Biochemistry

 Volume 23, Number 9 April 24, 1984

Accelerated Publications

Immunoaffinity Purification and Properties of a High Molecular Weight Calf Thymus DNA α-Polymerase†

Alan F. Wahl,* Stanley P. Kowalski, Lee W. Harwell, Edith M. Lord, and Robert A. Bambara‡

ABSTRACT: A rapid, three-step purification of DNA α-polymerase from calf thymus is described. The key feature is immunoaffinity chromatography using a column of immobilized monoclonal immunoglobulin G (IgG) developed against human KB cell α-polymerase. This step is followed by preparative sucrose gradient sedimentation. The highly purified polymerase has a specific activity of 35 000 nmol of nucleotide incorporated per hour per milligram. Its molecular weight is 404 000. This molecular weight is higher than observed in some earlier purifications, possibly because salt concentrations are kept at nearly physiological levels. Also, the rapidity of purification in the presence of multiple protease inhibitors minimizes degradation. The purified enzyme is inhibited by aphidicolin, *N*-ethylmaleimide, and the specific monoclonal IgG, thereby identifying it as DNA α-polymerase. ATP at 4 mM concentration stimulates enzymatic activity up to 4-fold on calf thymus DNA templates. The enzyme is also capable of priming single-stranded DNA with RNA. The procedure represents a significant advance from purifying α-polymerase from calf by conventional means, since it avoids ion-exchange chromatography and harsh conditions. It also minimizes the time required to produce sufficient quantities of purified high molecular weight polymerase for analysis.

The earliest purification of DNA α-polymerase from calf thymus to near homogeneity was that described by Holmes et al. (1974). The procedure relied on a series of salt elutions from ion-exchange resins, ammonium sulfate precipitation, gel filtration, and gradient sedimentation. The enzyme thus obtained required gel electrophoresis as a purification step before a nearly homogeneous product could be demonstrated. Characterization of this enzyme (Holmes et al., 1976; McKune & Holmes, 1979) showed it to consist of a large polypeptide (M_r 155 000) and at least three other polypeptides, M_r 50 000–70 000. More recently, Masaki et al. (1982) have purified the calf enzyme by similar methods. α-Polymerase was isolated by gradient salt elution from sequential columns of phosphocellulose, DEAE-cellulose, hydroxylapatite, and DNA–cellulose, followed by sucrose gradient sedimentation. Analysis of the resultant enzyme fraction by denaturing gel electrophoresis resolved a large polypeptide of M_r 140 000–150 000 and smaller peptides of M_r 43 000–50 000. Native molecular weights for calf polymerase range from M_r 200 000–250 000 after the procedures just described to greater than M_r 400 000 for the partially purified DNA polymerase (Grummt et al., 1979; Hübscher, 1983). Typical purifications take 1–2 weeks to complete and are hampered by proteolysis. Degradation of high molecular weight forms of α-polymerase occurs within days of isolation even in the presence of protease inhibitors (Holmes et al., 1977; Masaki et al., 1982; Sauer & Lehman, 1982).

Tanaka et al. (1982) developed several hybridoma cell lines producing monoclonal antibody against human KB cell DNA α-polymerase. They have used one of these to purify a KB cell α-polymerase–IgG[1] complex that is catalytically active (Wang et al., 1984). However, exposure to 1 M KCl under

† From the Departments of Biochemistry and Microbiology and the Cancer Center, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642. *Received January 23, 1984.* This research was supported by National Institutes of Health Grants GM24441 to R.A.B. and CA28322 to E.M.L. and Cancer Core Grant 5-P30-CA11198-14.

* Address correspondence to this author at the Department of Biochemistry, University of Rochester Medical Center. Supported by U.S. Public Health Service Grant T32-GM07102-08.

‡ Recipient of the American Cancer Society Faculty Research Award FRA220.

[1] Abbreviations: BME, 2-mercaptoethanol; BSA, bovine serum albumin; NEM, *N*-ethylmaleimide; IgG, immunoglobulin G; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris-HCl, tris(hydroxymethyl)-aminomethane; SDS, sodium dodecyl sulfate.

0006-2960/84/0423-1895$01.50/0

acid conditions is required to elute the antibody-complexed polymerase from its affinity support. We report here the use of another of these monoclonal antibodies to develop a unique purification scheme for the isolation of a high molecular weight form of α-polymerase from calf thymus. The distinct advantage of the methods we employ is the ability to rapidly purify the enzyme, free of the antibody, without subjecting it to higher than physiological levels of salt.

Materials and Methods

IgG Purification. A hybridoma cell line, SJK-287-38, producing monoclonal IgG antibody against human KB cell DNA α-polymerase (Tanaka et al., 1982) was obtained from the American Type Culture Collection (ATCC No. CRL-1644). The IgG product of this cell line was previously demonstrated to inactivate calf thymus α-polymerase (T. S. F. Wang, personal communication). Cells were cultured in Eagle's minimum essential media (Gibco) containing 10% fetal bovine serum (Sterile Systems, Inc.) as described by Harwell et al. (1976). IgG was obtained from cell culture supernatants by sequential centrifugation, first at 250*g* for 10 min to remove cells and then at 40000*g* for 30 min to clarify the fluid. The supernatant (225 mL) was dialyzed against two 2-L changes of 0.02 M potassium phosphate buffer, pH 8.0, containing 30 mM NaCl and 0.02% NaN_3, for 16 h. A 2.5 cm × 20 cm column of DEAE-Affi-Gel Blue (Bio-Rad) was equilibrated with this same buffer, and the dialyzate was applied twice at 40 mL/h. Between applications, the column was regenerated with 2 bed volumes of 3 M guanidine hydrochloride followed by 5 bed volumes of potassium phosphate buffer, pH 8.0. The second pass elution was concentrated to 5.0 mL in an ultrafiltration cell fitted with a PM-30 membrane (Amicon Corp.). This concentrate was centrifuged in 0.5-mL aliquots through 3.0-mL columns of Sephadex G-50 resin (Pharmacia) (Penefsky, 1977), equilibrated in 0.1 M sodium bicarbonate buffer, pH 8.0, to remove trace low molecular weight contaminants and to exchange buffers for later protein coupling to Affi-Gel 10. These techniques result in a highly purified, protease-free, IgG fraction.

Four milliliters of the IgG fraction (3 mg/mL) was combined with 30 mL of activated Affi-Gel 10 resin (Bio-Rad) and 10 mL of 0.1 M sodium bicarbonate buffer, pH 8.0. Under these conditions the immunoglobulin is covalently linked through its primary amines to the succinylated aminoalkyl Bio-Gel A matrix. The mixture was placed on a rocker table at 2 °C for 16 h to complete the coupling. The reacted resin was washed with homogenization buffer to remove uncoupled protein. The wash buffer was assayed for protein by the method of Bradford (1976) to determine the efficiency of coupling. Fractions of IgG and IgG resin were retained to test their ability to inactivate calf α-polymerase in a standard assay.

Purification of α-Polymerase. Freshly excised calf thymus glands (200 g), cleaned of connective tissue, were homogenized in a Waring blender for 2 min with 200 mL of frozen and 300 mL of cold liquid homogenization buffer. The homogenization buffer contained 50 mM Tris-HCl, pH 7.5, 5 mM dithiothreitol, 2 mM ATP, 0.5 M sucrose, 1 mM EDTA, 0.5 mM phenylmethanesulfonyl fluoride, 1 mg/mL bacitracin, and 0.5 μg/mL each of pepstatin A and leupeptin. All procedures were done at 2 °C. The homogenate was centrifuged in a Sorvall GS3 rotor for 35 min at 9000 rpm. The postmitochondrial supernatant solution was filtered through four folds of cheesecloth, reloaded into a Beckman type 35 rotor, and sedimented at 35000 rpm for 2 h. Fifty milliliters of this postmicrosomal supernatant solution was filtered through four folds of cheesecloth and combined with 15 mL of the IgG–Affi-Gel resin. This mixture was stirred slowly on ice for 2 h and then centrifuged in a Sorvall GSA rotor for 5 min at 3000 rpm. The supernatant solution was decanted and the resin resuspended in homogenization buffer; a transfer pipet was used to disperse the resin. A 2 cm × 5 cm column was poured and washed with homogenization buffer until no further decrease in the absorbance of the eluate at 280 nm could be detected. The column was eluted with a 120-mL linear gradient from 0 to 300 mM KCl in homogenization buffer. Fractions (1.5 mL) were collected and assayed for polymerase activity in a standard assay. Fractions containing the peak polymerase activity eluted between 110 and 120 mM added salt. Peak fractions were combined and concentrated 100-fold in an ultrafiltration cell fitted with a PM-30 membrane (Amicon Corp.) (see Table I).

Table I: Purification of DNA α-Polymerase from Calf Thymus

step	volume (mL)	protein (mg)	activity (units)	sp act. (units/mg)	yield (%)
postmicrosomal supernatant	25	275	1925	7	100
antibody–Affi-Gel chromatography	0.4	0.195	187	960	10
ultracentrifugation	0.1	0.0005	18	35 000	1

Preparative Sucrose Gradient Ultracentrifugation. Ultracentrifugation was carried out in a Beckman SW60 Ti rotor at 40000 rpm for 16 h. Replicate 5–20% (w/v) sucrose gradients were poured with a Beckman gradient former, and their linearity was verified with a refractometer. Centrifugation buffer contained 50 mM potassium phosphate, pH 7.8, 200 mM KCl, 2 mM BME, 0.5% Ampholine, pH 6–8 (LKB), and 1 mg/mL bacitracin. Samples (100 μL) of the polymerase concentrate were dialyzed against centrifugation buffer minus sucrose for 1 h prior to layering onto gradients. Molecular weight standards of thyroglobulin, M_r 680000, catalase, M_r 230000, and BSA, M_r 68000, each at 1 mg/mL, were layered onto parallel gradients. Gradient tubes were punctured at the bottom after sedimentation, and 29 fractions (0.15 mL) were collected and assayed for polymerase activity in a standard assay. The positions of the standard markers were determined by the Bradford protein assay (Bradford, 1976). Fractions of peak polymerase activity were pooled, concentrated with a Centricon-30 microconcentrator (Amicon Corp.) fitted with a PM-30 membrane, frozen in liquid N_2, and stored at −70 °C.

Gel Filtration. To determine the Stokes radius of the native enzyme, 200 μL of the purified enzyme was applied to a 1 cm × 111 cm calibrated Sephacryl S-300 column (Pharmacia), equilibrated in gel filtration buffer. This buffer contained 10 mM potassium phosphate, pH 7.8, 200 mM $(NH_4)_2SO_4$, 1 mM BME, 10 mM sodium bisulfite, 1 mM EDTA, and 10% glycerol. Ammonium sulfate was included to discourage aggregation. Thyroglobulin, catalase, and BSA were used as calibration standards. Blue dextran ($M_r > 10^6$) and Orange G (M_r 452) were used to determine V_0 and V_T, respectively. Fractions (0.6 mL) were collected at a rate of 6.0 mL/h and immediately assayed for polymerase activity (Figure 2A).

SDS–Polyacrylamide Gel Electrophoresis. Enzyme fractions were denatured and subjected to electrophoresis on a 7% acrylamide gel with 4.5% stacking using 0.4% sodium dodecyl sulfate as described by O'Farrell (1975). Protein was detected by silver staining using a modification of the method of Merril et al. (1981). Densitometry was performed on an LKB 2202 laser densitometer with peak integrator. To determine the protein concentration of the final enzyme fraction, the area

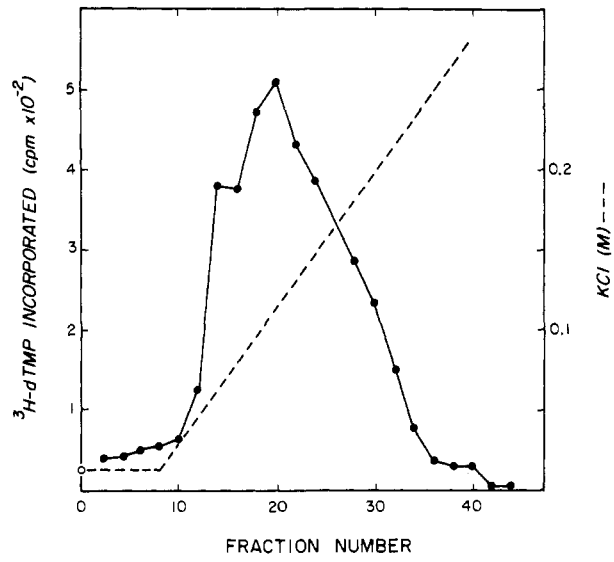

FIGURE 1: Anti-α-polymerase IgG–Affi-Gel column chromatography. A postmicrosomal solution (50 mL) containing 550 mg of protein and 3850 units of α-polymerase activity was mixed with 15 mL of anti-α-polymerase Affi-Gel, and a column was prepared as described under Materials and Methods. The washed column was eluted with a 120-mL linear gradient of 0–300 mM KCl in homogenization buffer. Fractions of 20 drops were collected and assayed for DNA α-polymerase activity in a standard assay. Salt concentrations were determined by measuring the conductivity of every tenth fraction.

under peaks from a laser densitometry scan of the α-polymerase was compared with the areas under peaks produced by electrophoresis of known concentrations of bovine serum albumin in the same gel. High molecular weight standards (Bio-Rad) consisting of myosin, β-galactosidase, phosphorylase B, bovine serum albumin, and ovalbumin were electrophoresed in parallel lanes to determine the molecular weight of enzyme subunits.

Assay. The standard polymerase assay (50 μL) contained 60 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 5 mM BME, 40 μM each of dCTP, dGTP, and dATP, 600 μM activated calf thymus DNA, 4 mM ATP, 500 μg/mL BSA, 25 μM (4 Ci/mmol) [^{3}H]dTTP, and 1–5 μL of enzyme fraction. Reactions were run for 30 min at 37 °C and terminated with 0.5 mL of 12% trichloroacetic acid and 0.5 mL of 30 μg/μL salmon sperm DNA. Samples were filtered onto glass fiber filters and counted in a scintillation counter. Analysis of synthesis on poly(dA)–oligo(dT) was done by centrifugation of reaction products through columns of Sephadex G-50 resin (Penefsky, 1977) under conditions in which labeled reaction precursors were included in the column and short products were excluded.

Results

Purification. DNA α-polymerase eluted as a single peak of polymerase activity between 110 and 120 mM added KCl from a 2 cm × 5 cm column of anti-α-polymerase IgG resin (Figure 1). Fractions of peak activity were pooled and concentrated to yield 187 units. Aliquots of 100 μL (40 units) were further purified by rate-zonal sedimentation in 5–20% sucrose gradients. Fractions of peak polymerase activity were pooled and concentrated to produce a total of 15 units of α-polymerase.

Characterization. As shown in Table II, the enzyme will utilize activated calf thymus DNA and several homopolymers for synthesis. Some synthesis is also observed on bacteriophage fd single-stranded DNA, possibly because of low-level primer contamination.

Table II: Properties of Immunoaffinity-Purified DNA α-Polymerase

reaction conditions[a]	amt of [^{3}H]dTMP incorpd (%)
activated calf thymus DNA	100
poly(dA)–oligo(dT) tailed[b]	38
poly(dA)–$(dT)_{10}$ (30:1)	41
alternating poly(dAdT)	11
bacteriophage fd single-stranded DNA	18
activated calf thymus DNA + 40 μg/mL aphidicolin	4
activated calf thymus DNA + 1 μL of IgG anti-α-polymerase antibody	3
activated calf thymus DNA + 10 mM *N*-ethylmaleimide	0.3
activated calf thymus DNA + 25 mM NaCl	45
activated calf thymus DNA + 50 mM NaCl	20
activated calf thymus DNA + 125 mM NaCl	19
activated calf thymus DNA + 4 mM ATP	369
poly(dA)–oligo(dT) tailed + 4 mM ATP	150
poly(dA)–$(dT)_{10}$ (30:1) + 4 mM ATP	56
bacteriophage fd single-stranded DNA + 200 μM each of ATP, GTP, UTP, and CTP	42

[a] Reaction conditions, other than the DNA substrate used and the additions described above, are given under Materials and Methods. DNA concentrations are 50 μM. [b] A gift of James J. Crute. $(dA)_{4000}$ was tailed at the 3'-hydroxyl end with oligo(dT) by the method of Kato et al. (1967).

The immunoaffinity-purified enzyme displays properties identifying it as DNA α-polymerase. It is inhibited more than 95% by 40 μg/mL aphidicolin, a selective inhibitor of α-polymerase (Ohashi et al., 1978). It is also inhibited more than 95% by the same IgG anti-α-polymerase antibody used in the purification. This antibody has been previously demonstrated to inactivate KB cell DNA α-polymerase (Tanaka et al., 1982). NEM sensitivity is also a characteristic of DNA α-polymerase (Grosse & Krauss, 1981) and has been observed with the immunoaffinity-purified enzyme.

The sensitivity of calf DNA α-polymerase activity to increases in salt concentration has previously been reported (Grosse & Krauss, 1981). Here we show that the immunoaffinity-purified enzyme is similarly sensitive to addition of NaCl.

Consistent with the findings of Wierowski et al. (1983), who used calf A form DNA α-polymerase (Holmes et al., 1974, 1976, 1977), the immunoaffinity-purified enzyme is stimulated as much as 4-fold by the presence of 4 mM ATP. Stimulation is observed on substrates having a poly(dA) template, indicating that ATP stimulation is independent of polymerase-associated primase activity.

Such primase activity is also detectable. Addition of the four ribonucleoside triphosphates to a reaction containing single-stranded fd DNA results in more than doubling of DNA synthetic activity. In a corroborative experiment (not shown), an associated primase activity was demonstrated by the incorporation of ^{3}H-labeled ribonucleotides onto the single-stranded bacteriophage fd DNA template.

Determination of the Native Molecular Weight. The native molecular weight of DNA α-polymerase was calculated from the sedimentation coefficient and Stokes radius by the method of Siegel & Monty (1966). Analysis of gel filtration of α-polymerase by a plot of its elution position relative to standards

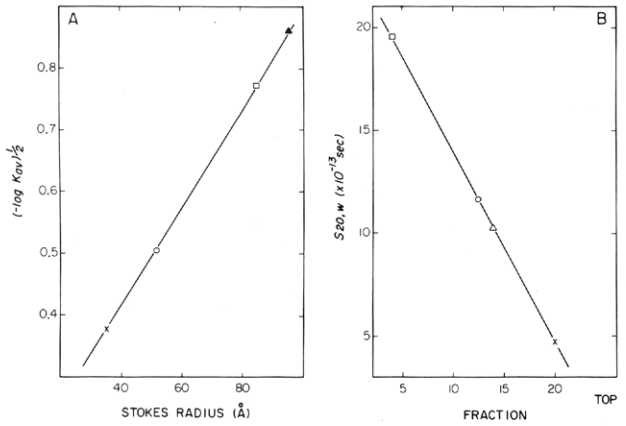

FIGURE 2: (A) Gel filtration of DNA α-polymerase for determination of Stokes radius. DNA α-polymerase (100 μL) (▲) was applied to a Sephacryl S-300 column as described under Materials and Methods and the elution position determined by standard polymerase assay. Standards shown were bovine serum albumin (×), catalase (○), and thyroglobulin (□). (B) Determination of the sedimentation coefficient of DNA α-polymerase. DNA α-polymerase (100 μL) (△) was sedimented through a 5–20% sucrose gradient as described under Materials and Methods. Standards that were sedimented in separate tubes in the same rotor were bovine serum albumin (×), catalase (○), and thyroglobulin (□).

(Figure 2A) yields a Stokes radius of 96 Å. Sedimentation in 5–20% sucrose gradients produces a single peak of polymerase activity at a sedimentation coefficient at 10.2 S (Figure 2B). From these data a M_r of 404 000 and a frictional coefficient of 1.96 were calculated.

Discussion

Immunoaffinity chromatography has proven to be a powerful new method for the purification of calf DNA α-polymerase. It has been incorporated into a three-step isolation scheme with gradient centrifugation as the third step. With this scheme, a high molecular weight polymerase has been purified to near homogeneity, as indicated by SDS gel electrophoresis. The monoclonal IgG used in our procedure (SJK-287-38) is capable of inactivating both human and calf DNA α-polymerases. Affinity of the antibody for KB cell α-polymerase is high ($K_d = 1.4 \times 10^{-9}$; Tanaka et al., 1982). We speculate that its use as a covalently immobilized ligand to bind the calf enzyme results in a reduced affinity that enables elution of calf α-polymerase from the IgG–Affi-Gel at only 110 mM added salt. The 100-fold concentration following elution from this column was found to be critical in maintaining enzymatic activity, since there is a rapid loss of activity at the low protein concentration of the effluent, probably from denaturation. The enzyme was further purified by sucrose gradient sedimentation in the presence of bacitracin and Ampholine. Consistent with the observation of Grosse & Krauss (1981), the presence of Ampholine in the centrifugation buffer is mandatory in order to retain enzymatic activity.

Analysis by SDS gel electrophoresis resolves the α-polymerase into three major subunits of M_r 170 000, 70 000, and 60 000 with minor bands evident at M_r 180 000 and 65 000 (Figure 3). These are similar to previously reported results (Grosse & Krauss, 1981) but represent some subunits of higher molecular weight.

Gel filtration of the enzyme indicated that DNA α-polymerase is larger than M_r 680 000 (thyroglobulin). The Stokes radius is 96 Å. Nonequilibrium sucrose gradient sedimentation showed the $s_{20,w}$ of the molecule to be 10.2×10^{-13} s. The anomalous gel filtration and sedimentation data result in a

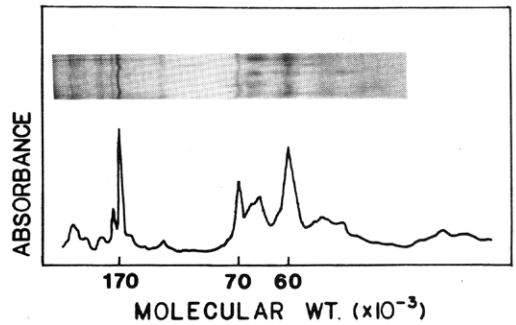

FIGURE 3: Densitometric scan and SDS–polyacrylamide gel. DNA α-polymerase (0.1 μg) was denatured and subjected to electrophoresis in a 7% polyacrylamide gel as described under Materials and Methods. Molecular weights of three major protein peaks are indicated.

frictional coefficient of 1.96, indicating a high degree of molecular asymmetry. A similar prolate ellipsoid polymerase was reported by Holmes & Johnston (1973). Using the correlation of Siegel & Monty (1966), which incorporates both Stokes radius and sedimentation values, we have estimated the native molecular weight to be 404 000.

Another in the collection of antibodies developed by Tanaka et al. (1982), SJK-237-71, has recently been used to purify the inducing antigen: human KB cell DNA α-polymerase (Wang et al., 1984). This IgG does not inactivate the KB polymerase; i.e., binding determinants do not affect the site of catalysis, but the higher affinity of antibody–antigen interaction ($K_d = 3.2 \times 10^{-9}$) necessitates purification of a polymerase–antibody complex. The complex is eluted with 1 M salt, pH 5.5, from a column of protein A–Sepharose. The IgG cannot be separated from the polymerase, so that it must be present in subsequent functional analysis of the polymerase.

The existence of high molecular weight forms of α-polymerase has been reported. Hübscher et al. (1982) reported the isolation of a calf thymus α-polymerase "holoenzyme" but had not purified it to the extent we report. The approach to purification taken here minimizes proteolysis and loss of accessory subunits by employing a minimum number of isolation steps in the presence of protease inhibitors. It has been shown (Holmes et al., 1977; Sauer & Lehman, 1982) that with time proteolysis will occur even in the presence of inhibitors. The use of ion-exchange resins and high salt for purification has been avoided in an attempt to prevent the dissociation of an enzyme complex.

Procedures reported here have not been optimized for yield. Because of ligand saturation of the antibody–Affi-Gel resin, only 10% of a polymerase present in the postmicrosomal supernatant solution is retained and consequently recovered from the affinity column. We have repeated this procedure using greater volumes of postmicrosomal supernatant with this fixed amount of resin. In each case between 170 and 190 total units were eluted. We anticipate that increasing the amount of anti-α-polymerase resin will result in a corresponding increase in total yield. The procedures outlined above, in concert with conventional purification techniques such as DNA–cellulose, could yield material of higher specific activity. These methods are in progress. Because of the mild conditions employed, it is possible that we have isolated a form of calf DNA α-polymerase that approximates the enzyme in vivo.

Acknowledgments

We thank Drs. Teresa S. F. Wang and David Korn for testing the specificity of human KB cell anti-α-polymerase for calf polymerases. We also thank Dr. Samuel Wilson for helpful discussions and for IgM supplied in the preliminary

development of this methodology and James J. Crute for useful suggestions.

Registry No. DNA polymerase, 9012-90-2.

References

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Grosse, F., & Krauss, G. (1981) *Biochemistry 20*, 5470–5475.
Grummt, F., Waltl, G., Jantzen, H., Hamprecht, K., Huebscher, U., & Kuenzie, C. C. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6081–6085.
Harwell, L., Kappler, J. W., & Marrack, P. (1976) *J. Immunol. 116*, 1379.
Holmes, A. M., & Johnston, I. R. (1973) *FEBS Lett. 29*, 1–6.
Holmes, A. M., Hesslewood, I. P., & Johnston, I. R. (1974) *Eur. J. Biochem. 43*, 487–499.
Holmes, A.M., Hesslewood, I. P., & Johnston, I. R. (1976) *Eur. J. Biochem. 62*, 229–235.
Holmes, A. M., Hesslewood, I. P., Wickremasinghe, R. G., & Johnston, I. R. (1977) *Biochem. Soc. Symp. 42*, 17–36.
Hübscher, U. (1983) *EMBO J. 2*, 133–136.
Hübscher, U., Gerschwiler, P., & McMaster, G. K. (1982) *EMBO J. 1*, 1513–1519.
Kato, K., Goncalves, J. M., Houts, G. E., & Bollum, F. J. (1967) *J. Biol. Chem. 242*, 2780–2789.
Masaki, S., Koiwai, O., & Yoshida, S. (1982) *J. Biol. Chem. 257*, 7172–7177.
McKune, K., & Holmes, A. M. (1979) *Nucleic Acids Res. 6*, 3341–3353.
Merril, C. R., Goldman, D., Sedman, S. A., & Ebert, M. H. (1981) *Science (Washington, D.C.) 211*, 1437–1438.
O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007.
Ohashi, M., Taguchi, T., & Ikegami, S. (1978) *Biochem. Biophys. Res. Commun. 82*, 1084–1090.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.
Sauer, B., & Lehman, I. R. (1982) *J. Biol. Chem. 257*, 12394–12398.
Siegel, L. M., & Monty, K. J. (1966) *Biochim. Biophys. Acta 112*, 346–362.
Tanaka, S., Hu, S., Wang, T. S., & Korn, D. (1982) *J. Biol. Chem. 257*, 8386–8390.
Wang, T. S. F., Hu, S. Z., & Korn, D. (1984) *J. Biol. Chem. 259*, 1854–1865.
Wierowski, J. V., Lawton, K. G., Hockensmith, J. W., & Bambara, R. B. (1983) *J. Biol. Chem. 258*, 6250–6254.

Articles

Escherichia coli DNA Topoisomerase III: Purification and Characterization of a New Type I Enzyme†

Kalkunte S. Srivenugopal, Daniel Lockshon, and David R. Morris*

ABSTRACT: A new topoisomerase capable of relaxing negatively supercoiled DNA in *Escherichia coli* has been identified during chromatography on novobiocin–Sepharose. A simple and reproducible purification procedure is described to obtain this enzyme, called topoisomerase III (topo III), in a homogeneous form. The protein is a single polypeptide with a molecular weight of 74000 ± 2000 and is a type I topoisomerase, changing the linking number of DNA circles in steps of one. It is present in deletion strains lacking the *top*A gene and further differs from the well-studied topoisomerase I (ω protein; Eco topo I) in (1) its requirement for K^+ in addition to Mg^{2+} to exhibit optimal activity and (2) its affinity to novobiocin–Sepharose. Positively supercoiled DNA is not relaxed during exposure to the enzyme. Topo III has no ATPase activity, and ATP does not show any discernible effect on the reduction of superhelical turns. The purified topoisomerase has no supercoiling activity and is unaffected by high concentrations of oxolinic acid and novobiocin in the relaxing reaction. Single-stranded DNA and spermidine strongly inhibit the topoisomerase activity.

DNA supercoiling, now recognized as a crucial factor in bacterial gene expression and in prokaryotic chromosome replication (Smith, 1981; Gellert, 1981a,b), is controlled by a ubiquitous and varied class of multifunctional enzymes known as topoisomerases (Wang & Liu, 1979). A change in the linking number (Lk) of closed circular DNA molecules by these enzymes is mediated through transient single-strand breaks (ΔLk = 1; type I enzymes, with no ATP requirement) or transient double-strand breaks (ΔLk = 2; ATP-dependent type II topoisomerases) followed by a strand-passing event (Liu et al., 1980). Hitherto, in *Escherichia coli*, three distinct and well-defined topoisomerase activities have been described. *E. coli* topoisomerase I, familiar in the literature as ω protein, is a 110000-dalton polypeptide (Wang, 1973) encoded by the *top*A gene mapped at 28 min near *cys*B (Sternglanz et al., 1981; Trucksis & Depew, 1981). It is a type I enzyme (Brown & Cozzarelli, 1979) that relaxes negatively supercoiled DNA efficiently but is totally inactive on overwound DNA (Wang, 1971). This enzyme appears to be dispensable for the growth of *E. coli*, but deletion strains lacking *top*A accumulate compensatory gyrase mutations, leading to reduced supercoiling in vivo (DiNardo et al., 1982; Pruss et al., 1982). These secondary mutations seem to modulate DNA gyrase by reducing its content and altering its catalytic properties (Gellert et al., 1982), thus suggesting an important role of topo I in regulation of supercoiling.

† From the Department of Biochemistry, University of Washington, Seattle, Washington 98195. *Received December 14, 1983.* This investigation was supported by a National Science Foundation grant (PCM-8301985) to D.R.M. A preliminary report of this work was presented in part at the Pacific Slope Biochemical Conference, Seattle, WA, June 1982.

0006-2960/84/0423-1899$01.50/0