EFFECT OF AURINTRICARBOXYLIC ACID ON IN VITRO DNA SYNTHETIC ACTIVITY OF MOUSE ASCITES SARCOMA CELLS

Shuji Seki, Ken Tsutsui, and Takuzo Oda

Department of Biochemistry, Cancer Institute,
Okayama University Medical School,
Okayama, Japan

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SUMMARY: Aurintricarboxylic acid inhibited replicative DNA synthesis in nucleotide-permeable mouse ascites sarcoma cells. DNA polymerase activity assayed with activated DNA template and DNA polymerase purified partially from sarcoma cells was also inhibited by aurintricarboxylic acid. The inhibition of DNA polymerase activity was probably due to the inhibitory interaction of aurintricarboxylic acid with DNA polymerase. The replicative DNA synthesis might be inhibited by aurintricarboxylic acid interacting with some essential protein component(s), such as DNA polymerase of the replication machinery.

Aurintricarboxylic acid* has been used successfully for examining the mechanism of RNA and protein synthesis in vitro (1-13) and for examining chromatin structure (13-15). These studies suggest that nucleic acid-binding proteins are generally sensitive to ATA. However so far as we know, no study dealing with the effect of ATA on DNA synthesis has been reported. The present study was undertaken to investigate ATA-effect on in vitro DNA synthesis and the usefulness of ATA as a probe for studying DNA replication.

MATERIALS AND METHODS

Ammonium aurintricarboxylate (formula weight: 473.44) was obtained from Wako Pure Chemicals Co., Tokyo.

Mouse ascites sarcoma (SR-C3H/He) cells (16) were made permeable to nucleotides by treatment with hypotonic buffer A (10 mM Tris-HCl, 4 mM $MgCl_2$, 1 mM EDTA and 6 mM 2-mercaptoethanol, pH 8.0) as described previously (17). To prepare DNA polymerase, SR-C3H/He cells were suspended in buffer A supplemented with 2 mM $CaCl_2$ and homogenized by a Dounce homogenizer (17). DNA polymerase α was partially purified from the ultracentrifuged supernatant of the homogenate by DEAE-cellulose and phosphocellulose column chromatographies (18).

Replicative DNA synthesis in permeable SR-C3H/He cells was measured essentially according to the method described previously (17). Permeable cells suspended at 2×10^6 in 0.34 ml of buffer A were mixed with 0.2 ml of

* abbreviated as ATA

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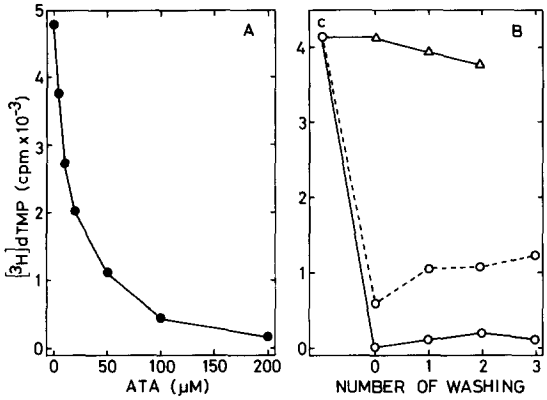

Fig. 1. Effect of ATA on replicative DNA synthesis in permeable SR-C3H/He cells. Preparation of permeable cells and assay of replicative DNA synthesis were conducted as described in "METHODS". Activity is expressed as cpm of [^{3}H]dTMP incorporated per 10^7 cells per 10 min.

(A) Effect of various concentrations of ATA on DNA synthesis. ATA was added in the assay mixture at the concentration indicated.

(B) Effect of washing ATA-treated permeable cells with buffer A on DNA synthesis. Permeable cells were distributed into assay tubes. The tubes were divided into 3 groups. After centrifugation, cells were treated with 0.4 ml of 0.05 mM ATA in buffer A in a group, treated with 0.4 ml of 0.2 mM ATA in buffer A in another group, or treated with 0.6 ml of buffer A in the remaining group. After treatment with ATA the cells were washed with 0.6 ml of buffer A at the number indicated. c, initial permeable cell preparation; (Δ), control (no treatment with ATA); (o---o), 0.05 mM ATA-treated cells; (o—o), 0.2 mM ATA-treated cells.

DNA replicase substrate mixture (pH 8.0) containing 0.1 M Tris-HCl, 7 mM $MgCl_2$, 0.3 M NaCl, 7.5 mM ATP, 0.05 mM dATP, 0.05 mM dCTP, 0.05 mM dGTP and 2.5 μM [^{3}H]dTTP (0.5 Ci/mmol). ATA dissolved in buffer A was added at 0.06 ml to the mixture. The suspension was incubated at 37° for 10 min. The reaction was terminated by chilling the tube in an ice-water bath and adding 2 ml of ice-cold 7.5 % trichloroacetic acid (TCA) containing 1 % sodium pyrophosphate. The precipitate was collected on a glass fiber disc (GF/C, Whatman) and washed with ice-cold 5 % TCA. Radioactivity was counted after drying the disc.

DNA polymerase activity was measured essentially according to the method described previously (18). The reaction mixture contained the following components in a total volume of 0.15 ml: 0.02 ml enzyme, 0.05 ml buffer A containing 30 μg activated calf thymus DNA (19), 0.03 ml of buffer A supplemented with or without ATA and 0.05 ml of polymerase substrate mixture (pH 8.0) containing 0.1 M Tris-HCl, 7 mM $MgCl_2$, 0.05 mM dATP, 0.05 mM dCTP, 0.05 mM dGTP and 2.5 μM [^{3}H]dTTP (0.5 Ci/mmol). The reaction mixture was incubated at 37° for 10 min, chilled in an ice-water bath, and 0.1 ml of bovine serum albumin solution (5 mg/ml) was added to the mixture. Radioisotope incorporated in the acid-insoluble fraction was determined by the filter disc method as described above.

RESULTS AND DISCUSSION

Our previous studies (17, 20) showed that DNA synthesis measured by the permeable cell system was replicative, because DNA synthesis was S-phase

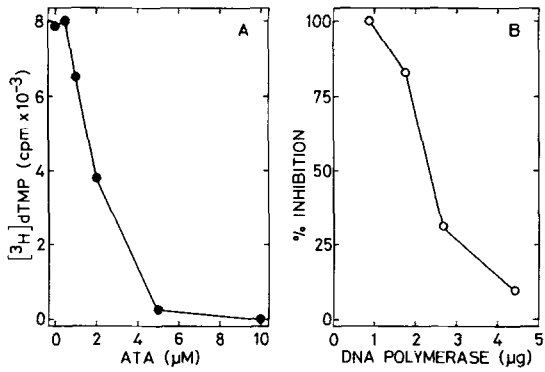

Fig. 2A. Effect of ATA on DNA polymerase activity. Partially purified DNA polymerase α was added at the concentration of 4.45 μg protein in 0.15 ml of the assay mixture. ATA was added in the assay mixture at the concentration indicated. DNA polymerase activity was measured as described in "METHODS". Activity is expressed as cpm of [^{3}H]dTMP incorporated per 10 min.
Fig. 2B. Inhibition of various concentrations of DNA polymerase α with ATA. Assay mixture contained 0.5 μM ATA and DNA polymerase preparation at the indicated amount with the constituents for assay of DNA polymerase activity. Data are expressed as per cent. of activity in ATA-free control.

specific, and the DNA synthesized in permeable cells was largely due to the elongation of strands initiated in vivo. Replicative DNA synthesis in permeable SR-C3H/He cells was inhibited by ATA, as shown in Fig. 1A. The inhibition was slightly reversed when ATA-treated cells were washed with buffer A to remove free ATA (Fig. 1B). Calf thymus histone which is known to combine with ATA (13) partially prevented ATA-inhibition of replicative DNA synthesis in permeable SR-C3H/He cells. ATA at the concentrations used in the present study did not inhibit the incorporation of [^{3}H]deoxythymidine into DNA in intact cells, possibly because of ATA impermeability to intact cells.

DNA polymerase activity measured with partially purified DNA polymerase α and activated DNA template was inhibited by ATA, as shown in Fig. 2A. The inhibition rate of DNA polymerase activity depended on the quantitative ratio of ATA-to-DNA polymerase preparation (Fig. 2B).

When DNA polymerase preparation and ATA were charged separately on a Sephadex G-50 column, DNA polymerase was eluted in the void volume, whereas ATA was weakly adsorbed on the column and the elution was retarded. When the

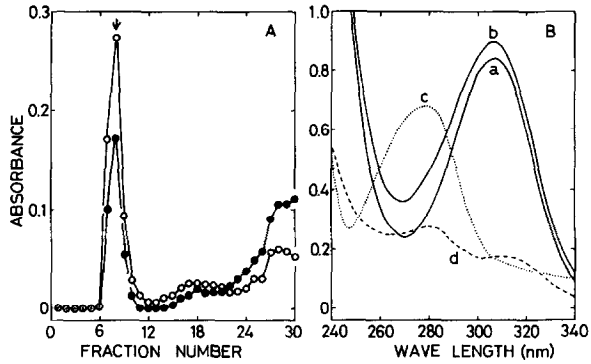

Fig. 3. Sephadex G-50 column chromatography (A) of a mixture of ATA and partially purified DNA polymerase, and absorption spectra (B) of the components and a fraction. ATA at the final concentration of 0.5 mM was added to partially purified DNA polymerase dissolved in a buffer containing 10 mM Tris-HCl, 1 mM EDTA and 6 mM 2-mercaptoethanol (pH 8.0). Protein concentration of the mixture was 760 µg/ml. A half milliliter of the mixture was applied to a Sephadex G-50 column (7.7 x 171 mm) previously equilibrated with the buffer containing Tris-HCl, EDTA and 2-mercaptoethanol, and was eluted with the buffer. Fractions of 0.56 ml were collected. Absorbances at 280 nm (o) and 310 nm (•) were measured. The arrow indicates void volume. B (absorption spectra): a, 0.1 mM ATA; b, a solution containing 0.1 mM ATA and partially purified DNA polymerase (152 µg protein/ml); c, partially purified DNA polymerase at 760 µg protein/ml; d, the peak fraction (fraction 8) shown in Fig. 3A.

mixture of ATA and DNA polymerase preparation was charged on the column and eluted, protein recovered in the void volume but no DNA polymerase activity was detected (Fig. 3A). The absorption spectrum, showing absorption peak at 280 nm and a shoulder at 310 nm, of the void volume fraction indicated the coelution of protein and a part of ATA (Fig. 3B). When activated DNA was mixed with ATA and applied to the Sephadex G-50 column, activated DNA having template activity was eluted in the void volume, while ATA was weakly adsorbed on the column. The gel filtration experiment suggests the direct inhibitory interaction (probably combining) of ATA with DNA polymerase α but not with activated DNA.

It was shown that ATA inhibited protein synthesis by preventing mRNA binding to ribosomes (1, 2, 4-7). Blumenthal and Landers (11) proposed that ATA combined with the template binding site of Qβ replicase, Escherichia coli RNA polymerase and T7 RNA polymerase to prevent initiation. Akiyama et al.

(12) reported that ATA interacted with mammalian RNA polymerizing enzyme to prevent the activity. Binding of ATA to histone was shown by Tsutsui (13). The present experiment showed that DNA synthetic activity was also inhibited by ATA, probably by the inhibitory interaction of ATA with DNA synthetic enzyme(s). ATA is thought to inhibit the function of most nucleic acid-binding proteins, as already suggested by Blumenthal and Landers (11).

In vitro replicative DNA synthesis of mammalian cells has been proved to date only in isolated nuclei and permeable cells. Reconstitution of DNA replicating activity from subnuclear components has rarely succeeded (18). ATA seems to be a useful probe for studying such complicated, labile replication machinery, because the ATA action is relatively mild and the reaction mechanism is understood fairly well. Reconstitution of replicative DNA synthesis from ATA-treated permeable cells and a nuclear salt extract (18) is presently being studied.

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