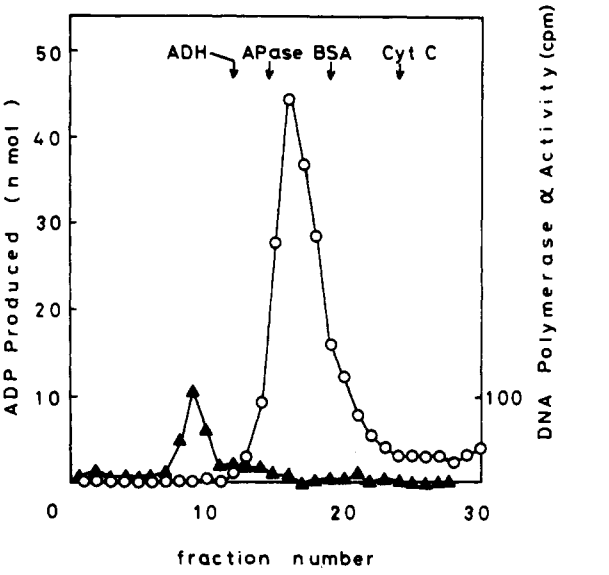

FIGURE 1: Glycerol gradient centrifugation. Fraction VII was dialyzed and layered onto a 15–35% glycerol gradient as described under Materials and Methods. Centrifugation was performed for 20 h at 258000*g* in a Hitachi RPS65-T rotor at 4 °C. Fractions were collected from the bottom of the tube. Fifteen and 5 μL of aliquots were assayed for DNA-dependent ATPase activity and DNA polymerase α activity, respectively, as described under Materials and Methods. Marker proteins used were alcohol dehydrogenase (ADH, 7.4 S), alkaline phosphatase (APase, 6.1 S), bovine serum albumin (BSA, 4.4 S), and cytochrome *c* (Cyt C, 1.8 S). DNA-dependent ATPase activity (○); DNA polymerase α activity (▲).

The purified DNA-dependent ATPase B sedimented at 5.5 S in a glycerol gradient containing 40 mM KCl (Figure 1). A similar result was obtained in the presence of 500 mM KCl (data not shown). The purified fraction contained no detectable DNA polymerase β, DNA polymerase γ, and topoisomerase type I and type II activities. It also showed no detectable endonucleolytic activity when assayed with pBR322 RFI DNA and single-stranded circular *fd* DNA. Furthermore, it had no measurable exonuclease activity when assayed with ^{3}H-labeled double- and single-stranded calf thymus DNA. A small but appreciable amount of DNA polymerase α activity was contained in fraction VII, but the activity was separated from ATPase B activity by glycerol gradient centrifugation in the presence of low salts (Figure 1).

RESULTS

Reaction Properties of DNA-Dependent ATPase B. The hydrolysis of ATP under optimal conditions was linear with

Table II: Effect of Inhibitors on DNA-Dependent ATPase B[a]

reagent	rel act. (%)	reagent	rel act. (%)
none	100	heparin (1.6 μg/mL)	53
ATP-γ-S (125 μM)[b]	30	aphidicolin (100 μg/mL)	104
AMP-PNP (125 μM)[b]	26	daunomycin (8 μM)	108
AMP-PCP (125 μM)[b]	44	ethidium bromide (1.6 μg/mL)	100
ouabain (2 mM)	95	actinomycin D (5 μg/mL)	74
oligomycin (10 μg/mL)	106	spermine (2.1 mM)	68
novobiocin (200 μg/mL)	98	spermidine (2.5 mM)	92

[a] The ATPase activity was assayed under the standard conditions as described under Materials and Methods in the presence of the indicated reagents. [b] The concentration of ATP was 1 mM.

time for up to 60 min at 25, 30, 37, and 45 °C and almost linear for up to 120 min at 25, 30, and 37 °C. The rate of hydrolysis of ATP increased with the temperature. The incubation temperature for the standard reaction was fixed at 37 °C.

The ATPase required a divalent cation for activity. In the presence of 5 mM ATP, the optimal concentrations of Mg^{2+}, Mn^{2+}, and Ca^{2+} were 5, 2.5, and 2.5–5 mM, respectively. The maximal activity obtained with Mn^{2+} was almost the same as that with Mg^{2+}. The efficiency of Ca^{2+} was lower than that of Mg^{2+}, and Ca^{2+}, at an optimal level, supported 31% of the maximal activity obtained with Mg^{2+}. The enzyme had a broad pH optimum from 7.5 to 9.0.

The enzyme activity with heat-denatured DNA as cofactor was not so much inhibited by KCl at concentrations up to 100 mM, and the inhibition became prominent at higher concentrations. A similar pattern of inhibition was observed with NaCl and Tris-HCl. KPO_4 buffer was much more inhibitory than the three salts, and only 15% of the original activity was obtained in the presence of 100 mM KPO_4. The ATPase activity with poly(dT) was more resistant to all salts tested than that with heat-denatured DNA, and inhibition was not observed with NaCl, KCl, or Tris-HCl up to 150 mM.

ATP was the most preferred substrate among ribo- and deoxyribonucleoside triphosphates tested. The relative rates of hydrolysis of dATP, CTP, GTP, and UTP were 88, 35, 45, and 37% of the rate of ATP, respectively. The K_m values for ATP and dATP were 0.75 and 0.56 mM.

The effect of various drugs on the ATPase activity with heat-denatured DNA as cofactor is shown in Table II. ATP analogues, ATP-γ-S, AMP-PCP, and AMP-PNP, inhibited the enzyme activity. Novobiocin, which is known to be a specific inhibitor of DNA gyrase and eukaryotic DNA topoisomerase II, did not inhibit the ATPase activity. Ouabain, a specific inhibitor of Na^+,K^+-ATPase, oligomycin, a specific inhibitor of mitochondrial FI ATPase, and aphidicolin, a specific inhibitor of DNA polymerase α, also did not inhibit the enzyme activity. An intercalating agent, ethidium bromide, had no effect, and actinomycin D reduced the enzyme activity only 26% when it was added at 5 μg/mL.

Polynucleotide Cofactor Requirements. The enzyme has an absolute requirement for a polynucleotide cofactor (Table III). Single-stranded circular *fd* DNA was most effective among natural DNAs. Almost no or very low activity was observed with ribonucleotide homopolymers, yeast tRNA, poly(dG), and poly[d(A-T)]. The most effective cofactor among the polynucleotides tested was poly(dT). Figure 2A shows ATP hydrolysis as a function of the concentration of natural DNAs. Heat-denatured DNA was preferred to activated and native DNA. The enzyme activity with native DNA was only 29% of the activity with optimal levels of heat-denatured DNA. This activity was abolished by the

Table III: Effect of Various Nucleic Acids on ATPase B Activity[a]

nucleic acid	rel act. (%)	nucleic acid	rel act. (%)
calf thymus heat-denatured DNA	100	poly(dA)·poly(dT)[b]	4
calf thymus native DNA	29	poly(dC) + poly(dT)[b]	146
calf thymus activated DNA	72	poly(rA)	2
S1 treated calf thymus DNA	2	poly(rC)	2
pBR322 RFI	22	poly(rG)	3
fd single-stranded circular DNA	116	poly(rU)	2
poly(dA)	58	yeast tRNA	2
poly(dC)	63	oligo$(dA)_{12-18}$	1
poly(dG)	16	oligo$(dT)_{12-18}$	15
poly(dT)	150	none	2
poly[d(A-T)]	14		

[a] ATPase B (fraction VII, 20 units) was assayed under the standard conditions for 90 min in the presence of 30 μM of the indicated nucleic acids. The values indicate percent of the activity in the presence of heat-denatured DNA as 100%. [b] Two homopolymers were mixed, incubated at 80 °C for 10 min, and cooled gradually.

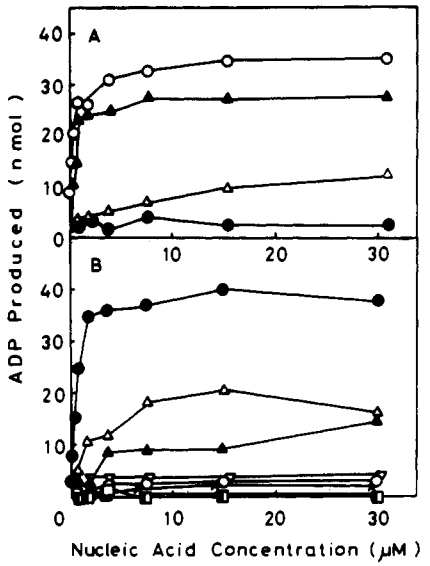

FIGURE 2: DNA-dependent ATPase B activity as a function of DNA concentration. (A) ATPase B (fraction VII, 20 units) was incubated for 90 min under the standard conditions in the presence of various concentrations of heat-denatured DNA (○), activated DNA (▲), native DNA (△), or S1 nuclease-treated native DNA (●). (B) ATPase B (fraction VII, 17 units) was incubated for 90 min under the standard conditions in the presence of various concentrations of poly(dT) (●), poly(dC) (△), poly(dA) (▲), poly(dA)·poly(dT) (▽), poly[d(A-T)] (○), poly(dG) (×), poly(A) (□), or poly(U) (■).

treatment of native DNA with S1 nuclease. Figure 2B shows the enzyme activity with various concentrations of homopolymers. The relative efficiencies of the synthetic polymers did not change at any concentration tested: poly(dT) > poly(dC) > poly(dA) ≫ poly(dA)·poly(dT), poly[d(A-T)], and poly(dG) > poly(A) and poly(U). It must be noted that poly(dT) alone and a noncomplementary combination of poly(dT) and poly(dC) supported very high activity, but the complementary combination of poly(dT) and poly(dA) was inactive (Table III).

ATP Alters Affinity of ATPase B for Single-Stranded DNA. During the purification of DNA-dependent ATPase B, we encountered the difficulties that the enzyme could not be recovered well from the single-stranded DNA–cellulose column and that the activity was distributed very broadly as shown in Figure 3A. The last sharp peak of activity shown in Figure 3A was obtained by eluting the column with the buffer containing 0.8 M KCl, 1 mM ATP, and 1 mM $MgCl_2$. Therefore, single-stranded DNA–cellulose column chroma-

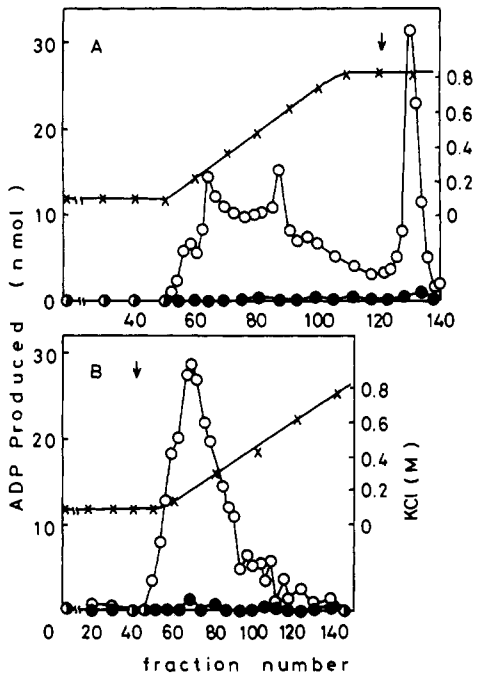

FIGURE 3: Single-stranded DNA–cellulose column chromatography. Two milliliters of the hydroxylapatite fraction (fraction V) was dialyzed against 0.1 M KCl in buffer 2 and applied onto a single-stranded DNA–cellulose column (2 mL) that had been equilibrated with 0.1 M KCl in buffer 2. (A) The column was washed with 2.5 bed volumes of the same buffer and eluted with 6 bed volumes of a linear gradient of KCl from 0.1 to 0.8 M in buffer 2. The proteins still bound to the column were eluted with 2.5 bed volumes of 0.8 M KCl in buffer 2 and then with 3 bed volumes of 0.8 M KCl in buffer 2 containing 1 mM ATP and 1 mM $MgCl_2$. The arrow indicates the position where elution buffer was changed to the buffer containing ATP and $MgCl_2$. (B) The column was washed with 3 bed volumes of 0.1 M KCl in buffer 2 and eluted with 12 bed volumes of a linear gradient of KCl from 0.1 to 1 M in buffer 2 containing 1 mM ATP and $MgCl_2$. Aliquots of each fraction were assayed for ATPase activity in the presence (○) and absence (●) of heat-denatured DNA as described under Materials and Methods.

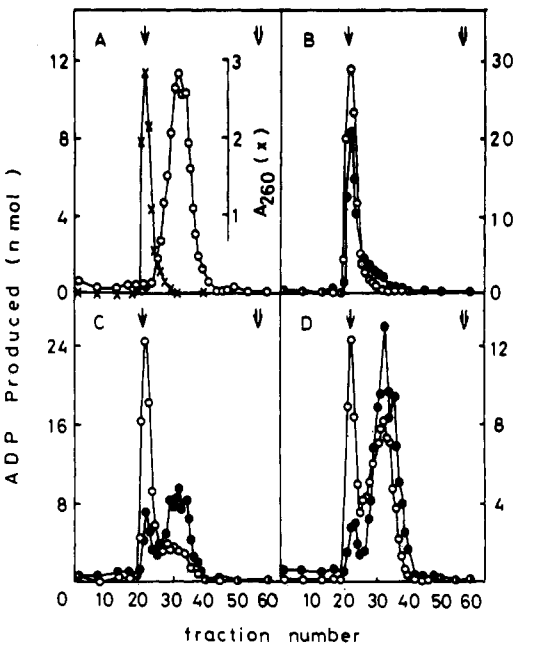

FIGURE 4: Effect of ATP on interaction of DNA-dependent ATPase B with single-stranded DNA at various concentrations of KCl. (A) Single-stranded circular *fd* DNA (90 μg) or ATPase B (600 units) was applied on a Sephadex G-200 column. The column was developed with buffer 4 containing 200 mM KCl. The figure is the composite illustration of two separate experiments. DNA-dependent ATPase activity (○); A_{260} (×). (B–D) Single-stranded circular *fd* DNA and ATPase B were mixed in the presence of 50 mM KCl and then the concentration of KCl was increased to 100, 150, or 200 mM by the addition of the buffer containing an appropriate concentration of KCl with or without 8.7 mM ATP and $MgCl_2$ (final concentration of 1 mM each) as described under Materials and Methods. The sample was applied onto a Sephadex G-200 column, and the column was developed in the presence (●) or absence (○) of 1 mM ATP and $MgCl_2$ with buffer 4 containing 100 mM (B), 150 mM (C), and 200 mM (D) KCl. The arrows indicate the positions where the markers, blue dextran (single-tailed arrow) and ATP (double-tailed arrow), are eluted.

tography was performed with the buffer containing 1 mM ATP and 1 mM $MgCl_2$ as described in the previous section. In the presence of ATP and $MgCl_2$, the ATPase activity was eluted from the column as a single peak with high recovery (Figure 3B).

In order to examine the effect of ATP on the interaction of ATPase B with single-stranded DNA, we performed a Sephadex G-200 gel filtration assay. Single-stranded circular *fd* DNA was eluted from the Sephadex G-200 column in the void fractions, and ATPase B was eluted in the included fractions as shown in Figure 4A (composite illustration of two separate experiments). When single-stranded circular *fd* DNA and ATPase B were mixed in the presence of 0.1 M KCl, loaded on a Sephadex G-200 column, and eluted with the buffer containing 0.1 M KCl, almost all of the ATPase activity was eluted with the DNA although a slight dissociation of the enzyme from the DNA was observed in the presence of 1 mM ATP and $MgCl_2$ (Figure 4B). In the presence of 0.15 and 0.2 M KCl, the amount of dissociated enzyme was greatly increased by the addition of ATP and $MgCl_2$ (Figure 4C,D). The effect was also observed with ATP alone in the presence of 0.15 M KCl (Figure 5A). The effect of dissociation of the enzyme from single-stranded DNA was not observed with other ribonucleoside triphosphates, CTP, GTP, and UTP (Figure 5B–D).

Discussion

We have purified and characterized in detail one of the four DNA-dependent ATPases from mouse FM3A cells, ATPase B. We paid attention to the separation of the ATPase from other DNA replication enzymes and proteins because the next step of our research is to develop an in vitro DNA replication system with purified enzymes and proteins from mammalian cells. The purified enzyme was devoid of contaminating or associated endo- and exodeoxyribnuclease, polymerase β and γ, or topoisomerase activities. The enzyme fraction was also devoid of other DNA-dependent ATPases, C_1, C_2, and C_3 (Tawaragi et al., 1984), and stimulating factors for DNA polymerase α activity on heat-denatured DNA (Kawasaki et al., 1982, 1984). Although a very low level of DNA polymerase α activity contaminated the purified fraction, the contaminating polymerase activity was separated from the enzyme by glycerol gradient centrifugation under low-salt conditions, (Figure 1).

ATPase B had a sedimentation coefficient of 5.5 S, and the estimated molecular weight of the native form of the enzyme was about 100 000. SDS–polyacrylamide gel electrophoresis revealed that the most purified fraction still contained many polypeptides, but only one polypeptide of M_r 58 000 was labeled with [α-^{32}P]ATP by photoaffinity labeling (data not shown). Although it seems likely that the polypeptide of M_r 58 000 carries the enzyme activity and the native form of the enzyme is a dimer of the polypeptide, it must be determined whether this polypeptide corresponds to the enzyme activity or not.

The enzyme has an absolute requirement for a polynucleotide cofactor for activity. The results shown in Table

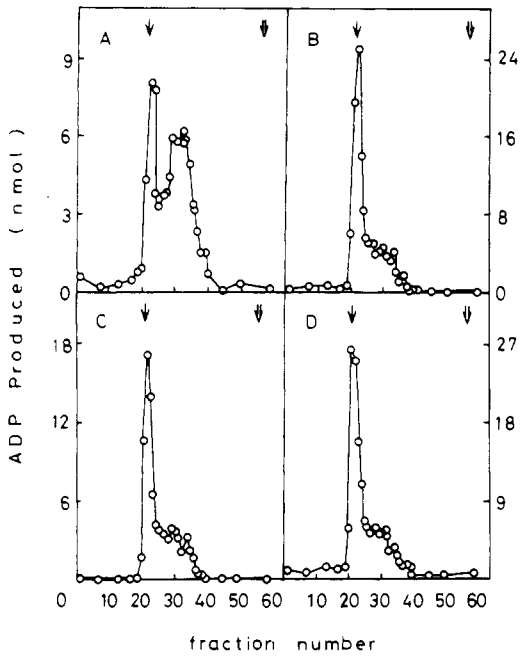

FIGURE 5: Effect of ribonucleoside triphosphates on interaction of DNA-dependent ATPase B with single-stranded DNA. Single-stranded circular *fd* DNA (90 μg) and ATPase B (600 units) were mixed in the presence of 50 mM KCl, and then the buffer containing 680 mM KCl and 8.7 mM of one of the four ribonucleoside triphosphates was added to the mixture in order to increase the concentration of KCl to 150 mM and to add the ribonucleoside triphosphate at a final concentration of 1 mM. The sample was applied onto a Sephadex G-200 column, and the column was developed with 0.15 M KCl in buffer 4 containing 1 mM ATP (A), CTP (B), GTP (C), or UTP (D). The arrows indicate the positions where the markers, blue dextran (single-tailed arrow) and ATP (double-tailed arrow), are eluted.

III and Figure 2 clearly demonstrated that the requirement was satisfied only by single-stranded DNA but not by double-stranded DNA or polyribonucleotides. The low activity with poly(dG) may be due to particular structural properties of the polymer because poly(dG) is known to have considerable secondary structure in solution. The high efficiency of single-stranded circular DNA suggests that free termini are not significantly involved in the interaction of the enzyme with single-stranded DNA.

ATPase B could hydrolyze four ribonucleoside triphosphates and dATP. This property distinguishes the enzyme from the other three ATPases from FM3A cells, C_1, C_2, and C_3, as described previously (Tawaragi et al., 1984). Among the DNA-dependent ATPases so far isolated from eukaryotic cells, the ATPase isolated from mouse myeloma by Hachmann and Lezius corresponds to ATPase B because of its sedimentation coefficient of 5.5 S, preference for single-stranded DNA, ability to hydrolyze four ribonucleoside triphosphates, and chromatographic behavior during the purification.

The results shown in Figures 3 and 4 demonstrated that ATP decreases the affinity of ATPase B for single-stranded DNA. The effect of ATP was observed both in the presence and absence of $MgCl_2$. Therefore, it seems likely that hydrolysis of ATP is not necessary for the effect of ATP and the binding of ATP to ATPase B alone causes the conformational change of the enzyme that decreases the affinity of the enzyme for single-stranded DNA. However, the possibility that ATP hydrolysis accelerates the dissociation of the protein from DNA cannot be completely excluded. Such acceleration of the dissociation of an enzyme from DNA by ATP hydrolysis has been observed with several prokaryotic DNA-dependent ATPases such as the *rep* protein (Arai et al., 1981) and *recA* protein (Menetski & Kowalczykowski, 1985).

The in vivo functions of ATPase B are presently unknown. Although the enzyme was found to be associated with DNA polymerase α during the early steps of the purification (Watanabe et al., 1982), it does not have any effect on DNA polymerase α activity assayed with various template-primers such as activated DNA, native DNA, and poly[d(A-T)]. The enzyme did not show the ability to promote renaturation of complementary single strands and the homologous pairing of single strands with duplex DNA when the activities were measured by the methods of Weinstock et al. (1979) and Shibata et al. (1979). The enzyme also failed to show unwinding activity when it was assayed by the method using nuclease S1 developed by Abdel-Monem and Hoffmann-Berling (1976). However, this assay system seems to be too insensitive to detect a limited unwinding of the helix. Therefore, we are now trying to develop a new assay system suitable for the detection of a limited unwinding. Experiments are also in progress to isolate monoclonal antibodies against the DNA-dependent ATPase.

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Registry No. ATP, 56-65-5; dATP, 1927-31-7; ATP-γ-S, 35094-46-3; AMP-PCP, 7292-42-4; AMP-PNP, 25612-73-1; ATPase, 9000-83-3.

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Affinities of tRNA Binding Sites of Ribosomes from *Escherichia coli*†

Roland Lill, James M. Robertson, and Wolfgang Wintermeyer*
Institut für Physiologische Chemie, Physikalische Biochemie, und Zellbiologie der Ludwig-Maximilians-Universität München, D-8000 München 2, Federal Republic of Germany

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ABSTRACT: The binding affinities of $tRNA^{Phe}$, Phe-$tRNA^{Phe}$, and *N*-AcPhe-$tRNA^{Phe}$ from either *Escherichia coli* or yeast to the P, A, and E sites of *E. coli* 70S ribosomes were determined at various ionic conditions. For the titrations, both equilibrium (fluorescence) and nonequilibrium (filtration) techniques were used. Site-specific rather than stoichiometric binding constants were determined by taking advantage of the varying affinities, stabilities, and specificities of the three binding sites. The P site of poly(U)-programmed ribosomes binds $tRNA^{Phe}$ and *N*-AcPhe-$tRNA^{Phe}$ with binding constants in the range of 10^8 M^{-1} and 5×10^9 M^{-1}, respectively. Binding to the A site is 10–200 times weaker, depending on the Mg^{2+} concentration. Phe-$tRNA^{Phe}$ binds to the A site with a similar affinity. Coupling A site binding of Phe-$tRNA^{Phe}$ to GTP hydrolysis, by the addition of elongation factor Tu and GTP, leads to an apparent increase of the equilibrium constant by at least a factor of 10^4. Upon omission of poly(U), the affinity of the P site is lowered by 2–4 orders of magnitude, depending on the ionic conditions, while A site binding is not detectable anymore. The affinity of the E site, which specifically binds deacylated $tRNA^{Phe}$, is comparable to that of the A site. In contrast to P and A sites, binding to the E site is labile and insensitive to changes of the ionic strength. Omission of the mRNA lowers the affinity at most by a factor of 4, suggesting that there is no efficient codon–anticodon interaction in the E site. On the basis of the equilibrium constants, the displacement step of translocation, to be exergonic, requires that the tRNA leaving the P site is bound to the E site. Under in vivo conditions, the functional role of transient binding of the leaving tRNA to the E site, or a related site, most likely is to enhance the rate of translocation.

Understanding the mechanism of protein biosynthesis on a molecular level requires—in addition to biochemical, structural, and kinetic information—the knowledge of the thermodynamic parameters of the individual steps of the process. Most important in that respect are the interactions between tRNA and its ribosomal binding sites. For charged tRNA, the ribosome possesses two of them, to which aminoacyl-tRNA (A site) and peptidyl-tRNA (P site) are bound in the state before peptide bond formation. An additional site (E site), which is accessible for deacylated tRNA only, was found for eucaryotic ribosomes (Wettstein & Noll, 1965) and has recently been shown to exist also on *Escherichia coli*

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