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Spectrochimica Acta Part A 74 (2009) 1145–1151

Contents lists available at ScienceDirect

Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

journal homepage: www.elsevier.com/locate/saa

Interaction of aurintricarboxylic acid (ATA) with four nucleic acid binding proteins DNase I, RNase A, reverse transcriptase and Taq polymerase

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ARTICLE INFO

Article history:
Received 27 January 2009
Received in revised form 17 August 2009
Accepted 12 September 2009

Keywords:
Aurintricarboxylic acid
Telomerase
DNase I
RNase A
Reverse transcriptase
Taq polymerase
Tryptophan fluorescence
Circular dichroism

ABSTRACT

In the investigation of interaction of aurintricarboxylic acid (ATA) with four biologically important proteins we observed inhibition of enzymatic activity of DNase I, RNase A, M-MLV reverse transcriptase and Taq polymerase by ATA in vitro assay. As the telomerase reverse transcriptase (TERT) is the main catalytic subunit of telomerase holoenzyme, we also monitored effect of ATA on telomerase activity in vivo and observed dose-dependent inhibition of telomerase activity in Chinese hamster V79 cells treated with ATA. Direct association of ATA with DNase I (K_d = 9.019 μM)), RNase A (K_d = 2.33 μM) reverse transcriptase (K_d = 0.255 μM) and Taq polymerase (K_d = 81.97 μM) was further shown by tryptophan fluorescence quenching studies. Such association altered the three-dimensional conformation of DNase I, RNase A and Taq polymerase as detected by circular dichroism. We propose ATA inhibits enzymatic activity of the four proteins through interfering with DNA or RNA binding to the respective proteins either competitively or allosterically, i.e. by perturbing three-dimensional structure of enzymes.

1. Introduction

The triphenylmethane dye aurintricarboxylic acid (ATA) has been reported to interact with a large number of protein molecules. The ATA molecule carries three carboxylic acid moieties that take negative charge at physiological pH. The structure of ATA was shown in Fig. 1. These charged groups are responsible for extensive ionic binding with positively charged groups on cellular proteins [1]. ATA was initially shown to inhibit template binding to QB replicase, *E. coli* RNA polymerase, T7 RNA polymerase and to prevent the binding of RNA to ribosome [2,3]. It was postulated that ATA would inhibit association of the nucleic acid binding proteins with nucleic acid. Subsequently, ATA's general inhibitory effect to nucleases was reported [4]. Inhibition of apoptosis by ATA was widely documented in various cell lines under different stimulations [5–10]. Our previous study showed that aurintricarboxylic acid (ATA) inhibited apoptosis induced by two different inducer gamma radiation and benzamide [an inhibitor of poly(ADP)-ribose polymerase] [11]. ATA reduced significantly nuclear fragmentation and nucleosomal ladder formation without affecting upstream events like cytochrome c release or caspase-3 activation in Chinese hamster V79 treated with gamma radiation or benzamide. Further, we observed treatment with protein kinase C (PKC) inhibitors (staurosporin and H-7) induced apoptosis (unpublished) and ATA pre-treatment reduced DNA ladder formation by PKC inhibitors without changing caspase-3 activation (unpublished). This result implicated that ATA might be interacting with down-stream events of caspase-3 in the apoptosis pathway, most likely with DNases involved in apoptosis. A large number of DNases localizing in cytoplasm are involved in apoptosis and their activity is either Ca^{2+}/Mg^{2+}-dependent or cation independent. For example, DNase II and L-DNase II are cation independent [12]. DNases translocate into nucleus during apoptosis and cleave DNA into nucleosomal ladder. DNase I, DNase-γ, DNAS1L3, NUC70, NUC18 are all cation-dependent endonuclease and gets activated in the process of apoptosis and cleaves the DNA into large and smaller fragments [13–16]. It has been reported that ATA inhibits endonucleases and promotes long-term survival of cells [6,7].

In modeling studies by docking, it has been shown that ATA can bind with several viral proteins like RNA-dependent RNA polymerase (RdRp), S1, HIV integrase and likely to alter their functions. The same study also reported that ATA bind to the catalytic domain of RdRp in severe acute respiratory syndrome (SARS) coronavirus [17,18]. Anti-viral activity of ATA has been shown due to ATA's ability to interfere with the attachment of viral mRNA to host polyribosome complex in cell free system [17–19]. Anti-reverse transcriptase activity of ATA has been demonstrated by in

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doi:10.1016/j.saa.2009.09.024

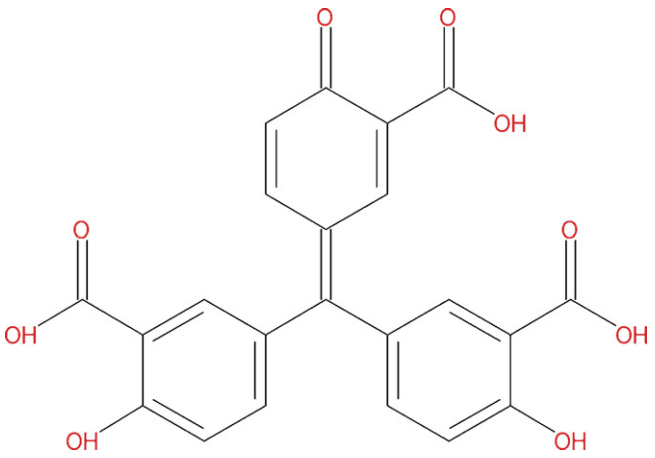

Fig. 1. Chemical structure of aurintricarboxylic acid (ATA).

vitro assay [20]. A potent inhibitor of RNA-dependent DNA polymerase (reverse transcriptase) is important for the discovery of therapeutic agents against retroviruses. ATA's anti-reverse transcriptase activity inspired us to investigate its role in regulation of telomerase which has telomerase reverse transcriptase (TERT), the catalytic subunit. Telomerase is a ribonucleo-protein complex having the main catalytic subunit – TERT [21–23], associated RNA template-telomerase RNA component (TERC or TR or TER) and other associated proteins like telomerase associated protein TEP1/TP1, HSP90, P23, etc. [21,24–26]. Besides, various other proteins have been identified that interact with the telomere. Role of these proteins and their interacting partners for maintenance of the telomere and regulation of telomerase activity is being discovered. The reverse transcriptase replicates the end of chromosomes for synthesizing the telomeric repeats with help of its own RNA molecule as the template [26]. Here we demonstrated inhibition of telomerase activity by ATA treatment in V79 cells.

Several in vitro studies reveal that ATA can inhibit activities of different DNA or RNA binding proteins such as restriction enzymes, DNA polymerase, RNA polymerase. It has been observed that restriction enzymes like Sal I, Bam H1, Pst I, Sma I are inhibited in vitro by ATA treatment [4]. In vitro DNA synthesis activity by DNA polymerase was also inhibited by ATA [27]. The activities of the purified human DNA polymerases alpha, beta, gamma, and DNA primase as well as those of DNA polymerase I and RNA polymerase from *Escherichia coli* and Rauscher leukemia virus are also inhibited by ATA [28]. ATA also inhibits amino acyl ^{t}RNA synthetase [29] and ribonucleotide reductase [30]. In protein synthesis ATA inhibits the interaction of mRNA with the ribosome complex has been modulated by ATA inhibiting the initiation and elongation of protein synthesis [31]. ATA also interfere DNA-NF-κB binding as detected by mobility shift assay [32].

Apart from its inhibitory effect on nucleic acid binding proteins to associate with nucleic acid, it plays significant role in phosphorylation. Apoptosis induced by TNF-alpha alone is suppressed by ATA and this event is paralleled by phosphorylation and nuclear translocation of Jak2, Stat2, Stat4 and NF-kB, along with inhibition of caspase activation in human B cells leading to promotion of survival [33,34]. ATA can also promote bcl2 phosphorylation at evolutionary conserved residue ser-70 whose phosphorylation is required for full anti-apoptotic function [35]. Increase in the phosphorylation of serine and tyrosine residues of proteins have been observed in cells treated with ATA [36–38].

In spite of large number of reports about the inhibitory effect of ATA with several nucleic acid binding proteins by biochemical assay, as well as modeling studies, no information is available about the direct interactions of ATA with the proteins. In this manuscript, we are presenting direct physical interaction of ATA with four biologically important nucleic acid binding proteins such as DNase I, RNase A, reverse transcriptase and Taq polymerase as detected by biochemical enzymatic assay and tryptophan fluorescence quenching study. Three dimension conformational analysis of DNase I, RNase A and Taq polymerase was also demonstrated using circular dichroism spectroscopy. In this connection we are also presenting inhibition of telomerase activity in V79 cells treated with ATA.

2. Materials and methods

2.1. Chemicals

Aurintricarboxylic acid (ATA), RNase A and DNase I were purchased from Sigma chemicals (USA). Medium MEM was procured from Hi-media, India. Goat blood was procured locally and processed in our laboratory for serum preparation as described [39]. M-MLV reverse transcriptase, first strand buffer and oligo dT were from Life Technologies (USA), gene specific oligonucleotides for PCR amplification were from MWG Biotech (Germany). Taq polymerase was purchased from Clontech.

2.2. Enzyme assay and cell culture

2.2.1. Gel electrophoresis

We have used 1.5% and 2% agarose gels in 0.5× TBE buffer for resolving PCR products and RNA, respectively. The gels were stained with ethidium bromide to visualize the DNA or RNA and photographed in ImageMaster VDS (Pharmacia Biotech). To check the purity of the enzymes we resolved in 12.5% SDS-PAGE. After completion of electrophoresis the protein bands were stained with coomasie blue and photographed (data not shown).

2.2.2. DNA digestion

PCR product of 315 bp DNA (obtained as described in the following PCR reaction section) was used as substrate for DNase I in a digestion buffer (40 mM Tris–Cl pH 8.0, 10 mM $MgSO_4$, 1 mM $CaCl_2$) in presence and absence of ATA at 37 °C for 15 min. The digested product was resolved in 1.5% agarose gel, stained with ethidium bromide, photographed under transluminator (data not shown).

2.2.3. RNA digestion

Total RNA was extracted from exponentially growing Chinese hamster V79 cells following the standard protocol and estimated spectrophotometrically. Five micrograms of RNA was digested with RNase A (4.5 μg/ml) in presence and absence of ATA (1, 10, 50 100, 150, 200 μM) in a digestion buffer (150 mM NaCl, 5 mM EDTA, 10 mM Tris–Cl pH 7.5) for 10 min at 37 °C. The product was resolved in 2% agarose gel and stained with ethidium bromide and photographed under transluminator (data not shown).

2.2.4. cDNA synthesis

We have used M-MLV reverse transcriptase for the preparation of cDNA from RNA isolated from V79 cells. We have followed the same method for cDNA preparation (RT product) as the manufacturer described. First, the reverse transcriptase taken in first strand buffer was incubated with different concentration of ATA for 15 min at room temperature. Five micrograms of RNA and 100 ng oligo dT was taken, heated at 70 °C for 10 min followed by chill on ice for 1 min. Then the reverse transcriptase with different concentrations of ATA was added to RNA solution along with DTT and dNTP (containing P^{32}α-dCTP). Then it was incubated at 37 °C for 1 h, heated at 90 °C for 5 min and chilled on ice for 10 min. Similarly RT product was produced using reverse transcriptase without ATA. The product was resolved in 10% non-denaturing acrylamide gel and

exposed with X-ray film for 48 h at −80 °C. The film was developed and autoradiograme (data not shown) was scanned and intensities of the bands obtained were calculated using the software Image-Master VDS.

2.2.5. PCR reaction

PCR reaction was carried out using the cDNA (as described in Section 2.2.4) as template and the oligos 5′-GAA GCA TTT GCG GTG GAC CAT-3′ and 5′-TCC TGT GGC ATC CAC CAA ACT-3′ as primers (these oligos are specific for β actin gene) in presence of different concentration of aurintricarboxylic acid (ATA) in PCR buffer for 30 cycles (94 °C, 45 s; 55 °C, 45 s; 72 °C, 90 s). The PCR product (315 bp) was resolved in 1.5% agarose gel, stained with ethidium bromide, photographed under transluminator (data not shown).

2.2.6. Cell culture

Chinese hamster V79 (male lung, fibroblast) was obtained from National Institute of Virology, Pune, India. V79 cells were routinely grown as monolayer in plastic petridishes using Eagle's minimal medium (MEM) supplemented with dialyzed goat serum (complete medium) [39] at 37 °C in humidified atmosphere containing 5% CO_2.

2.2.7. Determination of telomerase activity by PCR based telomerase repeat amplification protocol (TRAP)

V79 cells were treated with various doses of ATA (200- 600) μM for 48 h and processed for TRAP assay as described earlier [40]

2.3. Biophysical methods

2.3.1. Fluorescence measurements

Steady-state tryptophan (Trp) fluorescence was measured in a Hitachi 410 and Fluoromax 3 spectro-flurometer using a 1 cm path length quartz cuvette. The buffer used in the present study for four different proteins were same as their respective assay buffers as mentioned earlier. Small aliquots of an aqueous stock solution of ATA were added to the protein solutions for fluorescence measurements using excitation at 295 nm and emission at 340 nm. The slit width of bandpass was 5 nm for both excitation and emission channels. The final concentrations of RNase A, reverse transcriptase, DNase I and Taq polymerase used in measuring fluorescence were 3.52, 1.168, 1.33 and 0.4588 μM. Protein-free buffer containing different concentrations of ATA used as a reference blanks in all fluorescence measurements. All measurements were performed at 25 °C with multiple sets of samples.

2.3.2. Evaluation of binding constants

Any change in the fluorescence emission intensity of these proteins (λ_{ex} = 295 nm, λ_{em} = 340 nm) upon the progressive addition of the ATA results from the spectrofluorometric titrations were analyzed using the following relationship to evaluate the dissociation constants (K_d) for these protein–ATA interactions [41].

$$\frac{1}{\Delta F} = \frac{1}{\Delta F_{max}} + \frac{K_d}{\Delta F_{max}(C_L - C_P)}, \quad (1)$$

where ΔF is the change in fluorescence emission intensity at 340 nm (λ_{ex} = 295 nm) upon addition of each aliquot of the ATA and ΔF_{max} is the same parameter when the ligand (ATA) is totally bound to protein, C_L is the concentration of the ATA and C_P is the initial concentration of the protein. The equation is valid under the experimental conditions of $C_L \gg C_P$.

2.3.3. CD measurements

Circular dichroic (CD) spectra were recorded on a JASCO J-720 spectropolarimeter using cylindrical quartz cuvette (Hellma) of path length 1 mm over the wavelength range of 190–250 nm. For measurements in the near-UV range (250–310 nm) a cylindrical quartz cuvette of 1 cm path length was used. Each spectrum represents the average of five successive scans performed at a scan speed 20 nm/min and a bandwidth of 1 nm. The reported spectra are the average of five scans. Each spectrum was subtracted from the buffer baseline subtraction and smoothed within the permissible limits using the in-built software of the instrument.

3. Results and discussions

3.1. ATA specifically binds to DNase I and inhibited enzymatic activity of DNase I

We digested 315 bp DNA as obtained from PCR with 50 μg/ml DNase I in absence and presence of various concentrations of ATA (0–200 μM). DNase I activity decreased dose-dependently with increasing concentration of ATA as detected by the intensity of DNA band (315 bp) in gel (data not shown). The DNase I activity was completely inhibited at 200 μM ATA (data not shown).

We have also carried out association of ATA with DNase I as detected by Trp fluorescence and CD spectroscopy. To a fixed concentration of DNase I (1.33 μM) in assay buffer as used in biochemical assay of DNase I, increasing concentrations of ATA were added and subsequently tryptophan (trp) fluorescence was measured in Fluoromax3. The tryptophan fluorescence was quenched gradually with increasing concentration of ATA indicating the association of DNase I with ATA as shown in Fig. 2. The dissociation constant (K_d = 9.019 μM) was calculated from the double-reciprocal plot as shown inset in Fig. 2. The result implicated that ATA specifically binds DNase I. We have used CD spectroscopy to elucidate whether the association of DNase I with ATA results any structural change of the DNase I or not. Here in Fig. 3 shows the near-UV CD spectra (inset, far-UV CD spectra) of free DNase I (solid line) and DNase I with saturating ATA (44 μM) concentration (dotted line). It is known that near-UV and far-UV CD spectra correspond to tertiary structure and secondary structure of protein respectively. The CD spectrum of a protein in the near-UV spectral region (250–350 nm) is contributed only by the aromatic amino acids (phenylalanine, tyrosine, tryptophan) and disulfide bonds. So, the magnitude of the observed change in the near-UV

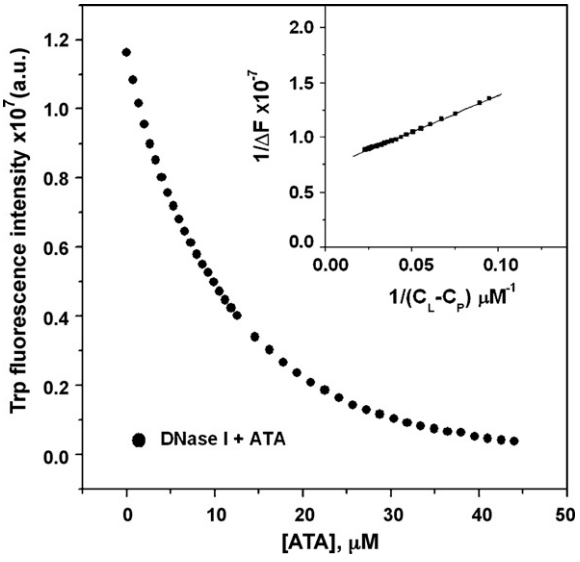

Fig. 2. Binding isotherm for the interaction of DNase I with ATA at 25 °C. The tryptophan fluorescence emission intensity (λ_{ex} = 295 nm, λ_{em} = 340 nm) was plotted as a function of ligand (ATA) concentration C_L. Inset shows the corresponding plot of $1/\Delta F$ (ΔF denotes the change in fluorescence) against $1/(C_L - C_P)$ to evaluate the dissociation constant by means of Eq. (1), where C_P is the protein concentration.

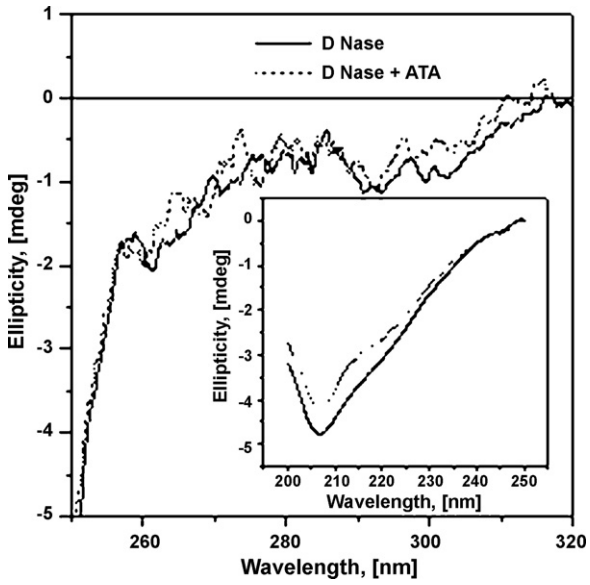

Fig. 3. The near-UV and far-UV (inset) CD spectra of 1.33 μM of DNase I as denoted by solid line and ATA (44 μM) bound DNase I as denoted by dotted line in DNase I assay buffer at 25 °C was measured in Hitachi 410.

spectra does not entirely reflect the actual changes occurring in the tertiary structure, which can be dominated by contributions from the non-aromatic residues but have no visible impact on the spectra. In our case, changes in the spectra were observed in the regions 260–280 nm and 295–320 nm. In addition, the CD data in the near-UV region is less noisy than in the far-UV. In light of the good signal-to-noise ratio of this data, it can be concluded that the small changes at 260–280 nm and 295–320 nm were not due to noise but due to the overall tertiary structure change of DNase I upon ATA binding. It is known that far-UV spectral region (190–250 nm) correspond to secondary structure of protein since radiation in this wavelength region is absorbed by peptide bonds of the protein. There was a significant change in far-UV region as shown in inset (noise corrected spectrum) indicating that secondary structure of DNase I was altered upon ATA binding at 44 μM ATA. Such perturbation of three-dimensional structure of enzyme most likely was responsible for inhibition of DNase I by ATA.

Our earlier study showed that ATA inhibited apoptosis induced by benzamide (a PARP inhibitor) or γ-radiation through the inhibition of nuclear fragmentation and DNA ladder formation without affecting the upstream cascades like caspase-3 activation or cytochrome c release [11]. Moreover, H-7 and staurosporin, the PKC inhibitors induced apoptosis through caspase-3 activation and DNA ladder formation, and ATA inhibited nucleosomal ladder formation by the inhibitors without changing the caspase-3 activity (unpublished). The mechanisms by which the PARP inhibitor (benzamide), γ-radiation and PKC inhibitors (H-7 and staurosporin) initiate apoptosis are likely to be different. However, the last step of apoptosis (nuclear fragmentation/nucleosomal ladder) is common to all agents. Since ATA inhibited nucleosomal ladder/nuclear fragmentation without affecting its upstream cascade like cytochrome c release or 13 activation, ATA likely modulates the DNases involved in apoptosis. In support, a large number of reports showed that ATA, a general nuclease inhibitor, inhibited DNases and apoptosis induced by different types of stimulus [4–8]. Various types of nuclease like DNase I, Dnase γ, caspase activated DNase (CAD) or DNaseIL3 [13–16] are involved in nucleosomal ladder formation. It has been observed that restriction enzymes like Sal I, Bam H1, Pst I, Sma I are inhibited in vitro by ATA treatment [4]. Here, we observed that DNA cleaving activity of DNase I, an important member of nucleases involving apoptosis, was inhibited by ATA. DNase I also physically interacted with ATA (K_d = 9.019 μM). ATA may inhibit DNase I activity after competitive binding or changing the three-dimensional conformation of DNase I. We propose that ATA might interact with other DNases involved in apoptosis and thus inhibited apoptosis.

3.2. ATA inhibited RNA digestion by RNase A

Total RNA was extracted from exponentially growing Chinese hamster V79 cells following the standard protocol and estimated the concentration spectrophotometrically. Five micrograms of RNA was treated with RNase A (4.5 μg/ml) in absence and presence of ATA (1, 10, 50 100, 150, 200 μM) in buffer (150 mM NaCl, 5 mM EDTA, 10 mM Tris–Cl pH 7.5) for 10 min at 37 °C. The intensity of undigested RNA increases with the ATA concentrations (data not shown), indicating that ATA inhibited RNase A activity.

Trp fluorescence study revealed that ATA physically interact with RNase A. RNase A (3.52 μM) was titrated with ATA and subsequently Trp fluorescence was measured in Hitachi 410. The result showed quenching of Trp fluorescence with the increase of ATA concentration implicating the association of RNase A with ATA as shown in Fig. 4. The dissociation constant (K_d = 2.33 μM) was calculated from the double-reciprocal plot as shown inset in the same picture. We have also used CD spectroscopy for studying structural perturbation, if any, of RNase A after binding ATA. The CD spectra of free RNase A was denoted by solid line where as ATA (9 μM) bound RNase A was denoted by dotted line as shown in Fig. 5. There was little change in the far-UV spectra of RNase A after saturating with 9 μM ATA as shown inset, indicating a slight change in secondary structure induced by ATA binding. On the other hand, a significant change in near-UV spectra indicated that ATA induced significant alteration of tertiary structure of RNase A upon binding. This result showed that competitive binding or alteration of three-dimensional conformation of RNase A by ATA most likely inhibited the RNA cleavage activity of RNase A.

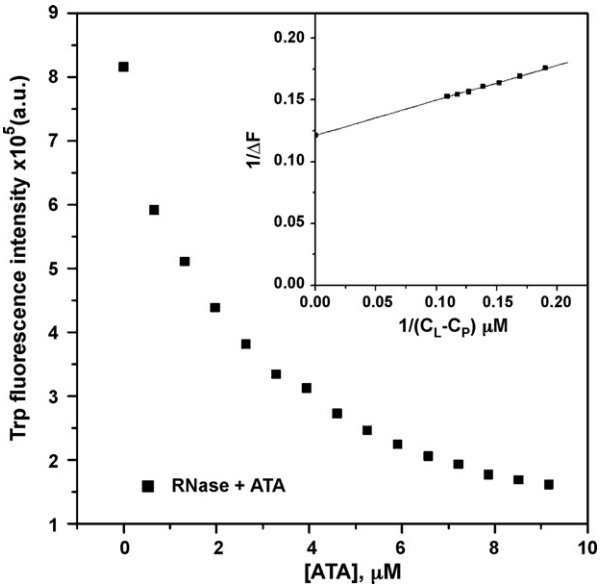

Fig. 4. Binding isotherm for the interaction of RNase A with ATA at 25 °C. The tryptophan fluorescence emission intensity (λ_{ex} = 295 nm, λ_{em} = 340 nm) was plotted as a function of ligand (ATA) concentration C_L. Inset shows the corresponding plot of $1/\Delta F$ (ΔF denotes the change in fluorescence) against $1/(C_L - C_P)$ to evaluate the dissociation constant by means of Eq. (1), where C_P is the protein concentration.

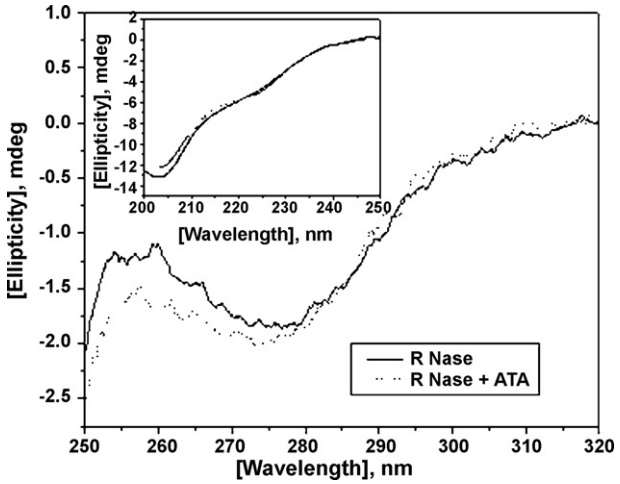

Fig. 5. The near-UV and far-UV (inset) CD spectra of 3.52 μM of RNase A (solid line) and 9 μM ATA bound RNase A (dotted line) in RNase assay buffer was measured in Hitachi at 25 °C.

3.3. Reverse transcriptase activity was inhibited by ATA

The cDNA was prepared with ^{32}P-α-dCTP along with cold dNTPs in presence of different concentration of ATA (0–100 μM). The amount of cDNA produced was decreased dose-dependently with ATA concentration as observed in autoradiogram (data not shown). This result indicated that ATA inhibited reverse transcriptase activity in vitro.

To the reverse transcriptase (1.168 μM) solution in first strand buffer as used in biochemical assay of reverse transcriptase, increasing concentration of ATA were added and subsequently Trp fluorescence was measured in Hitachi 410. The fluorescence was quenched with increasing concentration of ATA indicating the association of reverse transcriptase with ATA as shown in Fig. 6. The dissociation constant (K_d = 0.255 μM) was calculated from the double-reciprocal plot as shown inset in Fig. 6, implicating that ATA

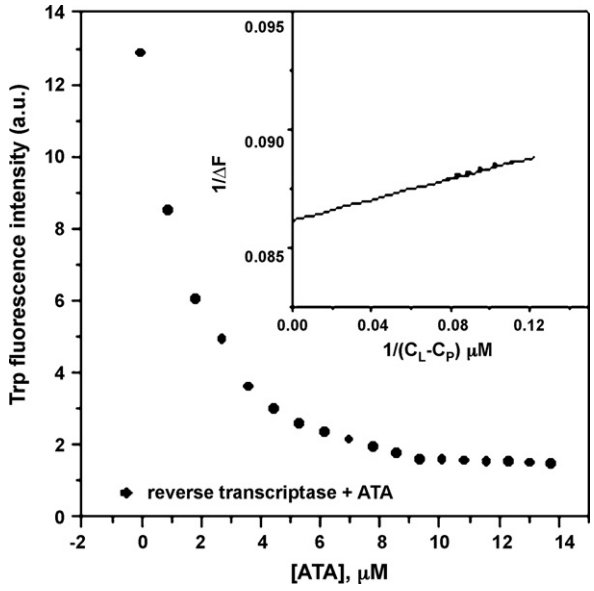

Fig. 6. The tryptophan fluorescence spectra of free M-MLV reverse transcriptase and ATA saturated (14 μM) reverse transcriptase in assay buffer at 25 °C. The tryptophan fluorescence emission intensity, (λ_{ex} = 295 nm, λ_{em} = 340 nm) was plotted as a function of ligand (ATA) concentration C_L. Inset shows the corresponding plot of $1/\Delta F$ (ΔF denotes the change in fluorescence) against $1/(C_L - C_P)$ to evaluate the dissociation constant by means of Eq. (1), where C_P is the protein concentration.

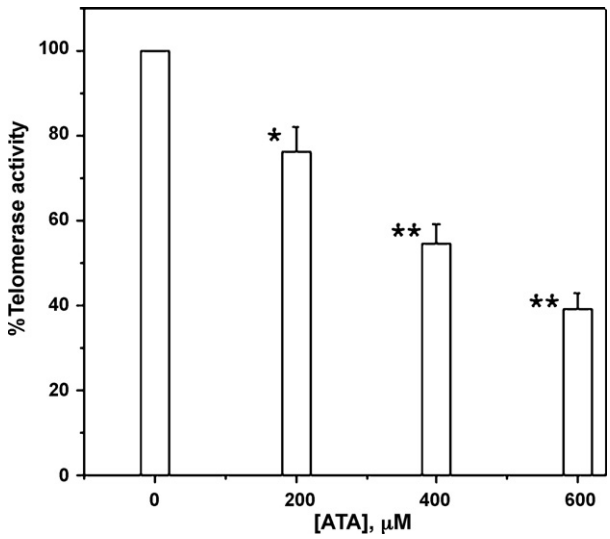

Fig. 7. Telomerase activity as detected by TRAP assay in V79 cells treated with different concentration of ATA. Histograms show the semi-quantitative representation of telomerase activity obtained by densitometric analysis of telomeric product obtained from three independent experiments. Standard deviations are shown by the vertical lines. The p-values $0.01 < p \leq 0.05$ and $0.001 < p \leq 0.01$ were denoted as '*' and '**', respectively.

has a strong affinity to the reverse transcriptase. Anti-viral activity of ATA was reported due to its ability to bind several viral proteins like RNA-dependent RNA polymerase (RdRp), S1, HIV integrase and likely to alter their functions [17,18]. Anti-reverse transcriptase activity of ATA has been demonstrated by in vitro assay [20]. This result indicated that inhibition of reverse transcriptase activity was mediated by direct interaction of ATA.

3.4. Inhibition of telomerase activity in Chinese hamster V79 cells treated with ATA

ATA's reverse transcriptase inhibition inspired us to investigate its effect on telomerase activity. TRAP assay was carried out using the V79 cell extracts prepared after treatment with different concentrations of ATA for 48 h. Detail methods for detection of telomerase activity were described in our earlier study [40]. Telomerase activity was reduced to 76 ± 6%, 54.5 ± 4.5% and 39 ± 4% of untreated control after treatment with 200 μM, 400 μM and 600 μM ATA, respectively, as shown by bar diagram in Fig. 7. The p-values $0.01 < p \leq 0.05$ and $0.001 < p \leq 0.01$ were denoted as '*' and '**', respectively. Telomerase activities after ATA treatment were significantly smaller than that of untreated control. We showed in Section 3.3 that ATA can strongly (K_d = 0.255 μM) bind to the reverse transcriptase and thereby inhibited enzymatic activity of reverse transcriptase. Since telomerase reverse transcriptase (TERT) is the main catalytic subunit of telomerase holoenzyme, possibly ATA binds to TERT and inhibited telomerase activity in cells.

3.5. ATA inhibited activity of Taq polymerase as detected by PCR

We have carried out PCR using cDNA (prepared from RNA) as template in presence and absence of ATA. The band intensity of PCR product decreased dose-dependently with the increase of ATA concentration (data not shown). This result indicated that ATA inhibited Taq polymerase activity in vitro.

Taq polymerase (0.4588 μM) was taken in PCR buffer and gradually ATA was added and subsequently Trp fluorescence spectra were measured in fluorescence spectrophotometer Hitachi 410. Successive quenching of tryptophan fluorescence was observed with increasing concentration of ATA as shown in Fig. 8. The

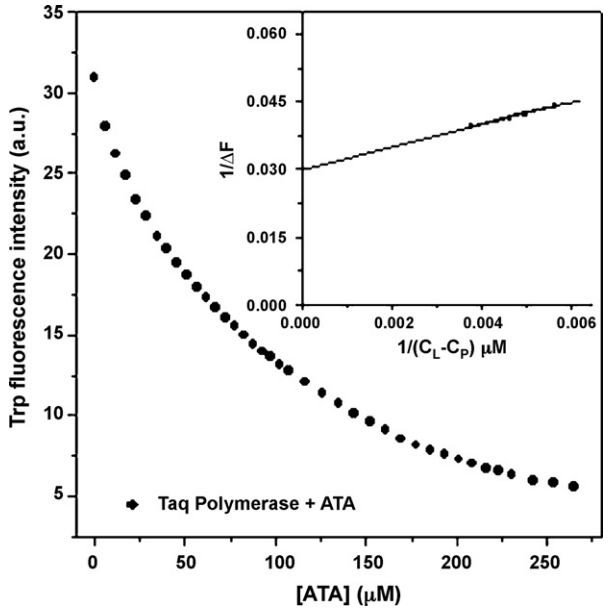

Fig. 8. The tryptophan fluorescence spectra of free Taq polymerase and ATA saturated (260 μM) Taq polymerase in PCR buffer at 25 °C. The tryptophan fluorescence emission intensity (λ_{ex} = 295 nm, λ_{em} = 340 nm) was plotted as a function of ligand (ATA) concentration C_L. Inset shows the corresponding plot of $1/\Delta F$ (ΔF denotes the change in fluorescence) against $1/(C_L - C_P)$ to evaluate the dissociation constant by means of Eq. (1), where C_P is the protein concentration.

dissociation constant (K_d = 81.97 μM) was calculated from double-reciprocal plot as shown in inset picture. The results indicated that ATA specifically binds Taq polymerase. The near-UV and far-UV CD (inset) spectra of Taq polymerase only (solid line) and ATA (260 μM) bound Taq polymerase (dotted line) as shown in Fig. 9 indicated that a significant change in both secondary and tertiary structure of Taq polymerase upon binding with ATA. This result again established that ATA inhibited Taq polymerase activity either competitive binding or allosterically like other nucleic acid binding proteins as mentioned above.

A number of report showed that ATA inhibited in vitro DNA synthesis activity by DNA polymerase [28]. The activities of the

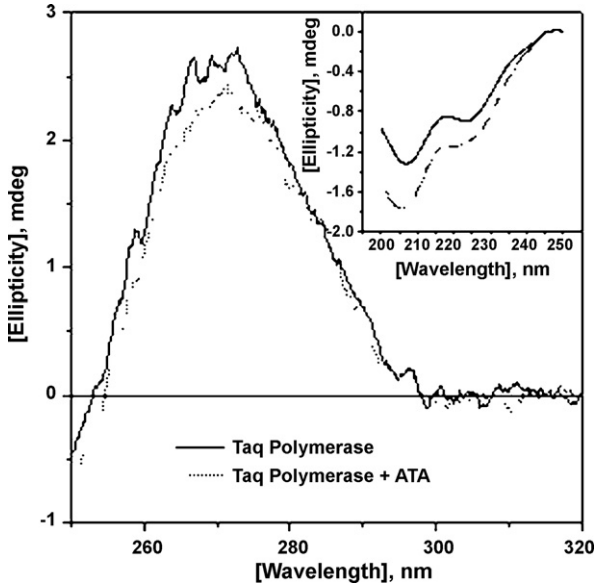

Fig. 9. The near-UV and far-UV (inset) CD spectra of 0.4588 μM of Taq polymerase (solid line) and 260 μM ATA bound Taq polymerase (dotted line) in PCR buffer were measured in Hitachi 410 at 25 °C.

Table 1
The dissociation constants (K_d) of various enzymes with ATA.

Protein (final conc.)	K_d (μM)
DNase I (1.33 μM)	9.019
RNase A (3.52 μM)	2.33
Taq polymerase (0.4588 μM)	81.97
Reverse transcriptase (1.168 μM)	0.255

purified human DNA polymerases alpha, beta, gamma, and DNA primase as well as those of DNA polymerase I and RNA polymerase from *E. coli* are also inhibited by ATA [29]. We observed that DNA polymerizing activity of Taq polymerase was inhibited by ATA. Trp fluorescence revealed that Taq polymerase physically interacted with ATA (K_d = 81.97 μM). Such ATA binding resulted alteration of three-dimensional conformation of polymerase (as detected by CD analysis) that might resulted the loss of its DNA polymerizing activity.

4. Conclusion

In vitro enzymatic activity of DNAse I, RNase A, reverse transcriptase and Taq polymerase was inhibited by ATA treatment in a concentration-dependent manner. Telomerase activity in Chinese hamster V79 cells was inhibited dose-dependently by ATA. The K_d values obtained from spectroscopic analysis at 25 °C were shown in Table 1. This data implicated that affinity of ATA was greater with reverse transcriptase (K_d = 0.255 μM) and less with Taq polymerase (K_d = 81.97 μM) compared with other enzymes studied. ATA interferes with DNA or RNA binding to the respective proteins either competitively or allosterically (i.e. by perturbing three-dimensional structures of enzymes). Further studies are required to look into the specificity of the enzymes for the interaction with ATA.

Acknowledgements

We are grateful to Prof. Soumen Basak, Chemical Sciences Division, Saha Institute of Nuclear Physics, 1/AF Bidhannagar, Kolkata 700064, India, for useful discussions in designing experiments and critical analysis of spectroscopic data. This work was partially funded by Department of Science and Technology (DST), Government of India.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.saa.2009.09.024.

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