Adriamycin Interactions with T4 DNA Polymerase

TWO MODES OF TEMPLATE-MEDIATED INHIBITION*

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We examined the effect of adriamycin on kinetics of DNA synthesis catalyzed by DNA polymerase purified from bacteriophage T4-infected Escherichia coli. Two distinct modes of enzyme inhibition occur: uncompetitive and competitive at "low" and "high" drug:DNA nucleotide molar ratios, respectively. Competitive inhibition is not observed unless an unblocked amino group is present on the sugar (daunosamine) moiety. A model is proposed to relate the enzyme inhibition kinetics to intercalative and ionic binding of adriamycin to DNA.

The anthracycline glycosides adriamycin and daunorubicin are clinically important anticancer drugs which bind avidly to DNA and inhibit DNA and RNA synthesis both in cell free systems (1, 2) and in intact cells (3, 4). This inhibition of nucleic acid synthesis might select preferentially against cell proliferation in tumors. Studies on the structure activity relationships of these anthracyclines reveal that an unblocked amino group is necessary for optimal binding to DNA (3). Evidence based on x-ray crystallography (5) and sedimentation velocity experiments (6) suggests that the primary interaction of daunorubicin and DNA is through intercalation between the parallel stacked bases in regions of stable double helical structure. The presence of a positively charged amino sugar may facilitate this intercalative binding but may also permit ionic binding (6-8) of the drug to single-stranded regions of DNA.

We observed that adriamycin, daunorubicin, and two other intercalating agents, ethidium bromide and 9-aminoacridine, exert a differential effect on the rates of DNA synthesis by bacteriophage T4 mutant (L56), wild type (T4D), and antimmunator (L141) DNA polymerases (9). The present studies are designed to investigate structure-activity relationships between the anthracycline analogs and purified mutant and wild type T4 polymerases in an attempt to elucidate further the mechanism of polymerase inhibition by anthracyclines.

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EXPERIMENTAL PROCEDURES

Nucleotides and Anthracycline Analogos—All nonradioactive nucleotides were purchased from P-L Biochemicals, and radioactive nucleotides were purchased from Schwarz/Mann. 2-Amino-6-chloropurine, purchased from Torra-Marine Bioresarch, La Jolla, Calif., was converted to 2-amino[3H]purine through catalytic reduction by ICN Pharmaceuticals, Inc., Irvine, Calif. 2-Amino[3H]purine deoxyxynucleotide triphosphate was synthesized enzymatically from the free base as described in Ref. 10. Adriamycin·HCl was obtained from Farmitalia Co., Milan, Italy. Daunorubicin·HCl, daunorubincil, daunorubicin benzoylhydrazine, N-acetyl daunorubicin, daunorubicin aglycone, and adriamycin octanoate were provided by Drug Research and Development Branch, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health. N-Formyl daunorubicin was generously provided by Dr. David W. Henry, Stanford Research Institute. Daunorubicinol and daunorubicin aglycone were prepared as described previously (11). The physical stabilities of the anthracyclines were chromatographically monitored on Silica Gel 60 plates (EM Laboratories Inc., Elmsford, N.Y.) in chloroform/methanol/water (80:20:3) (11). The concentrations of the antibiotics in aqueous solutions or in ethanol were determined spectrally from their absorbance at 485 nm (12).

Nucleic Acids—Salmon sperm DNA was obtained from Calbiochem. Partially digested DNA was prepared according to Oleson and Koerner (13).

Microorganisms—We are indebted to Dr. John W. Drake, University of Illinois, for all the temperature-sensitive mutants in gene 43 used in these studies. They are described in Refs. 14 and 15. Phage T4D were those used in Ref. 16.

T4 DNA Polymerases—Wild type and mutant T4 DNA polymerases used in this study were the phosphocellulose fractions described previously (17).

The conventional assays for polymerase are essentially the same as those described in Ref. 17. Misincorporation frequency is measured as the normalized incorporation of 2-amino[3H]purine deoxyxynucleotide monophosphate compared with [3H]dAMP into DNA. The assay for turnover (10) (DNA-dependent conversion of a deoxyxynucleotide triphosphate to the corresponding monophosphate) was modified as follows. After incubating the reaction mix (identical with the polymerase assay mix), 2-amino[3H]purine deoxyxynucleotide monophosphate and [3H]dAMP were separated from unreacted triphosphate substrates by ascending thin layer chromatography on cellulose polyethyleneimine-impregnated plastic backed sheets (Brinkmann Instruments, Inc.) and developed with 1 M LiCl (at room temperature).

The ultraviolet light absorbing spots were cut out and eluted in scintillation vials in 1 ml of 0.1 N HCl for 30 min at 37° while agitating continuously. This elution procedure reproducibly resulted in >99% of the radioactivity appearing in the liquid phase thereby.
minimizing possible error due to variable quench. A scintillation mixture (9 ml) containing 2 liters of toluene, 1 liter of Triton X-100, 16.5 g of 2,5-diphenyloxazole (PPO), and 0.375 g of 1,4-bis[2-(5-phenyloxazol)]benzene (POPOP) was added directly to the vials and counted.

RESULTS

We have shown previously that the ability of bacteriophage T4 wild type and temperature-sensitive gene 43 mutant DNA polymerases to incorporate nucleotides is impaired differentially by the anthracyclines adriamycin and daunorubicin (9). We have examined two classes of structurally modified anthracyclines (Fig. 1) in an attempt to localize the inhibitory interactions. In the first class, modifications on the side chain at C-9 of the anthracycline moiety were examined. The second group of analogues consisted of substances modified at the sugar moiety by either blocking the amino group or removing the sugar.

Inhibition of T4D DNA polymerase by C-9 analogs of adriamycin is qualitatively similar to inhibition by the parent compounds; adriamycin octanoate is shown as an example (Fig. 2). The octanoate derivative is able to inhibit the polymerase activity completely despite the presence of a lengthy 9-carbon side chain which reduces but does not abolish (18) binding to DNA. Several additional C-9 analogs (Fig. 1) were tested: daunorubicin, daunorubincinol, daunorubicin benzoylhydrozone, and adriamycinol. These could not be distinguished from adriamycin on the basis of quantitative dose-response differences.

A substantially different inhibition pattern results in the presence of a second class of anthracycline analogs which are modified at the sugar moiety by either blocking the amino group or removing the sugar. The general structure of these compounds is shown (NAME structure). The blocked amino compounds elicit a polymerase response which is diminished significantly in overall magnitude, saturating at about 70% of remaining incorporation activity for T4D polymerase. Daunorubicin aglycone, which lacks the sugar moiety and hence the amino group, shows a similar 70% saturating inhibition curve over the same 0 to 1.5 mM anthracycline concentration range.

The presence of an unblocked amino group on daunosamine attached to the anthracycline ring appears necessary to obtain monotonically increasing inhibition with increasing adriamycin concentrations. The inhibition patterns obtained for N-acetyl daunorubicin (Fig. 2) and daunorubicin aglycone are unaffected by the addition of free daunosamine over a 0 to 15 mM concentration range. A comparison of the polymerase inhibition patterns with adriamycin and N-acetyl daunorubicin indicates the possibility of a multiple response of T4D enzyme to the DNA-anthracycline interaction. The response appears to depend on the concentration of the drug relative to DNA and the presence of a charged ionic group in proximity to the site of enzyme action.

A 2-fold reduction in T4D polymerase activity (Fig. 2) corresponds to adriamycin:DNA-P molar ratios of approximately 1:15; the time course for nucleotide incorporation is linear under these conditions (data not shown). Inhibition in the range 0 to 50% is not reversible by the addition of excess DNA after incubation has begun. Following addition of new template at the 50% inhibition level (Table I), the adriamycin:DNA-P molar ratio is reduced to about 1.5 so that we would expect to reattain a fully uninhibited rate of synthesis if the polymerase were able to dissociate in active form. We have verified that addition of excess template and polymerase to the adriamycin inhibited reaction results in a return to the uninhibited initial DNA synthesis rate; thus, if active enzyme could dissociate from the template-adriamycin complex, we should indeed reverse the inhibition upon addition of excess DNA. Addition of excess polymerase or nucleotide substrates has no measurable effect on the inhibition data. For convenience, we designate the relative drug to DNA levels comprising the 0 to 50% inhibition range as "low" adriamycin:DNA-P ratios. It appears that polymerase bound to DNA in the pres-
Reversal of adriamycin inhibition of T4D polymerase by template add back

The standard polymerase assay was used to measure the incorporation of dTMP into DNA by T4D polymerase. The initial concentration of partially nicked DNA (1.32 mM) was saturating.

| Add back | Stimulation | Adriamycin: 
| DNA-P molar ratio at t = 0 |
|---|---|---|
| 0 | 4.40 | 0.0 |
| 1.36 | 4.60 | 0.0 |
| 2.39 | 1.34 | 0.0 |
| 2.39 | 1.34 | 0.0 |
| 1.89 | 1.96 | 3.7 |
| 1.19 | 1.96 | 13.1 |
| 0.56 | 0.87 | 55.9 |
| 0.12 | 0.20 | 65.6 |

* At 15 min, the reaction mix was adjusted with template buffer to equalize volumes in the -DNA and +DNA add back assays.
* At 15 min, 0.20 pmol of partially nicked DNA was added to assay to final concentration of 1.83 mM.
* At 15 min, 2.8 nw mol of partially nicked DNA was added to assay to final concentration of 10.7 mM.

In Fig. 4, inhibition of T4D polymerase by adriamycin at "high" adriamycin:DNA-P molar ratios. Incorporation of dTMP was measured in a standard polymerase assay run for 30 min at 30°C with partially nicked template-primer DNA. Concentrations of the inhibitor adriamycin were: 0 (O--O); 58.4 µM (●--●); 87.6 µM (x--x).

A plot of velocity versus DNA concentration (Fig. 3, inset) indicates significant positive cooperativity at low template concentrations in the presence of a fixed concentration of adriamycin. At elevated drug to DNA ratios (>1:15) adriamycin acts to inhibit DNA polymerase competitively; a competitive pattern of inhibition is indicated in the double reciprocal plot shown for T4D polymerase (Fig. 4). The adriamycin:DNA-P ratio at which measurable reversal of inhibition occurs following the addition of excess template correlates well with the range in which competitive inhibition patterns occur. Inhibition reversal data spanning low and high adriamycin:DNA-P ratios are given for T4D polymerase in Table I; the relative stimulation of nucleotide incorporation following the addition of excess template becomes more pronounced at higher levels of inhibition. As in the case of T4D (Fig. 4), competitive inhibition is observed at elevated adriamycin:DNA-P ratios for L56 and L141 polymerases (data not shown).

The T4D, L56, and L141 polymerases have been shown to exhibit differing nucleotide mismatch removal efficiencies in vitro (10) consistent with the phenotypic behavior of the respective phage. We observe that despite a large differential sensitivity of the mutant and wild type polymerases to inhibition of nucleotide incorporation rates by adriamycin (9), "misincorporation" frequencies for the nucleotide analog 2-amino-purine deoxynucleoside monophosphate in place of dAMP are not significantly different from those reported previously (10). In a drug/DNA-P range where polymerase incorporation rates are inhibited between 0 and 90%, we measure a concomitant reduction in nucleotide turnover (data not shown) resulting in unaltered editing efficiencies for each of the enzymes.

**DISCUSSION**

Two modes of template-mediated inhibition of T4 DNA polymerases are elicited by adriamycin. The type of inhibition observed depends on the adriamycin:DNA-P molar ratio. At
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relatively low adriamycin-DNA-P ratios the inhibition is uncompetitive and cannot be reversed by the later addition of a large excess of template DNA. Thus, it appears that polymerase bound to DNA in the "low" adriamycin-DNA-P region can initiate DNA synthesis at free 3'-OH ends, but the velocity of nucleotide addition is reduced and the enzyme is unable to dissociate in an active form to reinitiate synthesis. The failure of excess DNA to reverse the inhibition contrasts to what occurs at relatively high adriamycin-DNA-P ratios where the readdition of template partially reverses the inhibition. Consistent with the competitive inhibition kinetics observed, it appears the polymerase is prevented from binding to the template at high adriamycin concentrations. Based on anthracycline DNA binding studies and the intrinsic properties of T4 DNA polymerase, it is possible to propose a mechanism to account for the two types of adriamycin inhibition including the differential inhibitory responses of the mutant and wild type T4 DNA polymerases.

It is well established that adriamycin binds to DNA by intercalating between the flat base pairs of the DNA helix (5, 6). The role played by the daunosamine moiety in the intercalation process is still not clear. However, the binding of the chromophore to DNA is reduced significantly if either daunosamine is absent or if its amino group is blocked (3) resulting in a diminished overall magnitude of polymerase inhibition. We presume that in the case where the amino-sugar is blocked or absent or when the adriamycin-DNA-P ratios are low, an intercalative drug binding mode is favored in double stranded regions of the gapped primer template sufficiently removed from the 3'-OH primer ends to preclude significant interference in the initiation of DNA synthesis. Two consequences are drawn from the results of the template add-back experiments at "low" adriamycin-DNA-P ratios: (a) the rates of nucleotide addition are mainly affected by adriamycin intercalation, stimulated slightly for L56 and inhibited for T4D and L141 (b) polymerization in the presence of adriamycin is probably occurring processively since distributive synthesis would permit excess template to compete for polymerase thereby reversing the inhibition.

The partial reversal of the inhibition which occurs at "high" adriamycin-DNA-P molar ratios suggests that some fraction of the polymerase is free to initiate synthesis on freshly added template. The data appear consistent with a model in which any excess drug could bind ionically in the gapped region of the template once the primary intercalative binding sites are saturated. It appears that the positively charged amino group on daunosamine could become involved in binding the chromophore along the phosphate backbone. Such binding could directly interfere with polymerase binding to free 3'-OH primer ends. Since the daunosamine moiety (where the chromophore has been removed) does not measurably affect polymerase activity, the anthracycline ring structure is likely to play a fundamental role in inhibiting DNA synthesis when involved in ionic binding to template DNA.

Although the competitive inhibition data seem consistent with a model in which adriamycin may engage in both intercalative and ionic binding to double and single stranded regions of DNA respectively, the physical details of the adriamycin-DNA interaction cannot be inferred from our data. Waring has proposed (7) that a significant fraction of anthracycline bound to DNA need not be intercalated. We suggest that the multiple polymerase response reported here fits a model in which intercalative binding, favored at low adriamycin-DNA-P ratios, causes an uncompetitive inhibition of polymerase. As the drug concentration is increased and the sites for intercalation in the gapped template become saturated, excess drug is then able to establish a lower affinity binding along the phosphate backbone. This binding within a gap could competitively inhibit polymerase binding. The positive cooperativity in enzyme activity exhibited at low DNA concentrations in the presence of adriamycin (Fig. 3, inset) may be caused by a very high drug affinity for intercalative compared with ionic binding sites. Thus, at low DNA concentrations, both intercalative and ionic binding sites are occupied leaving the polymerase free in solution. As the template concentration is increased, the ionic sites become stripped of drug as new intercalation sites become available; the polymerase is now free to initiate synthesis. A cooperative increase in synthesis rates with increasing template concentration continues until intercalation sites are available to bind most of the drug.

Some aspects of this model may be relevant to understanding the exceptionally high antitumor activity of adriamycin in comparison with other intercalating compounds such as ethidium bromide and 9-aminoacridine which are not effective as anticancer drugs. Possibly irreversible polymerase-template binding at relatively low drug to DNA ratios is involved in obtaining antitumor activity at therapeutically administered drug levels. It will be interesting to determine if other intercalating compounds can also inhibit DNA polymerase uncompetitively by sequestering it to the template.

Adriamycin is also known to interfere with functioning of normal cells causing, among other problems, severe cardiotoxic effects in patients (10). This may be due, in part, to a failure of nondividing cells to repair drug-induced damage. For example, a selective attack by nucleases in regions where the helical structure is distorted by intercalation could result in the formation of a nicked or even partially gapped DNA molecule. If the DNA polymerase present in nondividing cardiac cells is inhibited in repair, then DNA breakdown could result. Chromosomal fragmentation has been observed in adriamycin treated cells in vivo (20) and could be a contributing factor to the cardiotoxic effect of the drug.

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