SPECIFIC AND REVERSIBLE INHIBITION BY APHIDICOLIN OF THE α-LIKE DNA POLYMERASE OF PLANT CELLS

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1. Introduction

Aphidicolin, a tetracyclic diterpenoid obtained from *Cephalosporium aphidicola* [1] is an inhibitor of the replication of nuclear and viral DNA in animal cells [2,3]. The drug interferes specifically with the replicative [4–11] DNA polymerase α of animal cells or with the virus-induced replicative DNA polymerase [3], while it has no effect on the β-polymerase [3,12] which is most likely involved in DNA repair [7,8,13], nor on the γ-polimerase [3,12], involved in animal mitochondrial DNA synthesis [7,8].

Plant cells contain at least two distinct DNA polymerases; we have named them α-like [15] and γ-like [16] because their properties closely resemble those of the α- and γ-polymerases present in animal cells [4]. The α-like DNA polymerase is the most abundant in cultured plant cells and responds to changes in the rate of cell multiplication [15], whereas the γ-like DNA polymerase is present in chloroplasts [16].

This paper describes the effect of aphidicolin both in vivo, on cultured plant cells, and, in vitro, on the activity of the α-like and γ-like plant DNA polymerases. The results show that aphidicolin depresses the incorporation of thymidine into the DNA of cultured plant cells whereas it has no effect on RNA and protein synthesis. The effect is reversible since DNA synthesis and cell growth resume upon removal of the drug. In vitro the drug inhibits the activity of the α-like but not of the chloroplasts, γ-like DNA polymerase. The inhibition is competitive with dCTP in analogy with the mode of inhibition of the animal α polymerase [14,17].

The results, besides adding further evidence on the similarity between the α-like plant DNA polymerase and the animal α-polymerase, provide a valuable tool for studying the in vivo role of plant DNA polymerases.

2. Materials and methods

2.1. *Chemicals*

Unlabelled deoxyribonucleoside triphosphates (dNTPs) were purchased from Boehringer, Mannheim. Dithiothreitol (DTT) was from Miles Lab., Kankakee, II. Bovine serum albumin (BSA), A grade, was from Calbiochem, Lucerne. Deoxy[^{3}H]ribonucleoside triphosphates, [*methyl*-^{3}H]thymidine (25 Ci/mmol), [5-^{3}H]uridine (25 Ci/mmol), L-[4,5-^{3}H]leucine (50 Ci/mmol) were from the Radiochemical Centre, Amersham. Poly(A) and oligo(dT)$_{12-18}$ were from PL-Biochemicals, Milwaukee, WI.

2.2. *Preparation of templates*

Activated calf thymus DNA was prepared as in [18], while poly(A) and oligo(dT)$_{12-18}$ were hybridized at a base ratio of 5:1 as in [19].

2.3. *Plant cell growth and enzymes purification*

Cell suspension cultures of rice (*Oryza sativa* L. cv Roncarolo) were established and maintained as in [20]. Cells were harvested during the early exponential phase of growth (usually 4–6 days after incubating into fresh medium) and immediately utilized either for the purification of the α-like DNA polymerase [15] or for the in vivo assays of macromolecular synthesis. The γ-like DNA polymerase was purified from chloroplasts prepared from garden-grown spinach leaves (*Spinacia oleracea* L.) [16].

2.4. *Assay of DNA polymerases*

The α-like DNA polymerase was assayed as in [15] at 37°C in the presence of 20 mM K-phosphate (pH 7.2), 0.1 mM EDTA, 0.5 mM DTT, 10 mM $MgCl_2$, 250 μg/ml BSA, 200 μg/ml of calf thymus activated DNA, and of the unlabelled and radioactive dNTPs at the indicated concentrations. The γ-like DNA polymerase was assayed as in [16] at 37°C in the presence of Tris–HCl (pH 8.5 at 4°C), 100 mM KCl, 0.5 mM $MnCl_2$, 1 mM DTT, 250 μg/ml BSA, 50 μg/ml oligo-$(dT)_{12-18}$, poly(A) and 30 μM d[^{3}H]TTP (1000 cpm/pmol). Acid-insoluble radioactivity was collected according to [21] and measured in a Tri-Carb Packard liquid scintillation spectrometer (efficiency 31%).

2.5. *Measurement of the rate of DNA, RNA and protein synthesis in suspension cultured rice cells*

The incorporation of [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]leucine into acid-insoluble material was used as a measure of DNA, RNA and protein synthesis, respectively. For each experimental point, 50 mg cells (fresh wt) in 0.5 ml R2 medium were incubated at 26°C on a rotary shaker (120 rev./min) in the presence of 2.5 μCi of the appropriate radioactive precursor. Incubation was stopped by adding 1 ml of 10% trichloroacetic acid; then the suspension was homogenized with a motor-driven teflon-in-glass homogenizer and the insoluble material collected and washed 9 times with 5 ml 5% trichloroacetic acid on a Whatman GF/C glass filter. Radioactivity was determined in an Insta-gel liquid scintillation cocktail.

3. Results

3.1. *Aphidicolin inhibits in vitro only the α-like DNA polymerase of plant cells*

Plant cells are endowed with two distinct DNA polymerases, named α-like and γ-like [15,16], in analogy with animal DNA polymerases [4]. Aphidicolin inhibits the incorporation of deoxyribonucleoside triphosphates by the α-like DNA polymerase purified from cultured rice cells (fig.1). The K_i is ~0.9 μM when the polymerase is assayed in the presence of 5 μM dCTP. On the other hand, the γ-like DNA polymerase purified from spinach chloroplasts is completely resistant even at the highest tested drug concentrations (150 μM) (fig.1). Similar results were obtained when both enzymes were assayed in crude extracts from rice cells or from spinach leaves (unpublished).

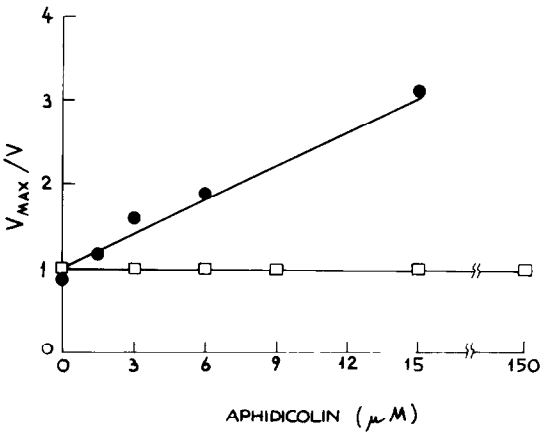

Fig.1. Effect of aphidicolin on the activity of plant α-like (●——●) and γ-like (□——□) DNA polymerases. The polymerases were assayed as in section 2 in the presence of the indicated concentrations of aphidicolin except that the α-like polymerase reaction mixture contained 100 μM dGTP and dATP, 5 μM dCTP and 30 μM d[^{3}H]TTP (1000 cpm/pmol). Incorporation of d[^{3}H]TMP in the absence of aphidicolin was 46 000 and 15 500 cpm/h for the α-like and γ-like DNA polymerase, respectively.

3.2. *The inhibition of the α-like DNA polymerase by aphidicolin is competitive with dCTP and non-competitive with the other three deoxyribonucleoside triphosphates*

To define the mechanism of inhibition of the α-like DNA polymerase, we have studied the effect of aphidicolin on the polymerization rates as a function of the concentration of each deoxyribonucleoside triphosphate. The results obtained when dGTP, dATP and dTTP were used, show that aphidicolin affects the slope of the lines of the double reciprocal plot but not the intercepts on the abscissa (fig.2A–C), thus indicating that the effect is purely non-competitive; i.e., aphidicolin does not affect the affinity of the α-like DNA polymerase for any of these three dNTPs. However, when dCTP was used, with saturating concentrations of dGTP, dATP and dTTP, the inhibition is apparently competitive (fig.2D) in analogy with the mode of inhibition of the animal replicative α-polymerase and of viral replicative DNA polymerases [14,17]. The data of fig.2 also allow the determination of the K_m values of the α-like DNA polymerase for each dNTP. They are 1.9, 2.7, 9.3 and 2.3 μM for dGTP, dATP, dTTP and dCTP, respectively, and represent another analogy with the animal α-polymerase [19].

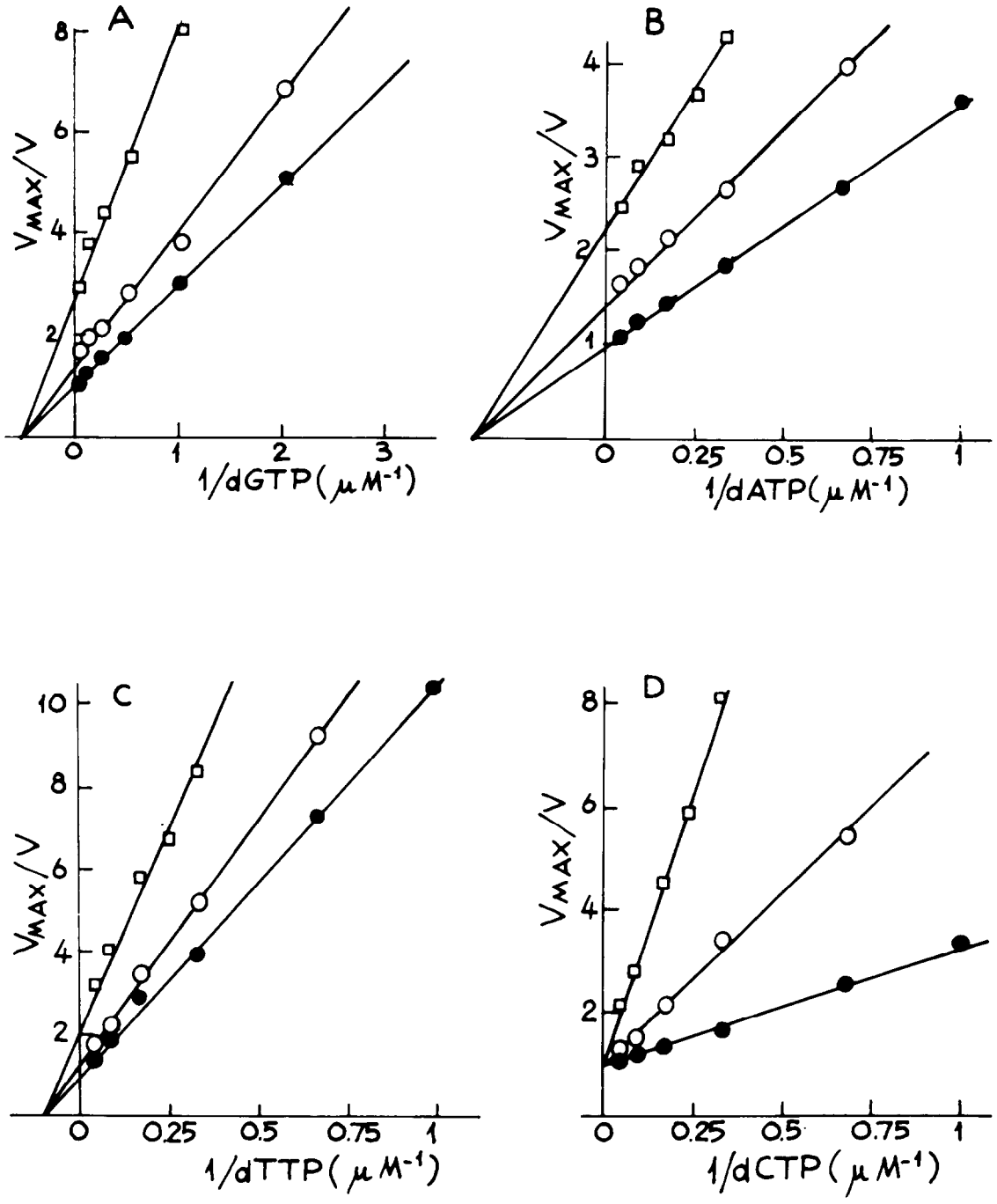

Fig.2. Double reciprocal plots of the effect of aphidicolin on the polymerization rate of the α-like DNA polymerase from *O. sativa* in the presence of varying concentrations of d[^{3}H]GTP (A), d[^{3}H]ATP (B), d[^{3}H]TTP (C) and d[^{3}H]CTP (D). The specific activity of each dNTP was 3500 cpm/pmol. In each case the other 3 non-radioactive dNTPs were 100 μM. Each point is the average of two determinations and all lines represent least squares fits of the experimental values. Time points were taken at 30 and 60 min and the kinetics was linear during this period. V_{max} is 22 000, 79 000, 92 000 and 42 000 cpm for A,B,C and D, respectively. (•) No aphidicolin; (○) 9 μM aphidicolin for A,B and C or 1.5 μM aphidicolin for D; (□) 36 μM aphidicolin for A,B,C or 15 μM aphidicolin for D.

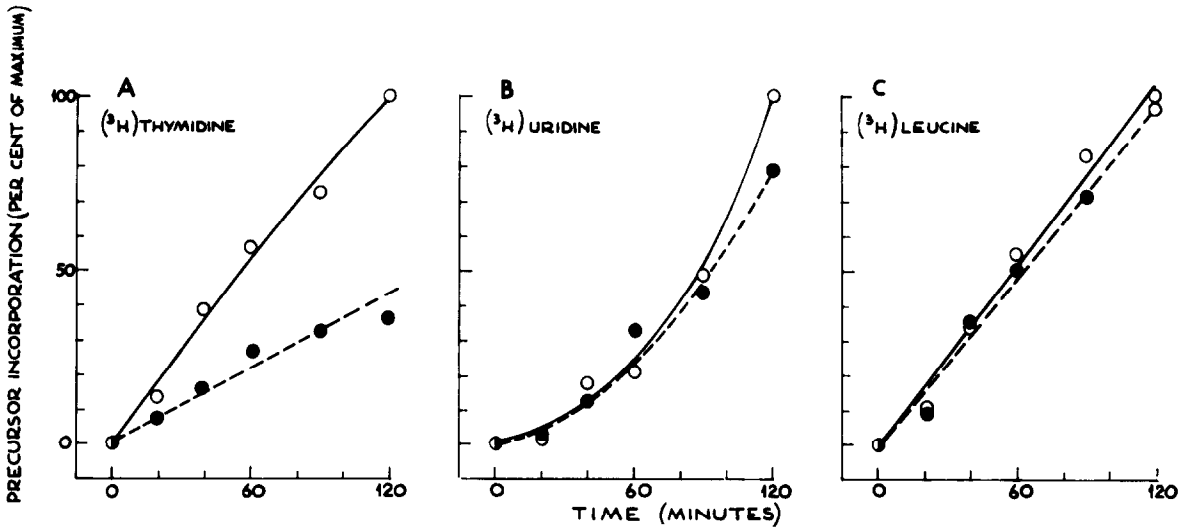

Fig.3. Effect of aphidicolin on DNA (A), RNA (B) and protein (C) synthesis in suspension cultured cells of *O. sativa*. The experiments were done as in section 2 in the presence of 15 μM aphidicolin. This concentration was chosen on the basis of the data of fig.4 and the results are expressed, for ease of comparison, as % of the maximum incorporation of the appropriate precursor in DNA, RNA or proteins. Maximum incorporation were 29 500, 40 600 and 136 000 cpm, respectively, in experiments A,B and C. Control values at zero time were <2% of the maximum and were subtracted from each sample. (•——•) 15 μM aphidicolin; (○——○) no aphidicolin.

3.3. *In vivo aphidicolin affects DNA synthesis but not RNA and protein synthesis of cultured* Oryza sativa *cells*

The specific inhibition of the α-like DNA polymerase γ.(Geuskens, Hardt, G. P.-N., S. S., in prepara- this drug affects DNA synthesis also in vivo i.e., in intact and proliferating plant cells, in analogy with its effect on DNA synthesis in animal cells [3]. If so, aphidicolin might be utilized as a convenient tool for several experimental purposes, in the research with plant cells (e.g., studies on the in vivo role of plant DNA polymerases, selection of resistant mutants, synchronization of the cell cycle). Fig.3 shows that 15 μM aphidicolin specifically inhibits cellular DNA synthesis with no effect on RNA and protein synthesis.

In the presence of 6 μM aphidicolin, the synthesis of DNA is depressed to ~30% of the control (fig.4). No further inhibition is observed at ⩽150 μM. The observed inhibition of DNA synthesis is probably due to the effect of aphidicolin on a single (most likely the α-like) enzyme. In fact, values corrected for the residual, drug resistant, DNA synthesis give a linear plot of V_{max}/V versus inhibitor concentration (fig.4 insert). Since in animal cells aphidicolin inhibits the replication of nuclear DNA but does not prevent replication of mitochondrial DNA by DNA polymerase γ (Geuskens, Hardt, G. P.-N., S. S., in prepara-

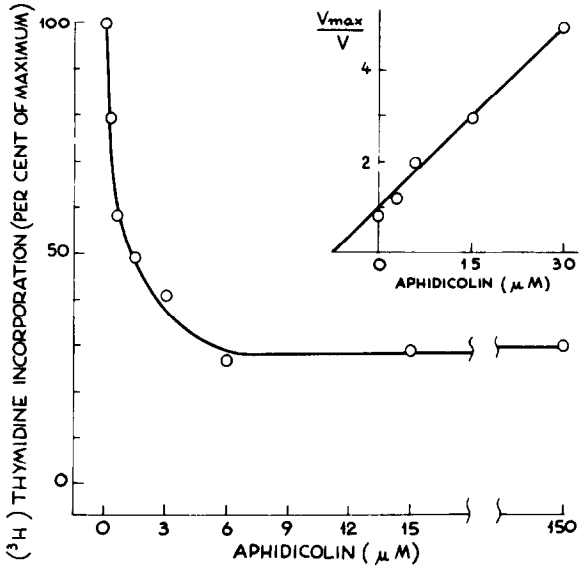

Fig.4. Effect of increasing concentrations of aphidicolin on DNA synthesis in suspension cultured cells of *O. sativa*. The experiments were performed as in fig.3, except that aphidicolin was present at the specified concentrations and incubation was for 60 min. The maximum incorporation of [³H]-thimidine was 48 000 cpm. Control values at zero time were <2% of the minimum and were substracted from each sample. The insert reports the plot of V_{max}/V versus inhibitor concentration of the values at low aphidicolin doses, after correction for the residual drug resistant DNA synthesis. V_{max} was 48 000 cpm.

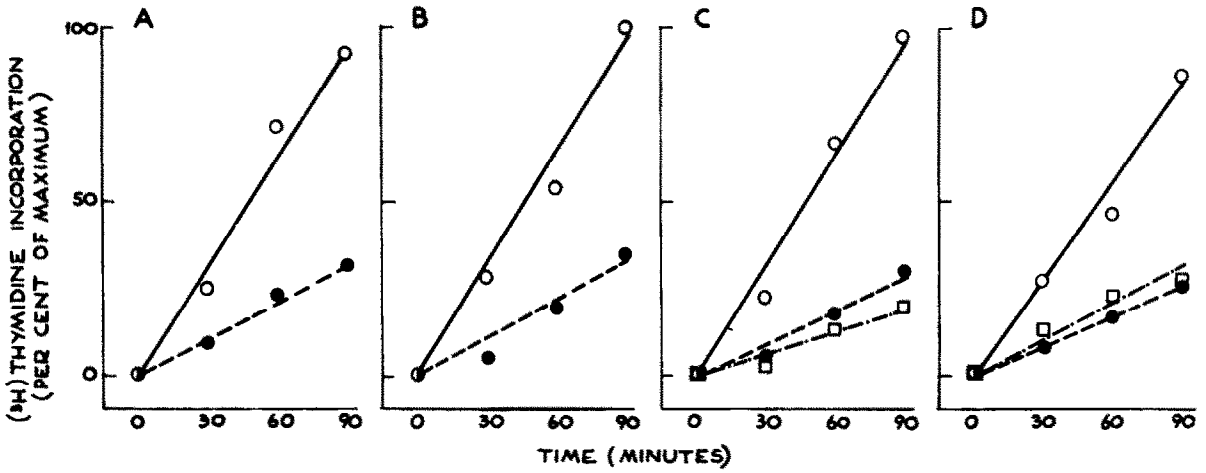

Fig.5. Reversibility of the inhibition of DNA synthesis in suspension cultured cells of *O. sativa*. Aphidicolin (15 μM) was added to a suspension culture of *O. sativa* cells in the early exponential phase of growth and incubation continued on a rotary shaker (120 rev./min) at 26°C. At time zero (A), 90 min (B), 24 h (C) and 48 h (D) a sample of the cell suspension was withdrawn, washed 10 times with fresh culture medium and the cells resuspended in fresh medium without (○——○) or with 5 μg aphidicolin/ml (●– – –●). At 24 h and 48 h, DNA synthesis was also assayed in unwashed cells, in the presence of the original culture medium (□–.–.–□). For ease of comparison, the results are expressed as % of the maximum recorded incorporation (37 400 cpm).

tion), we ascribe the residual aphidicolin-resistant 30% DNA synthesis in rice cells to the activity of the γ-like DNA polymerase(s) present in proplastids and possibly in mitochondria. In fact the residual activity is depressed to 10% of that of the control when both aphidicolin and ethidium bromide (30 μM) are present (unpublished). Ethidium bromide is known to interact preferentially with the organellar type DNA [22,23].

3.4. *The action of aphidicolin on intact cells is reversible*

To verify whether the action of aphidicolin on plant cells is reversible, a suspension culture of rice cells was incubated in standard growth conditions in the presence of 15 μM aphidicolin. Cell samples were withdrawn at time intervals, extensively washed to eliminate the drug and utilized for the assay of in vivo DNA synthesis. Fig.5 shows that the inhibition of DNA synthesis was immediately removed even from cells which had been exposed to the inhibitor for 2 days. Control experiments (fig.5C,D) also show that no extensive degradation of the drug occurred during the 48 h incubation with rice cells. It was also noticed that cells which had been in the presence of 15 μM aphidicolin for 48 h, resumed growth at the usual rate following removal of the drug and transfer into fresh culture medium.

4. Discussion

We have shown that aphidicolin inhibits the activity of the α-like DNA polymerase purified from rice cells. The inhibition is non-competitive with dGTP, dATP, dTTP and competitive with respect to dCTP as in the case of animal [14–17] and viral replicative DNA polymerases [14]. Thus the mechanism of inhibition is apparently similar in all sensitive eukaryotic replicative DNA polymerases and confirms our hypothesis that the drug must recognize a specific portion of the active site of the enzyme which is unique to these proteins. This binding site is probably near to, or even overlapping with, the binding site for dCTP since competitive interaction between aphidicolin and dCTP is observed [14]. In analogy with the animal mitochondrial DNA polymerase γ [3,12] the chloroplasts γ-like DNA polymerase [16] is resistant to the drug. Thus, the residual DNA synthesis which is observed in vivo in actively dividing cultures of rice cells, even at high doses of inhibitor, is most likely organellar DNA syn-

thesis resistant to aphidicolin. In fact this residual activity is sensitive to ethidium bromide, a drug that preferentially affects organellar DNA synthesis [22,23]. However, further work is required in order to confirm this interpretation and to assign unambiguously a function in replication of nuclear and organellar (chloroplast and, possibly, mitochondrial) DNA to the plant α-like and γ-like DNA polymerases. In analogy with the results obtained with human HeLa cells [5], both the inhibitory effect of aphidicolin on the α-like DNA polymerase and the reversibility of its action in vivo should allow the synchronization of plant cells in culture.

Acknowledgements

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