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CHROMATOGRAPHIC RESOLUTION OF TWO DNA POLYMERASE ACTIVITIES FROM BLOODSTREAM FORMS OF TRYPANOSOMA BRUCEI: DIFFERENTIAL RESPONSES TO EXOGENOUS POLYAMINE ADDITION

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SUMMARY. A single peak of DNA polymerase activity from extracts of T. brucei, obtained by DEAE-cellulose and phosphocellulose ion-exchange chromatography, was resolved into two peaks differing in KCl concentration necessary to elute them from a DNA-agarose column. Peak I (eluting at 0.2 M KCl) and Peak II (eluting at 0.4 M KCl), differed in response to increasing KCl concentrations, although both functioned optimally with Mg^{2+} as divalent cation when DNA synthesis was directed either by activated DNA or poly $(dC)\cdot(dG)_{12-18}$. Due to the potential significance of polyamines in the metabolism of T. brucei, the effect of exogenous polyamine on rates of DNA synthesis by the peak I and II enzymes was compared with that of murine DNA polymerase alpha. Only the peak I enzyme was significantly stimulated (up to 4-fold) by the biologically active polyamines spermine and spermidine at physiological concentrations. The response of the peak I enzyme resembled that of the alpha polymerase. This result suggests a possible functional difference between peak I and II enzymes, as well as a potential target site for trypanocidal drug development.

Mammalian cells are thought to contain three DNA polymerases, alpha, beta, and gama, distinguishable biochemically and immunologically (1); only the alpha is thought to be the true replicative DNA polymerase. In simple eukaryotes, the major DNA polymerase activity resembles that of the mammalian DNA polymerase alpha (2-9), although analysis of yeast (2-4) and Euglena gracilis (6,7) DNA polymerase activity chromatographically resolved two enzyme forms identical in native molecular weight but differing in biochemical properties. Characterization of DNA polymerases of pathogenic Trypanosomatidae is therefore important phylogenetically as well as in identifying potential targets for chemotherapy. An initial report (10) recently indicated that T. brucei contained a single major DNA polymerase activity resembling mammalian DNA polymerase alpha. We describe here for the first time the chromatographic resolution of two DNA polymerase activities from T. brucei bloodstream

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forms which differ significantly in their response to exogenous monovalent cation and to physiological levels of exogenous polyamines.

MATERIALS AND METHODS

Reagents: [^{3}H]dGTP was bought from New England Nuclear. Unlabeled deoxynucleoside triphosphates and poly (dC)·(dG)$_{12-18}$ were from P-L Biochemicals, Inc. The ratio of poly (dC) to oligo dG (mass ratio) was 1:1. Activated DNA was prepared as described by Aposhian and Kornberg (11). Heat-denatured calf thymus DNA was coupled to cyanogen bromide-activated agarose to form DNA-agarose as described for polycytidylate-agarose (11). Phosphocellulose and DEAE-cellulose were from Whatman, Inc.

Cell fractionation and enzyme purification: Bloodstream forms of T. brucei (EATRO isolate 110) were isolated from 72 h infections in rats (13). Cells were disrupted using an Aminco pressure cell (14,000 psi/15 min.) and the subsequent extract, prepared in 0.1 M Tris-HCl, pH 7.8, and 20% (v/v) glycerol was fractionated by centrifugation at 4° in the Sorvall SS-34 rotor at 10,000 x g for 1 h. The pellet was discarded, and the resulting supernatant fraction was made 1% (v/v) in respect to Triton X-100 and 0.4 M in respect to KCl. After gentle mixing the solution was centrifuged in the Beckman SW41 rotor at 100,000 x g for 45 min. at 4°. Any resulting pellet was discarded, and the supernatant fraction (S100) was then passed through a DEAE-cellulose column equilibrated with buffer containing 0.05 M KCl, 1 mM dithiothreitol, 0.4 M KCl and 10% glycerol to remove any associated nucleic acids. The DEAE flow-through fraction from the S100 was then diluted 4-fold with column wash buffer lacking KCl, and passed through a phosphocellulose column previously equilibrated with DEAE-cellulose column wash buffer containing 0.1 M KCl. Bound enzyme was eluted with a linear gradient of increasing KCl concentration; the major DNA polymerase peak fraction eluted at 0.3 M KCl as a single peak of activity. The phosphocellulose column fractions containing the peak DNA polymerase activity were pooled, diluted 10-fold with buffer lacking KCl, and passed over a DNA-agarose column at a flow rate of 18 ml/h. A linear KCl gradient was also used to elute bound enzyme from the DNA-agarose. The resulting two peaks of DNA polymerase activity, assayed using poly (dC)·(dG)$_{12-18}$, were pooled separately and used immediately or were stored at -70° in the presence of 0.01% (w/v) bovine serum albumin monomer. Murine DNA polymerase alpha was purified from AKR mouse thymus tissue by procedures described for isolating DNA polymerase from the cytoplasm of JLSC-10 tissue culture cells (12), except that DNA-agarose was used as the initial chromatography matrix followed by a phosphocellulose fraction step.

DNA polymerase assays: Reactions were in a total volume of 0.1 ml and consisted of 50 mM Tris-HCl, pH 7.8, 1 mM dithiothreitol and 10 µg of bovine serum albumin monomer together with 5 mM $MgCl_2$. Poly (dC)·(dG)$_{12-18}$ was used at 5 µg/ml, and activated DNA at 20 µg/ml final concentration (14). Poly (dC)·(dG)$_{12-18}$ stock solutions were annealed before use by heating them to 85° for 15 min. and then allowing them to cool slowly to room temperature. [^{3}H]dGTP (5 ci/mMole) was used at final concentration of 10 µM for both DNA-directed and synthetic template-primer-directed synthesis. Unlabeled deoxynucleoside triphosphates, when present, were added to a final concentration of 200 µM. Unless otherwise stated, reactions were initiated by the addition of enzyme fractions and were incubated at 37° for 20 min. Reactions were ended by addition of trichloroacetic acid solution containing Na pyrophosphate. Acid-insoluble material was collected by vacuum filtration onto Whatman glass fiber filters (GF/B) and, after drying, radioactivity was determined using toluene-based scintillation fluid in a scintillation counter (15).

RESULTS

Resolution of two peaks of DNA polymerase activity. In agreement with a report by Dube et al. (10), T. brucei DNA polymerase activity binding to phospho-

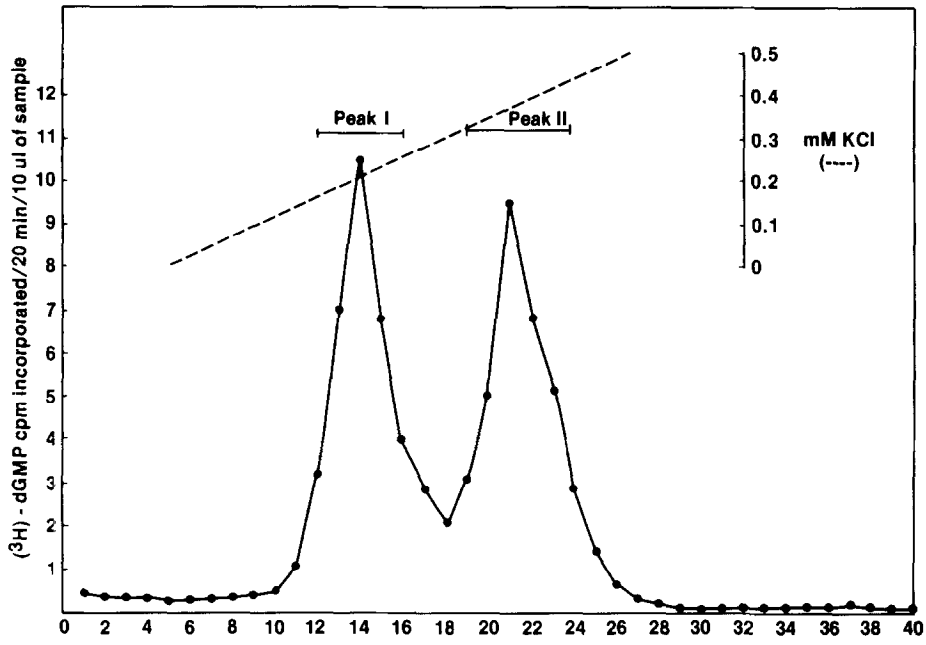

Fig. 1. Elution of DNA polymerase activity from a DNA-agarose column. The column was prepared and the T. brucei extract (containing 180 mg of protein) initially run through DEAE-cellulose and phosphocellulose as described in Materials and Methods. The DNA-agarose column was washed with a linear gradient of 0-0.8 M KCl to elute bound-enzyme activity. Fractions of 0.8 ml volume were collected and 10 µl of each fraction assayed for DNA polymerase activity as described in Materials and Methods.

cellulose eluted as a single major peak of activity at 0.3 M KCl (data not shown). Analysis of the apparent molecular weight of the DNA polymerase activity by sedimentation velocity experiments yielded a single major peak of polymerase activity sedimenting at 6-7S, as found previously (10). The sedimentation velocity did not vary appreciably in the presence or absence of 0.4 M KCl. In an effort to further purify the T. brucei DNA polymerase activity, the enzyme peak from the phosphocellulose column was passed through a DNA-agarose column. All enzyme activity bound to the DNA-agarose and elution of enzyme activity was carried out with increasing KCl concentration. The resulting fractions were assayed with poly (dC)·$(dG)_{12-18}$ as template primer. Two peaks of DNA polymerase activity eluted from the DNA-agarose column (Fig. 1), one at ∿0.2 M KCl and the other at 0.4 M KCl. Rechromatography of the 0.2 M KCl peak (Peak I) and 0.4 M KCl peak (Peak II) on DNA agarose resulted in their elution at the same initial concentration of KCl

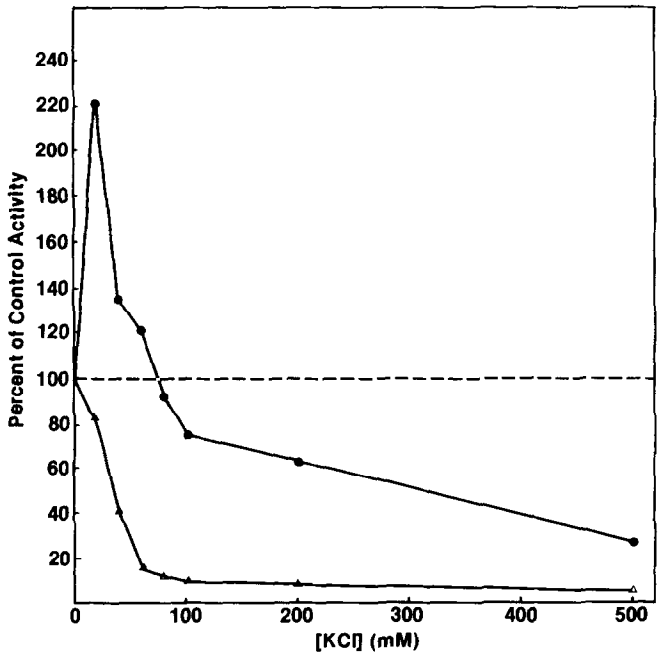

Fig. 2. Effect of increasing KCl on poly (dC)-directed DNA synthesis by T. brucei peak I and II DNA polymerase activities. Assay mixture containing enzyme was added to assay tubes containing various KCl concentrations. (Δ) = Peak I enzyme; (●) = Peak II enzyme. Control c.p.m. values in all experiments ranged from 5 to 10,000.

(data not shown). Final yields of enzyme activity were ∿20%, with ∿ a 500-fold increase in initial specific activity (data not shown).

General characteristics and response to KCl. Activated DNA and poly (dC)·$(dG)_{12-18}$ were the template-primers optimally utilized by both the peak I and II enzymes, with Mg^{2+} the preferred divalent cation with an optimal concentration of 5 mM for both template-primers. Poly (A)·$(dT)_{12-18}$, poly (dA)·$(dT)_{12-18}$, and poly (C)·$(dG)_{12-18}$ could not be used to direct DNA synthesis to any significant degree in the presence of Mg^{2+}. Since these template-primer and divalent cation preferences are similar to those of DNA polymerase alpha of mammalian cells (1), the response of the peak I and II enzymes to increasing KCl concentrations, which inhibit the alpha polymerase (1), was tested (Fig. 2). The peak I polymerase activity was inhibited by KCl concentrations as low as 20 mM, whereas peak II enzyme activity was significantly stimulated by <50 mM KCl. Peak II polymerase activity was inhibited by KCl >100 mM, although it was far less sensitive to

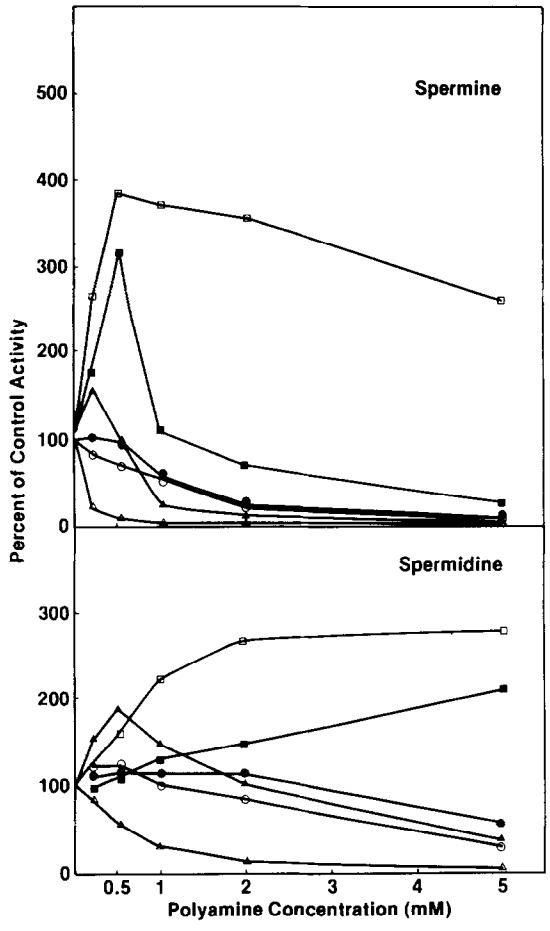

Fig. 3. Effect of increasing spermine and spermidine on DNA- and poly (dC)-directed DNA synthesis by T. brucei DNA-agarose peak I (◑) and peak II (■) and murine DNA polymerase alpha (▲) DNA polymerase activities. Filled symbols = DNA-directed reaction mixtures; open symbols = poly (dC)-directed reaction mixtures (see Materials and Methods). The presence of all four deoxynucleoside triphosphates in poly (dC)-directed reactions did not change these results.

inhibition than the peak I enzyme. In this respect therefore the peak I enzyme of T. brucei more closely resembles the alpha DNA polymerase (1) than does the peak II enzyme.

DNA polymerase responses to exogenously added polyamines. Because of the potential significance of polyamines both as activators of a critical respiratory enzyme in T. brucei (16) and as possible targets of cationic trypanocides (13, 17, 18), we determined whether exogenous polyamine affected DNA synthesis by the peak I and peak II enzymes and compared their responses with murine DNA polymerase alpha (Figs. 3 and 4). These studies were carried out with both activated DNA

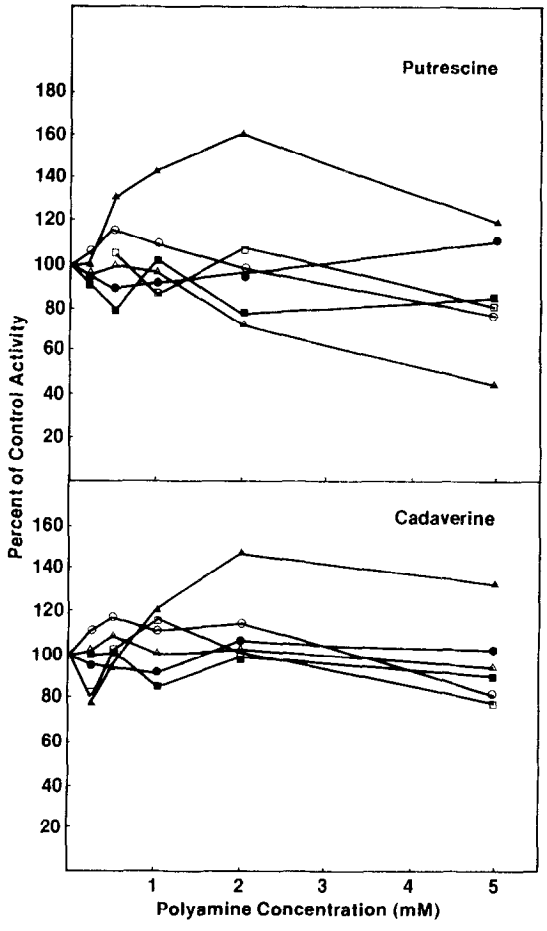

Fig. 4. Effect of increasing cadaverine and putrescine on poly (dC) and DNA-directed DNA synthesis by T. brucei peak I and II enzymatic activities and murine DNA polymerase alpha. Symbols the same as in the legend for Fig. 3.

and poly (dC)·$(dG)_{12-18}$ as template-primers. Responses to increasing spermine and spermidine are shown in Fig. 3. In agreement with a previous report (19), the reaction of DNA polymerase alpha to polyamine addition varied with the template primer. Both spermidine and spermine inhibited alpha-catalysed DNA synthesis on poly (dC) template, but synthesis on activated DNA was stimulated ∿2-fold by polyamine concentrations <0.5 mM, and inhibited at higher concentrations. T. brucei peak I and II DNA polymerase activities differed dramatically in responses to spermine and spermidine: peak I enzyme was either unaffected or inhibited by increasing polyamine concentration, regardless of the template-primer used. In

contrast, spermine at 0.5 mM stimulated poly (dC) and DNA-directed synthesis by the peak II enzyme 3- to 4-fold (Fig. 3), and increasing concentrations of spermidine also stimulated activity by this enzyme on both template-primers. Although optimal concentrations for observed stimulation by spermidine were 2 mM or more, T. brucei blood forms possess high concentrations of this polyamine (20). Increasing concentrations of putrescine (Fig. 4) affected the DNA polymerase alpha from mice in a manner similar to spermidine -- stimulation of DNA-directed synthesis and inhibition of poly (dC)-directed synthesis; increasing concentrations of cadaverine stimulated alpha-catalysed DNA-directed synthesis slightly, but did not significantly affect poly (dC)-directed synthesis. Neither increasing concentrations of putrescine nor cadaverine significantly affected DNA synthesis by either the peak I or peak II T. brucei enzyme activities (<20% stimulation or inhibition) (Fig. 4).

DISCUSSION

We have shown that chromatography on a DNA-agarose matrix can resolve two peaks of DNA polymerase from T. brucei, and that the two peaks of activity are further distinguishable by response to exogenous KCl or polyamine (Figs. 2-4). These two activities were not resolved by sedimentation velocity analysis, DEAE- or phosphocellulose chromatography, template-primer preference, or sensitivity to sulfhydryl reagents (10, also our unpublished observations). The peak I and peak II enzyme activities also have similar broad pH optima, appear about equal in quantity within cell extracts, and their proportion to each other is unaltered by inclusion of a protease inhibitor such as Trasylol (12) in the extraction buffers (unpublished observations).

Euglena gracilis possesses two forms of high-molecular weight DNA polymerase, differing only in position of elution from DEAE-cellulose, mobility on polyacrylamide gel electrophoresis, and presence of associated nuclease activity (6,7). Tetrahymena pyriformis may also contain two forms of DNA polymerase (5,8). None of the polymerase activity of these protozoa were considered to be of the beta type (9). The two high-molecular weight DNA polymerases of bakers' yeast differ in immunological cross-reactivity and associated nuclease activity, and are

separable by various chromatographic methods (2-4). The necessity of utilizing DNA-agarose for separation of the two T. brucei enzyme activities suggests that they may differ less in their structures than do the polymerases of the above mentioned organisms. The assignment of peaks I and II as truly separate entities, however, awaits their purification to homogeneity and their immunologic characterization. From our studies, it appears that the peak I enzyme is more "alpha-like" in its properties than the peak II enzyme. The specific polyamine stimulation of only the peak II enzyme may be a significant observation since these trypanosomatids may contain a uniquely polyamine-activated component in their energy-producing terminal respiratory pathway (13,16). Continued analysis of the polyamine activation of T. brucei peak II DNA polymerase should clarify its role in cell metabolism and may provide further evidence for a chemotherapeutically exploitable difference between host and parasite metabolism.

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