Deoxyribonucleic Acid Dependent Adenosinetriphosphatases from the Novikoff Hepatoma. Characterization of a Homogeneous Adenosinetriphosphatase That Stimulates DNA Polymerase β[†]

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ABSTRACT: Five chromatographically distinct DNA-dependent ATPase activities have been identified in high salt–detergent extracts of the Novikoff hepatoma. One of these, ATPase III, has been purified to apparent homogeneity as judged by polyacrylamide gel electrophoresis and has a specific activity of 12 μmol of ATP hydrolyzed min^{-1} (mg of protein)$^{-1}$. The enzyme, a dimer of M_r 65 000 subunits, has a sedimentation coefficient of 7.0 S in both high salt and low salt, a Stokes radius of 43 Å, and a frictional coefficient of 1.31. In the presence of Mg^{2+} ion and a polynucleotide effector, the enzyme catalyzes hydrolysis of ATP or dATP to a diphosphate with a K_m of 206 μM and 110 μM, respectively, for the two substrates. Although single-stranded effectors are preferred, the enzyme has significant activity with double-stranded effectors. The K_m for effector is 0.4 μM (nucleotide). The analogues adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS), dideoxyadenosine triphosphate (ddATP), and adenosine 5′-(α,β-methylenetriphosphate) (α,β-Me-ATP) are competitive inhibitors of the enzyme while adenosine tetraphosphate (ATP-P), 8-bromoadenosine 5′-triphosphate (8-Br-ATP), 5′-adenylyl imidodiphosphate (AMP-PNP), and adenosine 5′-(β,γ-methylenetriphosphate) (β,γ-Me-ATP) do not inhibit. The enzyme is insensitive to nalidixic acid, novobiocin, and berenil but is sensitive to *N*-ethylmaleimide. ATPase III is capable of stimulating DNA polymerase β on duplex DNA, but this effect is abolished in the presence of ATPγS. Polymerase stimulation is further enhanced in the presence of a single-stranded DNA-binding protein. These data suggest that ATPase III may play a role in DNA repair.

In recent years, DNA-dependent ATPases have been isolated and extensively characterized from bacterial sources. In *Escherichia coli* alone, there are at least 12 such enzymes reported. Most of these have been shown to play some role in one or more aspects of DNA metabolism. Among these are two enzymes, *dnaB* protein and protein n′ (also called factor Y), that are involved in initiation of replication (Ueda et al., 1978; Reha-Krantz & Hurwitz, 1978; Wickner & Hurwitz, 1975; Shlomai & Kornberg, 1980). DNA gyrase introduces superhelical turns into DNA (Gellert et al., 1978) while four different ATPases (helicases I, II, and III and *rep* protein) are involved in unwinding the helix (Abdel-Monem et al., 1976, 1977; Yarranton et al., 1979; Kornberg et al., 1978). *recA* protein and *recBC* nuclease both have ATPase activity, and both are involved in DNA recombination and repair (McEntee et al., 1980; Roberts et al., 1978; Shibata et al., 1979; Goldmark & Linn, 1972). In addition to these enzymes, three other DNA-dependent ATPases are known whose role in DNA metabolism has yet to be established. These include ATPases I and II described by Richet & Kohiyama (1976, 1978) and an ATPase associated with DNA polymerase III holoenzyme[1] (Meyer et al., 1979).

While similar enzymes should exist in higher systems, only a few reports have appeared in the literature, and the roles of these enzymes can only be surmised. DNA-dependent ATPases have been isolated from human KB cells (Boxer & Korn, 1980; DeJong et al., 1981), rat liver mitochondria (Yaginuma & Koike, 1981), calf thymus (Assairi & Johnston, 1979; J. W. Hockensmith and R. A. Bambara, unpublished results), yeast (Plevani et al., 1980), mouse myeloma (Hachmann & Lezius, 1976), EUE cells (Cobianchi et al., 1979), and lily (Hotta & Stern, 1978). Only three of these have been purified to homogeneity (Plevani et al., 1980; Boxer & Korn, 1980; J. W. Hockensmith and R. A. Bambara, unpublished results). The lily enzyme is the only one that exhibits significant helicase activity and is thought to be involved in recombination (Hotta & Stern, 1978). Recently Hockensmith & Bambara (1982) have purified an ATPase that can catalyze reannealing of complementary DNA, suggesting it may be a *recA* protein analogue. With regard to possible interactions with DNA polymerase, a limited degree of stimulation of DNA synthesis has been reported for both the KB and EUE cell enzymes (Boxer & Korn, 1980; Cobianchi et al., 1979). Clearly, a detailed analysis of the enzymological properties, physiological roles, and interactions of DNA-dependent ATPases with other replication and repair enzymes in mammalian systems remains to be done. With this in mind, we began a search for DNA-dependent ATPases in the Novikoff hepatoma, a tissue source from which this laboratory has previously purified several other enzymes involved in DNA metabolism (Probst et al., 1975; Stalker et al., 1976; Mosbaugh et al., 1977; Mosbaugh & Meyer, 1980).

In the present report, we demonstrate that there are at least five chromatographically distinct DNA-dependent ATPase activities in extracts of the Novikoff hepatoma. One of these, which we have designated ATPase III, has been purified to apparent homogeneity. This ATPase is capable of stimulating DNA synthesis by DNA polymerase β on duplex substrates. A purification and enzymological characterization of this enzyme are presented.

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[1] We have previously shown that highly purified, but not homogeneous, preparations of DNA polymerase III holoenzyme contain an M_r 83 000 DNA-dependent ATPase. At that time it was thought that this ATPase was the τ subunit of holoenzyme that also has an M_r of 83 000. Subsequent studies by McHenry and ourselves indicate that τ is not an ATPase but that the ATPase is a contaminant of holoenzyme. A detailed description of τ (C. McHenry, unpublished results) and this ATPase (R. R. Meyer, C. R. Brown, and D. C. Rein, unpublished results) will be published.

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Materials and Methods

Chemicals. All chemicals were of reagent grade. Unlabeled ribo- and deoxyribonucleoside diphosphates and triphosphates, adenosine tetraphosphate, Br-ATP,[2] AMP-PNP, α,β-Me-ATP, β,γ-Me-ATP, ATPγS, nalidixic acid, novobiocin, and *p*-nitrophenyl phosphate were purchased from Sigma, as well as the protein standards cytochrome *c*, myoglobin, yeast alcohol dehydrogenase, β-galactosidase, BSA, and ovalbumin. Enzyme-grade ammonium sulfate came from Schwarz/Mann. Tritium-labeled ribo- and deoxyribonucleoside triphosphates were products of ICN or New England Nuclear Corp; [^{35}S]ATPγS came from New England Nuclear Corp. Sodium dodecyl sulfate was obtained from Gallard-Schlesinger.

Chromatographic and Electrophoretic Supports. DEAE-Sephadex, QAE-Sephadex, and Sephacryl S-300 were obtained from Pharmacia, while phosphocellulose P-1 was obtained from Whatman. Bio-Rad was a source for polyacrylamide gel electrophoresis reagents, agarose, hydroxylapatite, and cellex-410. Brinkmann Instruments provided poly(ethylene imine)-impregnated cellulose thin-layer plates. DNA–cellulose was prepared according to Alberts & Herrick (1971). ATP–agarose came from Sigma.

Enzymes. DNase I, RNase A, and RNase T1 were purchased from Worthington, and nuclease S1 was purchased from Sigma. Novikoff hepatoma DNA polymerase β (Stalker et al., 1976) and *E. coli* single-stranded DNA-binding protein (Meyer et al., 1980) were prepared as described previously. Novikoff hepatoma DNA polymerase α was a gift from Dr. Diane Rein and DNA-binding protein a gift of T. J. Koerner of our department. *E. coli* DNA polymerase I was purchased from BRL.

Nucleic Acids. Calf thymus DNA was purchased from Sigma. Denatured DNA was prepared by boiling for 15 min and rapid chilling in ice. Activated calf thymus DNA and [^{3}H]DNA from *E. coli* were prepared as described previously (Stalker et al., 1976). Phage φX174 was prepared according to Eisenberg et al. (1975) while G4 DNA was a gift of Dr. Charles McHenry (University of Texas Health Science Center, Houston, TX). Phage φX174 restriction fragments were a gift of Dr. Thomas Kunkel (University of Seattle, Seattle, WA). Synthetic oligo- and polynucleotides were purchased from P-L Biochemicals. The purity of the oligonucleotides was confirmed by DEAE-cellulose chromatography by the method of Tomlinson & Tener (1963).

Buffers. TMEG buffer consists of 20 mM Tris-HCl (pH 8.0), 5 mM β-mercaptoethanol, 1 mM EDTA, and 10% (w/v) glycerol. PMEG buffer is 20 mM potassium phosphate (pH 7.5), 5 mM β-mercaptoethanol, 1 mM EDTA, and 10% (w/v) glycerol. PMG buffer is PMEG buffer with EDTA omitted. All buffers were prepared from 1.0 M stocks with pH adjustments made at 20 °C. Corrections for temperature and dilution coefficients have not been made.

Enzyme Assays. Standard ATPase assays were run in 25-μL volumes containing 20 mM potassium phosphate (pH 7.2), 2 mM dithiothreitol, 200 μg/mL BSA, 0.5 mM $MgCl_2$, 0.4 mM ATP, 5% (w/v) glycerol, 3.0 nmol of (nucleotide) G4 ssDNA, 2.0 μCi of [^{3}H]ATP, and enzyme. After incubation for 30 min at 37 °C, 1.0-μL aliquots were spotted on PEI–cellulose thin-layer strips (0.6 cm × 5.5 cm) previously spotted with a mixture of 10 mM ADP and 10 mM ATP as markers. The chromatogram was developed in 1.0 M formic acid–0.5 M LiCl and then dried. The ATP and ADP spots were located under UV illumination and cut out, and the radioactivity was determined by liquid scintillation counting. One unit of ATPase activity is defined as the hydrolysis of 1.0 pmol of ATP/min at 37 °C.

DNA polymerase β assays were carried out in 50-μL volumes containing 25 mM Tris-HCl (pH 8.4), 5 mM β-mercaptoethanol, 0.5 mM EDTA, 10 mM magnesium acetate, 0.015 mM each of dATP, dCTP, dGTP, and [^{3}H]dTTP (sp act. 325 mCi/mmol), 15% (w/v) glycerol, 6.25 μg of DNA, and 0.01–0.30 unit of DNA polymerase β fraction VI (Stalker et al., 1976). For DNA polymerase α assays, 25 mM potassium phosphate (pH 7.0) was substituted for Tris-HCl buffer. After a 30- or 60-min incubation at 37 °C, the reactions were precipitated with 10% trichloroacetic acid. Precipitates were collected on Whatman GF/C filters, washed, dried, and counted. One unit of DNA polymerase activity is defined as the incorporation of 1 nmol of total nucleotide/h at 37 °C.

Exonuclease assays were performed as described with ^{3}H-labeled *E. coli* DNA as substrate and in the presence or absence of 0.4 mM ATP (Mosbaugh & Meyer, 1980). Endonuclease assays were performed as described previously (Mosbaugh & Meyer, 1980) except that ^{3}H-labeled φX174 RFI supercoiled DNA was used as substrate and the reactions were carried out in the presence or absence of 0.4 mM ATP. The reaction products were analyzed on agarose gels.

Helicase assays using nuclease S1 sensitivity were performed according to Yarranton et al. (1979). The assay tubes contained, in a volume of 25 μL, the same components as the standard ATPase assay except that 1.8 nmol (nucleotide) of φX174 RFII [^{3}H]DNA (sp act. 13 200 cpm/nmol) was the DNA source. After incubation for 60 min at 37 °C, 25 μL of S1 buffer containing 30 mm sodium acetate (pH 4.5), 1 mM $ZnSO_4$, 5% (v/v) glycerol, and 100 units of nuclease S1 was added. After incubation for 45 min at 37 °C, 10 μg of calf thymus DNA was added as carrier, and the reaction was terminated with 1.0 mL of cold 10% trichloroacetic acid. The DNA was collected on GF/C filters and the remaining acid-insoluble radioactivity determined.

Topoisomerase reactions contained the standard ATPase components in 25 μL except that 1.5 nmol (nucleotide) of φX174 RFI DNA was used. Following incubation for 60 min at 37 °C, 10-μL aliquots were removed and brought to 1% sodium dodecyl sulfate, 5% (v/v) glycerol, 2.5 mM EDTA, and 0.01% bromphenol blue. The sample was then loaded onto a 0.8% agarose gel in 90 mM Tris, 90 mM boric acid, and 2.5 mM EDTA. Electrophoresis was carried out for 4 h at a constant voltage of 100 V. The gels were stained in 0.05% ethidium bromide and examined under UV light to determine whether there was any change in linking number of the DNA. Phosphatase activity was assayed colorimetrically by following the release of phosphate from *p*-nitrophenyl phosphate as described previously (Meyer & Simpson, 1970).

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Slab gels (11%) with a stacking gel (3%) were run after the method of

[2] Abbreviations: DEAE, diethylaminoethyl; QAE, [diethyl(2-hydroxypropyl)amino]ethyl; SSB, single-stranded DNA-binding protein; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; RF, replicative form (of phage DNA); SS → RF, single-stranded DNA to replicative form DNA; $NaDodSO_4$, sodium dodecyl sulfate; NEM, *N*-ethylmaleimide; PEI, poly(ethylene imine); EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; AMP-PNP, 5′-adenylyl imidodiphosphate; α,β-Me-ATP, adenosine 5′-(α,β-methylenetriphosphate); β,γ-Me-ATP, adenosine 5′-(β,γ-methylenetriphosphate); ATP-P, adenosine tetraphosphate; ddATP, dideoxyadenosine triphosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); Br-ATP, 8-bromoadenosine 5′-triphosphate; AP, apurinic/apyrimidinic; MYO, myoglobin; OVA, ovalbumin; ADH, yeast alcohol dehydrogenase; GAL, β-galactosidase; BSA, bovine serum albumin.

Laemmli (1970). Samples were heated 10 min at 90 °C in 0.06 M Tris-HCl (pH 6.8), 4% $NaDodSO_4$, 0.7 M β-mercaptoethanol, 12% (w/v) glycerol, and 0.001% bromphenol blue. After electrophoresis, gels were stained with Coomassie brilliant blue, destained, and scanned with a Helena Laboratories Quick-Scan R & D densitometer. Molecular weights were calculated relative to standards.

Velocity Gradient Centrifugation. Glycerol gradients of 10–30% (v/v) were prepared in buffer containing 20 mM Tris–HCl (pH 8.0), 5 mM β-mercaptoethanol, 1 mM EDTA, and either 0 or 0.5 M KCl. Some gradients also contained 0.5 mM ATP. Samples of ATPase III, yeast alcohol dehydrogenase (7.6 S), BSA (4.3 S), and myoglobin (2.0 S) were layered on the gradient in a final volume of 100 μL. Centrifugation was carried out for 24 h at 40 000 rpm and 2 °C in a Beckman SW 50.1 rotor. Fractions of 200 μL were collected from the bottom and assayed immediately. Sedimentation coefficients were determined by the method of Martin & Ames (1961).

Sephacryl S-300 Gel Filtration. A 0.5 cm^2 × 100 cm Sephacryl S-300 column was poured and equilibrated in TMEG buffer plus 0.5 M NaCl. The column was calibrated with blue dextran for the void volume and cytochrome *c*, myoglobin, ovalbumin, BSA, yeast alcohol dehydrogenase, and β-galactosidase as markers. A sample of 0.8 mL of ATPase III was applied, and fractions of 0.8 mL were collected. The Stokes radius was determined by the method of Ackers (1964). Using the sedimentation coefficient and Stokes radius, we calculated the native molecular weight according to Siegel & Monty (1966).

Protein Determination. Protein was measured by the procedure of Lowry et al. (1951) with BSA as a standard.

Maintenance of the Tumor. The Novikoff hepatoma was maintained and the cells were harvested and washed as previously described (Stalker et al., 1976). The cells were usually stored as a frozen pellet for at least 1 week before use.

Results

Multiple DNA-Dependent ATPases in the Novikoff Hepatoma. Crude extracts of the tumor cells contain several DNA-dependent ATPases. However, these are masked by the large amount of DNA-independent ATPases. A similar problem was encountered when various ammonium sulfate fractions were analyzed. It was only after chromatographing each ammonium sulfate fraction on DEAE-Sephadex that DNA-dependent activity could be detected. Figure 1 is presented to demonstrate the existence of several chromatographically distinct ATPases. When a 25–45% ammonium sulfate fraction is chromatographed (Figure 1A), two major and two minor peaks are observed. Better resolution of ATPases II and III can be achieved on subsequent columns. Similarly, the peaks labeled IV + V can be resolved into two distinct peaks on DNA–cellulose (Figure 1B). The 25–45% ammonium sulfate fraction is used here because it contains enough of each enzyme to illustrate heterogeneity. However, ATPases II and III are more predominant in other ammonium sulfate fractions that have been used for purification of these enzymes (see Table I for ATPase III). We have numbered these ATPases in their order of elution from these two columns. Each of these enzymes is chromatographically distinct and elutes in the same position upon rechromatography, suggesting they may be different enzymes. However, purification and extensive physical and enzymological studies will be necessary to confirm this. We have purified the first of these enzymes, ATPase III, to homogeneity.

Purification of DNA-Dependent ATPase III. All steps were carried out at 0–4 °C. Usually 240–300 g (wet weight) of cells was used. A typical purification scheme is given in Table I. Harvesting of the tumor cells and preparation of a high salt–detergent extract (fraction I) were carried out as previously described (Stalker et al., 1976). Solid ammonium sulfate was added slowly to fraction I to 35% saturation. The precipitate was collected by centrifugation, and the supernatant was brought to 60% saturation. Proteins precipitating between 35% and 60% saturation were collected, dissolved in TMEG buffer, and dialyzed overnight. This constitutes fraction II. Due to contaminating DNA and the presence of other ATPases, DNA-dependent activity cannot be determined at this step.

Fraction II was loaded onto a 8 cm^2 × 27 cm DEAE-Sephadex A-50 column that had been previously equilibrated

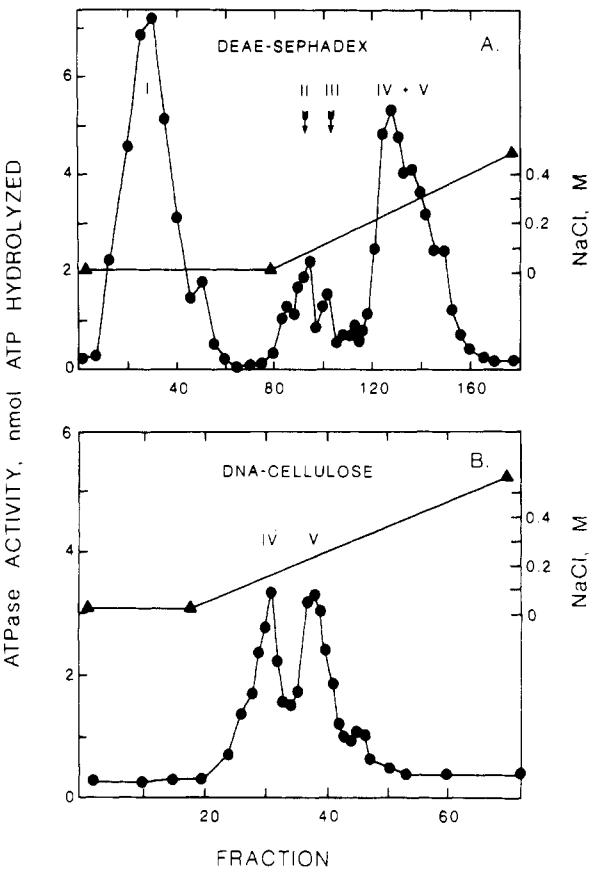

FIGURE 1: Chromatographic heterogeneity of DNA-dependent ATPases from the Novikoff hepatoma. (A) A 25–45% ammonium sulfate fraction was prepared from 250 g (wet weight) of tumor cells. Approximately 2180 mg of protein was loaded onto an 8 cm^2 × 30 cm DEAE-Sephadex A-50 column previously equilibrated with TMEG buffer. The column was washed with 100 mL of TMEG buffer and the proteins were eluted with an 800-mL gradient of 0–0.6 M NaCl in TMEG buffer at a flow rate of 75 mL/h. The fractions were assayed for DNA-dependent ATPase activity as described under Materials and Methods. This particular ammonium sulfate fraction was chosen to illustrate multiple species of ATPases. The peaks have been numbered in their order of elution. Although peaks II and III appear small, they are enriched in a 35–60% ammonium sulfate fraction that has been used for their purification (see text). (B) Peaks IV and V, which are not resolved on DEAE-Sephadex (as shown in panel A) or on hydroxylapatite or phosphocellulose (data not shown), can be resolved on DNA–cellulose. Approximately 4 × 10^6 units of ATPases IV and V contained in 7.0 mg of protein was loaded onto a 0.63 cm^2 × 10 cm column of DNA–cellulose equilibrated with TMEG buffer. After being washed with 20 mL of starting buffer, the column was developed with a 300-mL gradient of 0–0.6 M NaCl in TMEG buffer at a flow rate of 40 mL/h. Fractions were assayed for ATPase as described under Materials and Methods. ATPase IV elutes at 0.12 M NaCl while ATPase V elutes at 0.22 M NaCl.

Table I: Purification of Novikoff Hepatoma DNA-Dependent ATPase III[a]

fraction	protein (mg)	total units $\times 10^{-3}$	sp act. $\times 10^{-3}$ (units/mg)	purification (x-fold)	yield (%)
I, cell extract	10400	(2930)[b]	(0.28)	(1.00)	(100)
II, ammonium sulfate	2790	(2930)	(1.05)	(3.75)	(100)
III, DEAE-Sephadex	290	2930	10.1	36.1	100
IV, hydroxylapatite	61.2	4230	69.1	247	144
V, phosphocellulose	18.0	3620	201	718	123
VI, DNA-cellulose	0.345	750	2170	7750	25.6
VII, QAE-Sephadex	0.090	690	7670	27400	23.5
VIII, ATP-agarose	0.045	540	12000[c]	42900	18.4

[a] The starting material was 250 g (wet weight) of Novikoff hepatoma cells. [b] Fractions I and II contain so much DNA-independent ATPase activity that accurate measurements cannot be made. We have estimated the values in parentheses by assuming 100% recovery of the total units and calculating the specific activity on the basis of the loss of protein at these steps. One unit of ATPase activity is defined as the hydrolysis of 1 pmol of ATP/min at 37 °C. [c] With several different preparations the final specific activity varied from (7.5–15) $\times 10^6$ units/mg.

with TMEG buffer. After being loaded, the column was washed with 100 mL of TMEG buffer, and the proteins were eluted with a 700-mL gradient of 0–0.5 M NaCl in TMEG buffer at a flow rate of 75 mL/h. ATPase III elutes at 0.13 M NaCl and is heavily contaminated with ATPase II. At this stage, the DNA dependence of the reaction can be clearly demonstrated. The peak fractions were pooled and dialyzed overnight against PMG buffer. This constitutes fraction III.

Fraction III was loaded onto a 2 $cm^2 \times 17$ cm column of hydroxylapatite equilibrated with PMG buffer. The column was washed with 75 mL of buffer and developed with a 500-mL linear gradient of 0–0.4 M potassium phosphate in PMG buffer at a flow rate of 40 mL/h. The ATPase eluted at 0.23 M. Peak fractions were pooled and dialyzed overnight in PMEG buffer. This constitutes fraction IV. The increase in total units at this step is due to the removal of an inhibitor.

Fraction IV was loaded onto a 2 $cm^2 \times 16$ cm phosphocellulose column. The column was washed with 50 mL of PMEG buffer, and the proteins were eluted with a 500-mL gradient of 0–0.4 M potassium phosphate in PMEG buffer at a flow rate of 40 mL/h. The enzyme eluted at 0.13 M. The peak fractions, pooled and dialyzed overnight against TMEG buffer, represent fraction V.

Fraction V was loaded onto a 0.63 $cm^2 \times 14$ cm column of DNA–cellulose equilibrated with TMEG buffer. After being washed with 20 mL of starting buffer, the column was developed with a 400-mL gradient of 0–0.5 M NaCl in TMEG buffer at a flow rate of 40 mL/h. ATPase III elutes at 0.3 M. A second peak, which is ATPase II, elutes at 0.2 M. Both peaks were pooled separately and dialyzed against TMEG buffer. The ATPase peak represents fraction VI. The large apparent loss in total units (Table I) is due both to the instability of ATPase III at this step and to the complete resolution of ATPase II from ATPase III. In fraction V we estimate that approximately 40% of the total units are due to ATPase II activity. Thus the true recovery of ATPase III is on the order of 35% for DNA–cellulose chromatography.

Fraction VI was loaded onto a 0.63 $cm^2 \times 6$ cm column of QAE-Sephadex equilibrated with TMEG buffer. After a 20-mL wash with TMEG buffer, the proteins were eluted with a 300-mL linear gradient of 0–0.3 M NaCl in TMEG buffer at a flow rate of 40 mL/h. The ATPase peak, eluting at 0.13 M, was pooled and dialyzed against TMEG buffer and constitutes fraction VII. This step was useful in removing trace contaminants of DNA that elute from the column.

Fraction VII was loaded onto a 0.5 $cm^2 \times 1.5$ cm ATP–agarose column equilibrated with TMEG buffer. After being washed with 10 mL of starting buffer, a 250-mL gradient of 0–0.3 M NaCl in TMEG buffer was used to elute the enzyme at a flow rate of 30 mL/h. The ATPase peak elutes at 0.12 M. These fractions were pooled and concentrated against TMEG buffer with a Micro ProDiCon vacuum concentrator. The concentrate represents fraction VIII.

Stability and Purity of DNA-Dependent ATPase III. The enzyme is stable at 4 °C through all steps of purification except the last. Fraction VIII has a half-life of about 24 h at 4 °C but is stable at −70 °C for at least 3 months. It is also stable to freeze–thawing. Addition of 1 mg/mL BSA, 0.5 M NaCl or additional glycerol failed to enhance the stability at 4 °C.

The ATPase has been purified over 42 000-fold in an 18% yield through an eight step procedure (Table I). Electrophoresis of fraction VIII on $NaDodSO_4$–polyacrylamide gels shows a single stainable band. Densitometer scanning of the gel shows a single peak of >96% homogeneity at M_r 65 000 relative to standards. Analysis of proteins on $NaDodSO_4$ gels using samples from fractions across the peaks of the last three columns (DNA–cellulose, QAE-Sephadex, and ATP–agarose) revealed that this M_r 65 000 band closely follows the ATPase activity. Thus, we feel that the ATPase has been purified to near homogeneity. Unfortunately we were unable to recover enzymatic activity from nondenaturing gels, which would have strengthened this conclusion. We have examined fraction VIII for the presence of trace contaminants by enzymatic assay. The ATPase is free of DNA polymerase, exonuclease, endonuclease, and nonspecific phosphatase activities at the level of 10^{-5}–10^{-7} relative to the ATPase rate. However, some preparations contained a weak endonuclease activity in fraction VIII. This endonuclease is very heat labile relative to ATPase III, and the nuclease can be inactivated by heating for 4 min at 44 °C.

Physical Properties of ATPase III. Some of the physical and enzymological properties of ATPase III are summarized in Table II. The Stokes radius of the enzyme is 43 Å as determined from gel filtration on Sephacryl S-300. On gradient centrifugation, ATPase III was found to have a sedimentation coefficient of 7.0 S. The sedimentation is unaffected by changes in ionic strength, with the enzyme sedimenting identically in the absence of salt or in the presence of 0.5 M KCl (data not shown). However, addition of 0.5 mM ATP causes the enzyme to sediment slower at 6.1 S (Figure 2), indicating the ATPase undergoes a conformational change that makes it less compact in the presence of substrate. This effect is interesting but less dramatic than the change observed by J. W. Hockensmith and R. A. Bambara (unpublished results) for calf thymus ATPase where ATP promotes dissociation of a dimer into a monomer. Using the method of Siegel & Monty (1966), we calculate a native M_r of 123 000 (in the absence of ATP). With a denatured M_r of 65 000 (Figure 2), these results indicate the enzyme is composed of two equal-sized and probably identical subunits.

Table II: General Properties of ATPase III

M_r (NaDodSO$_4$-polyacrylamide gel electrophoresis)	65 000
s value (glycerol gradient centrifugation) (S)	7.0
Stokes radius (Sephacryl S-300) (Å)	43
frictional coefficient	1.31
native M_r (from s value and Stokes radius)[a]	123 000
Q_{10}	3.3
K_m for ATP (μM)	206
K_m for dATP (μM)	110
K_i for ATPγS (μM)	11
K_i for ddATP (μM)	720
K_i for α,β-Me-ATP (mM)	2.2
K_m for G4 DNA (μM)	0.4 (nucleotide)
K_m for poly(dT) (μM)	0.4 (nucleotide)
turnover number for ATP min^{-1}	1560

[a] Calculated according to Siegel & Monty (1966).

Table III: Reaction Requirements for ATPase III

additions or deletions	control activity (%)
complete	100
minus DNA	<1
minus Mg^{2+}	<1
minus Mg^{2+}, plus 0.5 mM Mn^{2+}	81
minus Mg^{2+}, plus 0.5 mM Ca^{2+}	52
plus 200 mM NaCl	100
plus 300 mM KCl	51
plus 200 mM potassium phosphate	53
plus 10 mM NEM	<1
plus 100 μM nalidixic acid	99
plus 100 μM novobiocin	100
plus 10 μM berenil	101

General Properties of ATPase III. The reaction requirements of ATPase III are shown in Table III. The enzyme is a nucleotide triphosphatase that hydrolyzes ATP or dATP to a diphosphate plus P_i. Production of monophosphates was not detected. The enzyme has an absolute requirement for a polynucleotide effector and a divalent cation (Table III). Magnesium is preferred at an optimum of 0.5 mM (data not shown), but the enzyme has significant activity with either Mn^{2+} or Ca^{2+} at a similar optimum. The ATPase has a broad pH optimum from pH 7.0 to pH 10.0. It is unaffected by salts up to 200 mM but slowly loses activity when the concentration is raised above this level (Table III). Although phosphate is a product of the reaction, it does not strongly inhibit the reaction except at high concentrations. The enzyme is sensitive to sulfhydryl blocking agents, showing complete inhibition at 10 mM NEM (Table III).

The rate of hydrolysis of ATP is linear until approximately 50% of the substrate has been hydrolyzed. The enzyme is most active at 42 °C. Some prokaryotic ATPases such as *rep* protein and the ATPase associated with DNA polymerase III holoenzyme are active at 0 °C (R. R. Meyer, C. R. Brown, and D. C. Rein, unpublished results). However, the Novikoff hepatoma enzyme is completely inactive at 0 °C. At 5 and 10 °C, there is a significant rate of hydrolysis. ATPase activity is directly proportional to the amount of enzyme added up to 200 units/25-μL assay. Between 200 and 400 units/assay, there is only a slight deviation from linearity (data not shown). Above 400 units, the reaction is nonlinear.

Substrates for ATPase III. The Novikoff hepatoma DNA-dependent ATPase uses either ATP or dATP equally well as a substrate (Table IV). Other ribonucleoside triphosphates and deoxyribonucleoside triphosphates are poor

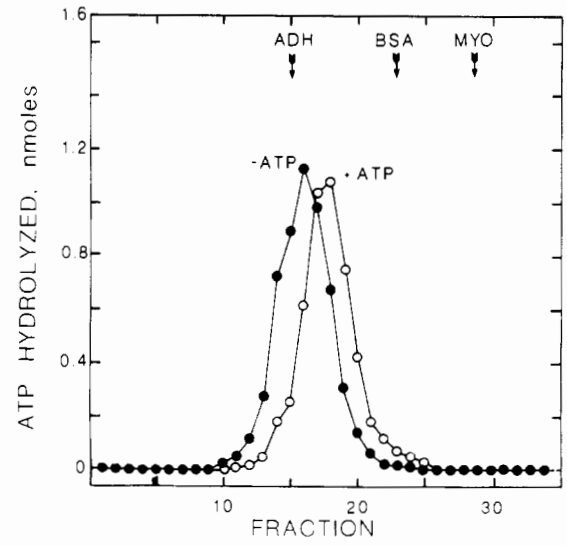

FIGURE 2: Glycerol gradient centrifugation of Novikoff hepatoma ATPase III. Approximately 1200 units of fraction VII ATPase III was layered onto a 10–30% glycerol gradient in 20 mM Tris-HCl (pH 8.0), 5 mM β-mercaptoethanol, 1 mM EDTA, and 0.5 M KCl. Centrifugation was carried out as described under Materials and Methods. Each gradient contained three internal markers, alcohol dehydrogenase (ADH, 7.4 S), BSA (4.3 S), and myoglobin (MYO, 2.0 S). Half of the gradients contained no ATP (●) or 0.5 mM ATP (○). In all gradients, >85% of the ATPase activity applied was recovered. Identical results were obtained with gradients lacking KCl. ATPase III sediments at 7.0 S in the absence of ATP but at 6.1 S in the presence of substrate.

Table IV: Polynucleotide Effectors for ATPase III[a]

effector	ATPase activity (pmol hydrolyzed)	rel act. (%)
none	10	<1.0
DNAs		
G4 ssDNA	4860	100
ϕX174 ssDNA	6070	125
denatured calf thymus DNA	4050	83
calf thymus dsDNA	2290	47
ϕX174 RFI supercoiled dsDNA	4780	98
ϕX174 *Hae*III fragment Z_2	5590	115
ϕX174 *Hae*III fragment Z_9	5750	118
synthetic polydeoxyribonucleotides		
poly(dA-dT)	1300	27
poly(dA)·oligo(dT)$_{12-18}$ (1:1)	2270	47
poly(dA)	1910	39
poly(dC)	2790	57
poly(dG)	984	20
poly(dI)	1890	39
poly(dT)	5830	120
oligodeoxyribonucleotides		
dTpdT	4540	93
$(dT)_2$	2860	59
$(dT)_4$	1100	23
$(dT)_6$	777	16
$(dT)_8$	624	13
$(dT)_{12}$	729	15
$(dT)_{16}$	2440	50
$(dT)_{300}$	4720	97
synthetic polyribonucleotides		
poly(A)	741	15
poly(C)	688	14

[a] ATPase assays were carried out as described under Materials and Methods except that the nucleotide effector was as listed in the table and used at a concentration of 1.5 nmol (nucleotide) per assay. Approximately 200 units of fraction VIII ATPase III was used per assay. Relative activity is expressed as the percent of activity obtained compared to that obtained with G4 DNA as the effector.

substrates. For example, CTP, dTTP, dCTP, and GTP are utilized at a rate of 8.7%, 6.4%, 4.6%, and 3.2%, respectively, compared to that of ATP hydrolysis. No activity at all could be detected with UTP or dGTP. The K_ms for the two preferred substrates ATP and dATP were determined from a Lineweaver–Burk plot. While the V_{max} values for both are identical, it is interesting that the K_m for dATP is much lower than that for ATP (110 μM vs. 206 μM, Table II). This K_m for substrate is considerably lower than that reported for other mammalian ATPases. The turnover number of the purified enzyme is 1560 mol of ATP min^{-1} (mol of enzyme)$^{-1}$.

Analysis of Nucleotide Effector Requirement for ATPase III. ATPase III has an absolute requirement for a polynucleotide effector (Table IV). Duplex DNA is also very active; double-stranded calf thymus DNA supports the reaction at about 50% of the rate of denatured DNA. Supercoiled ϕX174 RFI DNA supports the reaction as efficiently as single-stranded DNA, and the *Hae*III restriction fragments, which contain no ssDNA regions, are excellent effectors. Synthetic polynucleotides, with the exception of poly(dT), are less effective than natural ssDNA effectors (Table IV). Poly(dT) consistently gives higher rates of hydrolysis than all other polynucleotides tested and supports ATPase activity as well as or better than natural DNAs. ATPase III is capable of using polyribonucleotides as effectors but at low efficiency.

The results with purified oligonucleotides are interesting. Oligo(dT)$_8$ is the poorest effector, but the ability to support the reaction increases with increasing chain length (Table IV), a phenomenon observed with most prokaryotic and eukaryotic DNA-dependent ATPases. It is surprising, however, that as the chain length decreases below eight residues, activity increases. Indeed, the dinucleotide dTpdT, which lacks a 5′-phosphate, supports ATPase III at nearly 100% efficiency in comparison to the dinucleotide (dT)$_2$ containing the 5′-phosphate. Mononucleotides are totally inactive. Although these data are difficult to interpret at present, they may suggest that the active site for the effector could be a dinucleotide. However, more extensive studies will be required to substantiate this.

The K_m for nucleotide effectors was determined from Lineweaver–Burk plots with both G4 DNA and poly(dT). The K_m for both is identical at 0.40 μM (nucleotide) (Table II); however, the V_{max} is 20% higher for poly(dT).

Single-stranded DNA-binding protein from *E. coli* inhibits many DNA-dependent ATPases by competing more effectively for single-stranded DNA (Kornberg et al., 1978; Meyer et al., 1980; R. R. Meyer, C. R. Brown, and D. C. Rein, unpublished results). We have examined the effects of SSB on ATPase III. As shown in Figure 3, *rep* protein, used here as a control, is rapidly inhibited by SSB with complete inhibition at a concentration in which the G4 DNA effector is completely coated with SSB (Kornberg et al., 1978). While ATPase III is also inhibited by SSB, higher concentrations are required, and complete inhibition is never attained (Figure 3). One possible explanation for this difference may lie in the ability of ATPase III to use dsDNA very efficiently as an effector as compared to *rep* protein. The origin of G4 DNA for SS → RF replication is thought to be a duplex loop on SSB-coated G4 DNA (Rowen & Kornberg, 1978). This double-stranded region may act as an effector for ATPase III but not *rep* ATPase.

Effects of ATP Analogues on ATPase III Activity. A variety of ATP analogues were tested for their ability to inhibit ATPase III as shown in Figure 4. The four analogues adenosine tetraphosphate, AMP-PNP, 8-Br-ATP, and β,γ-

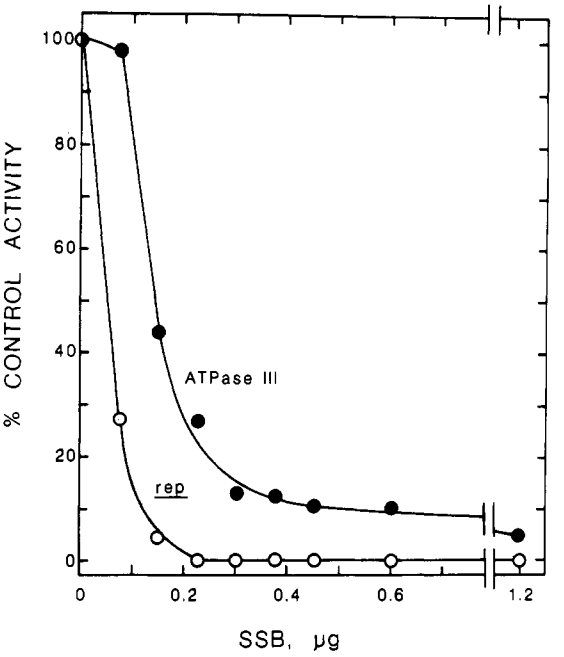

FIGURE 3: Inhibition of ATPase III and *rep* ATPase by *E. coli* single-stranded DNA-binding protein. Approximately 40 units of fraction VIII ATPase was incubated with 230 pmol (nucleotide) of G4 DNA and increasing concentrations of SSB as shown in the figure. For *rep* ATPase, 300 units was used as a comparison for ATPase III.

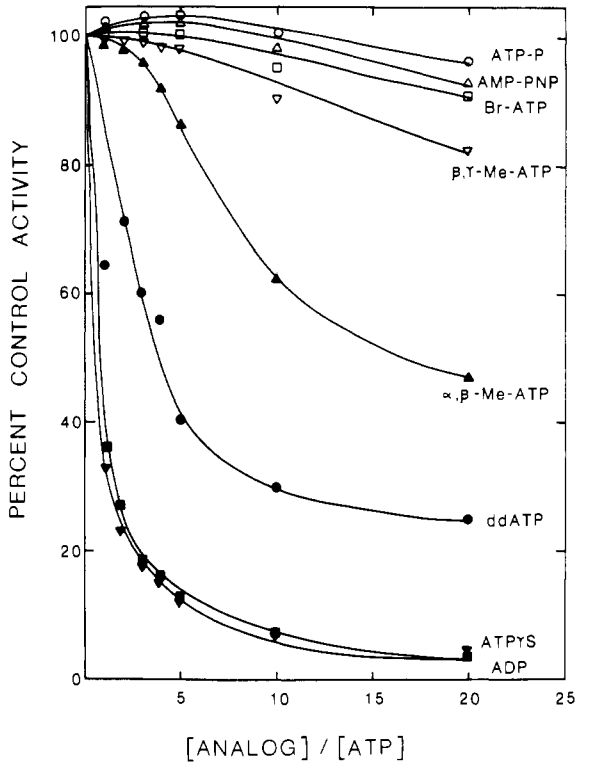

FIGURE 4: Inhibition of ATPase III by ATP analogues. Approximately 20 units of fraction VIII ATPase III was incubated with 0.4 mM ATP and various analogues at increasing concentrations up to 20 times that of ATP. Inhibitors used included adenosine tetraphosphate (ATP-P), 5′-adenylyl imidodiphosphate (AMP-PNP), 8-bromoadenosine 5′-triphosphate (Br-ATP), adenosine 5′-(β,γ-methylenetriphosphate) (β,γ-Me-ATP), adenosine 5′-(α,β-methylenetriphosphate) (α,β-Me-ATP), 2′,3′-dideoxyadenosine triphosphate (ddATP), adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS), and adenosine 5′-diphosphate (ADP).

Me-ATP do not significantly inhibit the enzyme at concentrations up to 20 times that of the ATP substrate. A low level

Table V: Stimulation of DNA Polymerase β by ATPase III and a Single-Stranded DNA-Binding Protein Isolated from the Novikoff Hepatoma[a]

additions	DNA polymerase activity: gapped DNA (pmol)	DNA polymerase activity: duplex DNA (pmol)
experiment I		
none	40.3	0.5
ATPase III	46.2	2.5
DNA-binding protein	54.6	2.2
ATPase III and DNA-binding protein	69.2	5.7
experiment II		
none		0.9
ATPγS		0.9
ATPase III		3.8
ATPase III and ATPγS		1.4
DNA-binding protein		7.2
DNA-binding protein and ATPγS		7.5
ATPase III and DNA-binding protein		17.8
ATPase III, DNA-binding protein, and ATPγS		9.6

[a] Polymerase assays were carried out as described under Materials and Methods, except that all tubes contained 0.4 mM ATP and 0.04 unit of fraction VI DNA polymerase β. Incubation was at 37 °C for 60 min. For ATPase III, 50 units of fraction VIII per assay was used, and for the DNA-binding protein, 0.18 μg per assay was used. When included, the ATPγS concentration was 0.8 mM. Experiments I and II were carried out with different preparations of DNA, ATPase III, and DNA-binding protein.

of inhibition is observed at high concentrations. However, ATP itself at these concentrations gives a similar level of inhibition (data not shown). The enzyme is strongly inhibited by ADP, an end product of the reaction. The three analogues ATPγS, ddATP, and α,β-Me-ATP inhibit to varying degrees. A Lineweaver–Burk plot of the kinetic data obtained in the presence of each inhibitor (data not shown) indicates that all are acting as competitive inhibitors. Using the method of Dixon & Webb (1979), we calculated a K_i for each analogue. The values are 0.011 mM for ATPγS, 0.72 mM for ddATP, and 2.2 mM for α,β-Me-ATP. Using [^{35}S]ATPγS, we found the enzyme unable to hydrolyze this analogue.

Interaction of ATPase III with Novikoff Hepatoma DNA Polymerases. In an attempt to define a biological role for ATPase III, we have examined its effects on the homologous DNA polymerases. ATPase III had no effect on the activity of DNA polymerase α (data not shown). However, the ATPase is able to stimulate DNA polymerase β severalfold (Table V). On activated (gapped) DNA, which is the preferred substrate for this polymerase, there is only a slight effect. This is probably due to the availability of sufficient productive sites for synthesis. On duplex calf thymus DNA, which the polymerase uses very poorly, ATPase III stimulates synthesis 4–5-fold. This occurs in the presence of 0.4 mM ATP (Table V) or 0.4 mM dATP (not shown). Particularly interesting is the effect of adding SSB[3] from Novikoff hepatoma cells. This DNA-binding protein also stimulates DNA polymerase severalfold on duplex DNA. When both ATPase III and SSB are used together, the stimulation is greater than the additive effect of the two individual proteins. Addition of 0.8 mM ATPγS, which is sufficient to inhibit the ATPase by 80% (see Figure 4), reduces the ATPase-dependent stimulation by roughly 80% (Table V). This is observed when ATPase III is used either alone or in combination with SSB. ATPγS, however, has no inhibitory effect on either the polymerase or the DNA-binding protein (Table V), indicating the effect is due solely to inhibition of the ATPase. No stimulation by ATPase III is observed if the DNA is pretreated with this enzyme and then heat treated (65 °C, 10 min) to inactivate the ATPase. We have not been able to detect DNA helicase activity directly for ATPase III. However, these assays are not very sensitive, so that it is possible that ATPase III may have a weak helicase activity that opens only a few base pairs of DNA. Such a weak activity would not be detected.

[3] An M_r 48 000 DNA-binding protein has been purified from the Novikoff hepatoma. This protein differs from two other binding proteins previously reported from rat liver. A detailed characterization of this protein will be published (T. J. Koerner and R. R. Meyer, unpublished results).

Discussion

Multiple species of DNA-dependent ATPases have been identified in prokaryotic systems and shown to be involved in DNA replication, repair, and recombination. Thus, it is not suprising to find a variety of such enzymes in eukaryotes. So far two DNA-dependent ATPases have been described in yeast (Plevani et al., 1980), three in KB cells (DeJong et al., 1981), four in calf thymus (J. W. Hockensmith and R. A. Bambara, unpublished results), and five here in the Novikoff hepatoma (Figure 1). Extensive purification and a detailed enzymological and physical characterization will be necessary to show that these chromatographically distinct activities are, in fact, different enzymes and to elucidate their precise role in DNA metabolism.

In the current report we have presented an initial description of Novikoff hepatoma ATPase III. However, identification of the function of this enzyme in vivo, as well as that of the ATPases isolated from other mammalian sources, has so far been elusive. ATPase III probably is not a topoisomerase, since it did not change the linking number of ϕX174 RFI DNA, and the ATPase is resistant to the antibiotics nalidixic acid, novobiocin, and berenil, which inhibit known topoisomerases. Similarly, we have been unable to show that ATPase III is a helicase with nuclease S1 sensitivity as an assay, although, as we pointed out, this assay may be too insensitive to detect a limited unwinding of the helix. If ATPase III is a weak helicase and capable of unwinding only a few base pairs, these short single-stranded regions would probably be too short to detect by our assay or might readily reanneal again. The presence of a DNA-binding protein may be necessary to stabilize these regions and promote limited synthesis from nicks. DNA polymerase β is stimulated by both ATPase III and SSB (Table V). The stimulation is minimal on gapped DNA but 4–5-fold on native DNA containing a few nicks. When both proteins are used together, the effect is more than additive with synthesis nearly 12 times that using the polymerase alone. The ATP analogue ATPγS abolishes the stimulation with ATPase III, suggesting the reaction may be biologically relevant. In this context, two other ATPases have been shown to stimulate polymerases. Boxer & Korn (1980) purified an M_r 75 000 ATPase from KB cell nuclei that stimulates DNA polymerase α about 5-fold and DNA polymerase β about 3-fold. The KB cell enzyme requires ATP for this stimulation in contrast to ATPase III, which stimulates in the presence of either dATP or ATP. Similarly, Cobianchi et al. (1979) partially purified an ATPase from human EUE cells that stimulates calf thymus DNA polymerase α. It is interesting that this ATPase appears to interact with an M_r 20 000 DNA-binding protein purified from calf thymus. When used together, these two enzymes have an additive effect on α polymerase stimulation. The

authors propose a model in which these proteins facilitate movement of the polymerase into duplex structures for replication.

Recently, J. W. Hockensmith and R. A. Bambara (unpublished results) have purified an ATPase to homogeneity from calf thymus. This ATPase promotes reannealing of duplex DNA, a reaction similar to one catalyzed by *recA* protein of *E. coli* (Weinstock et al., 1979). This ATPase may be a eukaryotic analogue to *recA* protein and may be involved in recombination. Further work will be necessary to establish this, however.

Finally, it is interesting that our ATPase is active with DNA polymerase β, the putative repair polymerase. The major thrust of our work over the past several years has been to develop a system in vitro, with purified proteins from mammalian cells, for the repair of damaged DNA. Excision repair is thought to be a multienzyme process involving recognition of the altered nucleotide, endonucleolytic incision, exonucleolytic gap expansion, resynthesis of the DNA, and ligation. We have already demonstrated that Novikoff hepatoma DNA polymerase β forms a complex with DNase V, a bidirectional double-strand exonuclease (Mosbaugh et al., 1977; Mosbaugh & Meyer, 1980; Meyer & Mosbaugh, 1980). More recently, it has been shown that DNase V is capable of converting a 3′-OH apurinic/apyrimidinic cleavage site into a competent primer for DNA polymerase β (P. Small, R. R. Meyer, and D. Rein, unpublished results; D. W. Mosbaugh and S. Linn, personal communication). However, the 5′ cleavage site, containing a damaged or altered nucleotide such as a thymine dimer, may require another nuclease for repair. One possible model involving ATPase III would be for this enzyme to catalyze a limited displacement on the 5′ side of the AP nick, perhaps in advance of synthesis. The short displaced strand, containing the damaged base, would now be susceptible to exonucleolytic attack by a single-stranded nuclease such as DNase VIII (Grossman, 1981). Gap filling by DNA polymerase β and ligation would complete the repair process. This model is, of course, highly speculative at this time. Further experimentation will be necessary to demonstrate the precise role of ATPase III. However, the ability of this enzyme to stimulate DNA polymerase β, and apparently to interact with SSB, suggests that it may play a physiological role in DNA repair.

Acknowledgments

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References

Abdel-Monem, M., Dürwald, H., & Hoffmann-Berling, H. (1976) *Eur. J. Biochem. 65*, 431–449.

Abdel-Monem, M., Dürwald, H., & Hoffmann-Berling, H. (1977) *Eur. J. Biochem. 79*, 39–45.

Ackers, G. K. (1964) *Biochemistry 3*, 723–730.

Alberts, B., & Herrick, G. (1971) *Methods Enzymol. 21*, 198–217.

Assairi, L. M., & Johnston, I. R. (1979) *Eur. J. Biochem. 99*, 71–79.

Boxer, L. M., & Korn, D. (1980) *Biochemistry 19*, 2623–2633.

Cobianchi, F., Riva, S., Mastromei, G., Spadari, S., Pedrali-Noy, G., & Falashi, A. (1979) *Cold Spring Harbor Symp. Quant. Biol. 43*, 639–647.

DeJong, P. J., Tommassen, J. P. M., van der Vliet, P. C., & Jansz, H. S. (1981) *Eur. J. Biochem. 117*, 179–186.

Dixon, M., & Webb, E. C. (1979) *Enzymes, 3rd Ed.*, 345–352.

Eisenberg, S., Harbers, B., Harus, C., & Denhardt, D. T. (1975) *J. Mol. Biol. 99*, 107–123.

Gellert, M., Mizuuchi, K., O'Dea, M. H., Ohmori, H., & Tomizama, J. (1978) *Cold Spring Harbor Symp. Quant. Biol. 43*, 35–40.

Goldmark, P. J., & Linn, S. (1972) *J. Biol. Chem. 247*, 1849–1860.

Grossman, L. (1981) *Arch. Biochem. Biophys. 211*, 511–522.

Hachmann, H. J., & Lezius, A. G. (1976) *Eur. J. Biochem. 61*, 325–330.

Hotta, Y., & Stern, H. (1978) *Biochemistry 17*, 1872–1880.

Kornberg, A., Scott, J. F., & Bertsch, L. L. (1978) *J. Biol. Chem. 253*, 3298–3304.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Martin, R. G., & Ames, B. N. (1961) *J. Biol. Chem. 236*, 1372–1379.

McEntee, K., Weinstock, G. M., & Lehman, I. R. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 857–861.

Meyer, R. R., & Simpson, M. V. (1970) *J. Biol. Chem. 245*, 3426–3435.

Meyer, R. R., & Mosbaugh, D. W. (1980) *ICN-UCLA Symp. Mol. Cell. Biol. 19*, 639–648.

Meyer, R. R., Shlomai, J., Kobori, J., Bates, D. L., Rowen, L., McMacken, R., Ueda, K., & Kornberg, A. (1979) *Cold Spring Harbor Symp. Quant. Biol. 43*, 289–294.

Meyer, R. R., Glassberg, J., Scott, J. V., & Kornberg, A. (1980) *J. Biol. Chem. 255*, 2897–2901.

Mosbaugh, D. W., & Meyer, R. R. (1980) *J. Biol. Chem. 255*, 10239–10247.

Mosbaugh, D. W., Stalker, D. M., Probst, G. S., & Meyer, R. R. (1977) *Biochemistry 16*, 1512–1518.

Plevani, P., Badaracco, G., & Chang, L. M. S. (1980) *J. Biol. Chem. 255*, 4957–4963.

Probst, G. S., Stalker, D. M., Mosbaugh, D. W., & Meyer, R. R. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1171–1174.

Reha-Krantz, L. J., & Hurwitz, J. (1978) *J. Biol. Chem. 253*, 4043–4057.

Richet, E., & Kohiyama, M. (1976) *J. Biol. Chem. 251*, 808–812.

Richet, E., & Kohiyama, M. (1978) *J. Biol. Chem. 253*, 7490–7495.

Roberts, J. W., Roberts, C. W., & Craig, N. L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4714–4718.

Rowen, L., & Kornberg, A. (1978) *J. Biol. Chem. 253*, 758–764.

Shibata, T., Cunningham, R. P., DasGupta, C., & Radding, C. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5100–5104.

Shlomai, J., & Kornberg, A. (1980) *J. Biol. Chem. 255*, 6789–6798.

Siegel, L. M., & Monty, K. J. (1966) *Biochim. Biophys. Acta 112*, 346–362.

Stalker, D. M., Mosbaugh, D. W., & Meyer, R. R. (1976) *Biochemistry 15*, 3114–3121.

Thomas, D. C., & Meyer, R. R. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 1875.

Tomlinson, R. V., & Tener, R. R. (1963) *Biochemistry 2*, 697–702.

Ueda, K., McMacken, R., & Kornberg, A. (1978) *J. Biol. Chem. 253*, 261–269.

Weinstock, G. W., McEntee, K., & Lehman, I. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 126–130.
Wickner, S., & Hurwitz, J. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3342–3346.
Yaginuma, K., & Koike, K. (1981) *Nucleic Acids Res. 9*, 1949–1961.
Yarranton, G. T., Das, R. H., & Gefter, M. L. (1979) *J. Biol. Chem. 254*, 11997–12006.

Inhibition of Dihydrofolate Reductase: Effect of Reduced Nicotinamide Adenine Dinucleotide Phosphate on the Selectivity and Affinity of Diaminobenzylpyrimidines†

David P. Baccanari,* Susan Daluge, and R. W. King

ABSTRACT: The selectivity of benzylpyrimidines for bacterial dihydrofolate reductases was studied by using equilibrium and kinetic techniques. Trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine] and a series of close structural analogues with different methoxy group substitutions on the benzyl showed in vitro *Escherichia coli* antibacterial activities that varied according to their degree of substitution. Trimethoprim, the most potent analogue tested, was 400-fold more active than benzylpyrimidine, and the monomethoxy and dimethoxy analogues were of intermediate antibacterial activity. The relative antibacterial potencies of all the compounds were directly proportional to their *E. coli* form 1 dihydrofolate reductase K_i values, as determined by classical enzyme kinetics. Inhibition of the enzyme assay is a measure of the E–I–NADPH ternary complex, and the K_i values ranged from 670 nM for the unsubstituted benzylpyrimidine to 1.3 nM for trimethoprim. However, equilibrium dialysis and fluorescence studies with the *E. coli* and *Lactobacillus casei* enzymes performed in the absence of NADPH showed that the dissociation of inhibitors from the EI binary complex did not vary as widely as kinetic K_i values and that these binary constants were not directly related to either kinetic K_i or antibacterial activity. NADPH increased the affinity of the bacterial enzymes for inhibitors, and this increased affinity in the E–I–NADPH ternary complex (cooperativity) varied with the degree of methoxy substitution (up to 230-fold for trimethoprim and the form 2 *E. coli* enzyme). Contrary to this, all the compounds were weak inhibitors of the mammalian enzyme (SR-1 rodent lymphoma), and none showed more than 8-fold binding cooperativity with NADPH. Therefore, NADPH cooperativity is an important factor in the high affinity of *E. coli* dihydrofolate reductase for trimethoprim, and the lack of cooperativity with the mammalian enzyme is important in the selectivity of trimethoprim as an antibacterial.

Dihydrofolate reductase (EC 1.5.1.3) catalyzes the NADPH-dependent reduction of dihydrofolic acid. The product, tetrahydrofolate, and other reduced folates are essential for the biosynthesis of purines, thymidylate, and several amino acids (Blakley, 1969). Inhibitors of dihydrofolate reductase are effective in the treatment of cancer (methotrexate), malaria (pyrimethamine), and bacterial infections (trimethoprim).[1] Since the latter two are clinically useful because of their marked selectivity for the pathogen dihydrofolate reductases (Burchall, 1974), there is much interest in determining their mechanisms of binding. NMR spectroscopy and equilibrium and kinetic analyses have shown that inhibitor binding can be a complex process involving a variety of protein conformations with different inhibitor affinities (Baccanari & Joyner, 1981; Cayley et al., 1981; Gronenborn et al., 1981). Also, several types of cooperative ligand interactions have been observed. For example, Perkins & Bertino (1966) used fluorescence titrations to show that the L1210 dihydrofolate reductase dissociation constant for triamterene is decreased 60-fold in the presence of NADPH. Similarly, Otting & Huennekens (1972) demonstrated that the binding of methotrexate to dihydrofolate reductase is enhanced by NADPH. Cooperative interactions are also evident from the fact that the covalent binding of active site directed irreversible inhibitors is augmented in the presence of NADPH (Freudenthal et al., 1970). More recently, Birdsall et al. (1977) reported that *p*-aminobenzoylglutamate and 2,4-diaminopyrimidine (regarded as "fragments" of methotrexate) bind cooperatively in the absence of coenzyme. NADPH increases the binding of both fragments without altering the cooperativity between them. Birdsall et al. (1980) also studied the effect of $NADP^+$, NADPH, and a series of coenzyme analogues on the binding of methotrexate and trimethoprim to the *Lactobacillus casei* enzyme. The reduced coenzymes showed significantly more cooperativity than their oxidized counterparts, and dissociation rate constants for reduced 3-acetylpyridine adenine dinucleotide phosphate decreased up to 2200-fold in the ternary complex with methotrexate. Trimethoprim cooperative effects have also been observed with both isozymes of *Escherichia coli* RT 500 dihydrofolate reductase (Baccanari et al., 1981a). In the present study, equilibrium dialysis and fluorescence kinetic analyses were used to assess the effect of NADPH on the binding of trimethoprim and a series of close structural analogues to both bacterial and mammalian dihydrofolate reductases. It was shown that cooperativity (enhanced inhibitor binding in the presence of NADPH) is an important factor in the selectivity of trimethoprim as well as its tight binding to the *E. coli* enzyme and that *E. coli* dihydrofolate

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[1] Abbreviations: trimethoprim, 2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine; TLC, thin-layer chromatography; Me_2SO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

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