Inhibition of DNA Polymerase from L1210 Murine Leukemia by a Sulfhydryl Reagent from Agaricus bisporus

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SUMMARY

The 490 quinone, a natural sulfhydryl-arylating reagent from the mushroom, Agaricus bisporus, markedly inhibited L1210 murine leukemia DNA polymerase α while resulting in little inhibition of DNA polymerase β from this source. This quinone was more strongly inhibitory than p-chloromercuribenzoate or N-ethylmaleimide and was less readily neutralized by sulfhydryl-containing molecules such as dithioerythritol. Preliminary experiments indicate that DNA protects DNA polymerase α from inhibition by the 490 quinone. The inhibition of DNA synthesis by quinone 490 may contribute significantly to the cytotoxicity of this compound and to the potential of γ-L-glutaminyl-4-hydroxybenzene as an antitumor agent.

INTRODUCTION

Oxidative products of the colorless phenol, γ-L-glutaminyl-4-hydroxybenzene, are apparently responsible for the induction of the dormant state in the spores of the mushroom, A. bisporus (14). One product, a pink quinone with absorbance maximum at 490 nm, can be produced from the phenol by oxidation with tyrosinase or isolated directly from the mushroom. It is a strong sulfhydryl reagent which, by arylating cysteiny1 residues in succinic dehydrogenase, α-ketoglutaric dehydrogenase, and pyruvic dehydrogenase, markedly inhibits energy production in the presporulating zygote (14, 16). Although reported to be γ-L-glutaminyl-3,4-benzoquinone, its molecular structure remains uncertain (15). Mild oxidation of the 490 quinone yields a brown compound with absorption measured at 360 nm. The 360 compound inhibits ribosomal protein synthesis and, in Escherichia coli, RNA polymerase (8, 13). Thus the cryptobiotic state of the spore apparently results from the additive effects of the 490 and 360 compounds on energy, protein, and nucleic acid synthesis.

These inhibitors are cross-reactive with prokaryotic and eukaryotic cells (12, 13). The 490 quinone inhibits energy production by rat liver mitochondria (14), and the 360 compound notably retards protein synthesis by ribosomes of rat liver and E. coli (8, 13). Together, they inhibit the incorporation of [3H]thymidine, uracil, and amino acids into DNA, RNA, and protein by a variety of bacteria and murine ascites tumor cells (12, 13). While the 490 and 360 inhibitors are too unstable for in vivo testing, the precursor of these inhibitors, γ-L-glutaminyl-4-hydroxybenzene, is stable and has demonstrated an in vivo effect with the B16 murine melanoma. The potential for this compound in the therapy of melanoma derives from its specificity for tyrosinase-containing cells and its apparent lack of systemic toxicity.

Evidence for a primary effect on DNA synthesis by the 490 quinone was contained in the observation that exposure of murine tumor cells to the 490 quinone in tissue culture resulted in greater inhibition of [3H]thymidine incorporation than that of [3H]uracil or 3H-labeled amino acids (12). To elucidate the mechanism of this inhibition, 2 species of DNA polymerase were isolated from L1210 leukemia cells and evaluated for sensitivity to the purified 490 quinone.

MATERIALS AND METHODS

Isolation of the 490 Quinone. As outlined previously (12), gill tissues from A. bisporus were homogenized in water, and the supernatant was passed through 5×100-cm columns of Sephadex G-25 fine (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), eluting with water. The 3rd colored band to elute after the protein and ion peaks, the pink 490 quinone, with a molar extinction coefficient at 490 nm of 10,000 (16), was saturated with nitrogen, shell-frozen, and stored in liquid nitrogen, where it was stable for at least 2 years. The 490 quinone so isolated was free from contamination by ions or other organic compounds.

Preparation of Templates. Calf thymus DNA was purchased from Sigma Chemical Co. (St. Louis, Mo.), and poly[d(A-T)]* was from Biopolymers (Chagrin Falls, Ohio). Denaturation of DNA was achieved by heating at 100°C for 10 min, followed by rapid cooling. Activation of calf thymus DNA and poly[d(A-T)] followed the method of Olson and Koerner (9) utilizing dilutions of pancreatic DNase (Worthington Biochemicals, Freehold, N. J.) which gave optimal activity for L1210 DNA polymerase α after 15 min of digestion at 37°C. Partial digestion of poly[d(A-T)] by pancreatic DNase resulted in a doubling of its activity with polymerase α but caused a slight reduction in its activity with polymerase β.

* The abbreviations used are: poly[d(A-T)]; DTE, dithioerythritol; NEM, N-ethylmaleimide; PCMB, p-chloromercuribenzoate.

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2 To whom requests for reprints should be addressed.

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Isolation of DNA Polymerases. The L1210 leukemia was maintained in DBA/2J mice from the Jackson Laboratory, Bar Harbor, Maine, by the i.p. injection of 250,000 cells at 8-day intervals. The method outlined by Smith and Gallo (11) for human lymphocyte polymerase II (β) was followed for the isolation of both L1210 DNA polymerases. Briefly, 12 g of L1210 cells were harvested from 100 mice, extracted twice with 0.8 M KCl buffer, and dialyzed, and the supernatants were passed through a DEAE-cellulose column to remove nucleic acids, eluting with 0.3 M KCl. Fractions containing DNA polymerase activity were pooled, dialyzed, absorbed onto a phosphocellulose column, and eluted with a buffered KCl gradient from 0.1 to 0.7 M. The 2 peaks of DNA polymerase activity were dialyzed against 50% glycerol with 50 mM Tris, 1 mM DTE, and 1 mM EDTA, pH 7.8, and stored at −20°C.

Assays for DNA Polymerase Activity. DNA polymerase assays followed methods outlined by Hurwitz and Leis (5). Final volume of 50 μl contained 2.5 μg of activated calf thymus DNA or 0.02 A₄₅₀ units of poly[d(A-T)]; 50 mM Tris-HCl (pH 8.0); 0.8 to 2.2 mM MgCl₂; 25 or 50 mM KCl; 5 mM DTE; 80 μM dATP, dCTP, and dGTP (Sigma); 7 μM [³H]TTP (12,600 dpm/μmole; New England Nuclear, Boston, Mass.); and 1 to 10 μl of DNA polymerase. After 30 min at 37°C, the reaction was terminated by placing the tubes on ice and adding 0.1 ml 0.1 M NaPP₃, 1 drop of heat-denatured salmon sperm DNA (Sigma; 30 A₄₅₀ units/ml), and 5 ml of 5% trichloroacetic acid. The precipitate was collected on Whatman GF/C glass fiber filters, washed with 15 ml of 1% trichloroacetic acid and 3 ml of 95% ethanol, dried, and digested with 0.5 ml of NCS and counted in 15 ml of toluene with POPOP (4 g/liter) and POPOP (0.1 g/liter) by the procedure outlined by Schrier and Wilson (10). Counting efficiency for tritium, using a Beckman LS 150 scintillation spectrometer, averaged 48%. One polymerase unit was defined as the amount of enzyme catalyzing the incorporation of 1 n mole of deoxyribonucleoside monophosphate into DNA per 30 min at 37°C.

Protein Quantitation. Protein concentration was determined as described by Hartree (4) using bovine serum albumin standards dissolved in the appropriate concentrations of glycerol.

RESULTS

Two peaks of DNA polymerase activity were resolved after phosphocellulose chromatography. The 1st eluted at KCl concentrations between 0 and 0.2 M; the 2nd, between 0.3 and 0.5 M. As estimated by glycerol gradient centrifugation, peak I had a molecular weight of 176,000, while peak II had a molecular weight of 43,000 (Chart 1). By convention, the 1st was identified as DNA polymerase α and the 2nd as DNA polymerase β (17).

DNA polymerase α had a Mg⁺⁺ optimum of 1.5 mM, with either an activated calf thymus DNA or poly[d(A-T)] template and demonstrated highest activity with 25 mM KCl and Tris buffer at pH 8.0. Optimal conditions for DNA polymerase β were Mg⁺⁺ concentrations of 2.5 mM with activated DNA or 0.8 mM with poly[d(A-T)], 50 mM KCl, and glycinate buffer, pH 10, or Tris buffer at pH 8 to 8.5.

Micromolar concentrations of the 490 quinone caused progressive inhibition of L1210 polymerase α, while polymerase β was only minimally affected (Chart 2). The inhibition of polymerase α was enhanced by preincubating the 490 quinone with the enzyme for 15 min but not by preincubation of the 490 quinone with DNA and deoxynucleoside triphosphates (Table 1). The greater degree of inhibition by 490 quinone in the latter experiment is a reflection of a 10-fold greater dilution of DNA polymerase α. These data provided evidence that the inhibition of L1210 DNA polymerase α by 490 quinone was directed toward the enzyme rather than DNA.

Whereas dATP in concentrations from 10⁻⁴ to 10⁻⁴ M resulted in no protection of polymerase α from 490 inhibition, increasing concentrations of activated calf thymus DNA resulted in a reduction in the amount of inhibition achieved by the 490 quinone (Chart 3). If the sigmoidal shape of the curves in Chart 3 is taken as an artifact of the logarithmic scale, one could conclude that DNA and the 490 quinone compete for the same binding site on DNA polymerase α. Another interpretation of these data would suggest that a critical concentration of DNA induces a confor-
Effect of preincubation on inhibition of L1210 DNA polymerase α by 490 quinone

The 1st set of tubes was assayed without preincubation. In the 2nd, all components except DNA polymerase were preincubated for 10 min at 37° before the enzyme was added. In the 3rd set of tubes, activated calf thymus DNA and dNTP were omitted until after 10 min at 37°. Each assay contained 1 μL (0.565 μg protein) of DNA polymerase α.

<table>
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<tr>
<th>Components preincubated with 490</th>
<th>1 mM DTE</th>
<th>2 μM DTE</th>
<th>Units/mg protein</th>
<th>% Specific activity</th>
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<tr>
<td>None</td>
<td>+</td>
<td>−</td>
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<td>+</td>
<td>0.34</td>
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</tr>
<tr>
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<td>−</td>
<td>+</td>
<td>0.04</td>
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</table>

* dNTP, deoxynucleoside triphosphate.

Preincubation of the 490 quinone with polymerase α resulted in greater inhibition of the enzyme. When preincubation of 3 to 12 min was followed by the addition of sufficient of enzyme protein added was reduced to 10% of that in the previous experiment and the remaining DTE was removed by dialysis against glycerol buffer. As an inhibitor of L1210 DNA polymerase α in the absence of DTE, the 490 quinone had the same activity as PCMB but was several times more effective than NEM (Chart 5). While 1 mM DTE neutralized NEM and PCMB as DNA polymerase inhibitors, neutralization of the 490 quinone occurred only with great excesses of DTE (see Table 1). With ratios of DTE to 490 quinone of 50 or 100 to 1, little neutralization was achieved. This suggested either that the 490 quinone binds to DTE and retains the capacity to inhibit the enzyme, or that it possesses an unexpected affinity for the active site of L1210 DNA polymerase α.

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DTE (final concentration, 4 mM) to neutralize the 490 quinone, PCMB, or NEM, before the addition of activated DNA and deoxynucleoside triphosphates, progressive inhibition was achieved with all 3 sulfhydryl reagents (Chart 6). The inhibition by NEM and the 490 quinone appeared to be independent of whether a massive excess of DTE was added. This was consistent with irreversible covalent binding to the enzyme. On the other hand, the binding of polymerase α by PCMB appeared to be partially reversed by the addition of DTE.

**DISCUSSION**

A variety of sulfhydryl reagents have been evaluated for possible use as antitumor agents (6). It is presumed that such compounds are likely to act at many sites within the cell, but the consistent susceptibility of the α class mammalian DNA polymerases to these agents (7) points to inhibition of these enzymes as a critical mode of action. There is considerable evidence that DNA polymerase α plays a more major role in DNA replication than DNA polymerase β. Thus, inhibition of the former is presumed to have greater cytological significance (1).

The present studies disclose that a naturally occurring sulfhydryl-aryating reagent from the mushroom, A. bisporus, markedly inhibits DNA polymerase α from the murine L1210 leukemia while minimally inhibiting DNA polymerase β from this source. The actions of the 490 quinone parallel other sulfhydryl reagents, PCMB and NEM. These observations indicate that the bond between the quinone and cysteinyl residues of DNA polymerase α is covalent (16), as with NEM (3), and not reversible, as is the ionic bond formed by PCMB (2).

It is clear from these studies that the 490 quinone is, on one hand, more effective than PCMB or NEM as an inhibitor of DNA polymerase α, and, on the other, less readily neutralized by compounds containing free sulfhydryl groups, such as DTE. The latter property seems best explained by the presence of a specific binding site on DNA polymerase α for the 490 quinone. The preliminary experiments reported in this paper suggest a relationship between the locus of inhibition by the 490 quinone and the binding site for DNA on L1210 DNA polymerase α, since this enzyme is protected from 490 quinone inhibition by DNA but not by deoxynucleoside triphosphates. Such inhibition should markedly retard DNA synthesis and significantly modify the capacity of a cell for replication.

**REFERENCES**


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