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Mechanism of Action of Polymeric Aurintricarboxylic Acid, a Potent Inhibitor of Protein–Nucleic Acid Interactions†

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ABSTRACT: The mechanism of inhibition of protein–nucleic acid complex formation by polymeric aurintricarboxylic acid (ATA) was investigated by proton magnetic resonance spectroscopy. The approach was the synthesis of totally deuterated ATA, followed by a 100-MHz proton magnetic resonance study of its interaction with bovine pancreatic ribonuclease A (RNase), a model nucleic acid binding protein. The binding of ATA to RNase elicited chemical shift changes and line broadening in the C(2)–H resonances of histidyl residues 12 and 119, both of which are located in the active site, whereas that of histidyl residue 105, which resides on the exterior of the protein structure, is unaffected. (Histidyl residue 48 is not observed under our conditions except at high pH.) The ϵ-methylene protons of the lysyl side chains were also broadened upon the binding of ATA. Polymeric ATA displaces cytidine 2′-monophosphate and cytidine 3′-monophosphate from the active site of the enzyme as revealed by nuclear magnetic resonance spectroscopy. These observations suggest that the mechanism of action of ATA involves competition between the nucleic acid and the polymeric ATA for binding in the active site of the protein. Electron spin resonance spectroscopy reveals that polymeric ATA is a stable free radical, thus accounting for the major line broadening effect upon binding to protein. This finding may provide a powerful means of probing the nucleic acid binding site of proteins by proton magnetic resonance spectroscopy.

Aurintricarboxylic acid (ATA)[1] has been extensively utilized by molecular biologists as a powerful inhibitor of proteins whose biological function depends on the formation of a complex with nucleic acid. Numerous studies have employed ATA in the investigation of both prokaryotic and eukaryotic systems [for reviews, see Apirion & Dohner (1975), Grollman & Huang (1976), and Schleich et al. (1978)]. Interpretation of the mechanism of inhibitory activity of ATA has been difficult because commercially available preparations of this reagent contain numerous components, not all of which possess inhibitory activity (Huang & Grollman, 1972; Givens & Manly, 1976; Steward et al., 1977; Blumenthal & Landers, 1973; Tsutsui et al., 1978). Recent work in our laboratory revealed that the active components present in ATA preparations are a collection of heterogeneous polymers of the phenol–formaldehyde type (Schleich et al., 1978; González et al., 1979).

The objective of the present study was the elucidation of the mechanism by which ATA prevents the formation of a protein–nucleic acid complex. Our approach was the synthesis of totally deuterated ATA, followed by a proton magnetic resonance study of its interaction with ribonuclease A (RNase A), a model nucleic acid binding protein (Jensen & von Hippel, 1976, and references cited therein). RNase A was chosen because of its well-known ability to bind both RNA and DNA, because of its ready availability, and because this protein has been extremely well characterized by a variety of physical techniques including ^{1}H nuclear magnetic resonance (NMR) spectroscopy [for reviews, see Roberts & Jardetzky (1970) and Benz & Roberts (1973)].

Materials and Methods

Bovine pancreatic RNase, type XII-A, the free acids of cytidine 2′-monophosphate (2′-CMP), and cytidine 3′-monophosphate (3′-CMP) were obtained from Sigma Chemical Co. "Aluminon" grade ATA was purchased from Aldrich (lot 061757). Nitrobenzene-d_5 (99 atom % ^{2}H), paraformaldehyde-d_2 (98 atom % ^{2}H), methanol-d_4 (99 atom % ^{2}H), and sulfuric acid-d_2 (99 atom % ^{2}H) were obtained from Merck Sharpe & Dohme Canada Ltd.

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[1] Abbreviations used: ATA, aurintricarboxylic acid; 2′-CMP, cytidine 2′-monophosphate; 3′-CMP, cytidine 3′-monophosphate; RNase, bovine pancreatic ribonuclease A (EC 3.1.4.22); NMR, nuclear magnetic resonance; ESR, electron spin resonance; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; HMDS, hexamethyldisiloxane; ppm, parts per million.

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Deuterated ATA was prepared from salicylic acid-d_4 and formaldehyde-d_2. The salicylic acid-d_4 was prepared as follows. Nitrobenzene-d_5 was reduced catalytically to aniline-d_5 by using 10% palladium on charcoal under an atmosphere of hydrogen, from which phenol-d_5 was synthesized according to Vogel (1972). Salicylic acid was prepared from this material according to Gottesman & Chin (1968). It gave a melting point of 157 °C. Formaldehyde-d_2 was prepared by refluxing paraformaldehyde-d_2 in ^2H_2O for 48 h. Methanol-d_4 (10% w/w) was then added and the mixture was allowed to reflux for an additional 12 h. (The methanol was added to approximate commercial formalin preparations.) The deuterated ATA was synthesized according to Welcher (1947) using salicylic acid-d_4, the deuterated formalin solution, sulfuric acid-d_2, and $NaNO_2$. The product gave an ^1H NMR spectrum which revealed the residual protons resonating as a broad band centered at 6.7 ppm downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS).

Both the deuterated and commercial preparations of ATA were fractionated according to published procedures (Schleich et al., 1978; González et al., 1979) using dialysis (Spectra Por tubing, 3500 M_r cutoff) and ultrafiltration (Amicon Diaflo UM 05 ultrafilter, 500 M_r cutoff). The highest molecular weight fraction (>3500) was designated fraction I and the intermediate molecular weight fraction (500–3500) was designated fraction II.

The labile protons of RNase were exchanged for deuterium as follows. RNase was dissolved in ^2H_2O (1% w/w) and allowed to stand at room temperature for 24 h. The solution was then concentrated by ultrafiltration (Amicon Diaflow UM 2 ultrafilter, 1000 M_r cutoff) to 10% of the original volume; ^2H_2O was again added to approximate a 1% RNase solution and the procedure was repeated, followed by lyophilization and storage at 4 °C in a desiccator.

All solutions for the NMR experiments contained 0.2 M NaCl. The RNase concentration in all experiments was 1.5×10^{-3} M which was determined spectrophotometrically (Sage & Singer, 1962). ATA stock solutions were prepared by weight by using the free acid, and enough NaO^2H was added to bring the acid form of the dye into solution. The free acids of 2′-CMP and 3′-CMP were dissolved in a similar fashion. All pH adjustments were made by using NaO^2H and ^2HCl (5%) in ^2H_2O; pH values given represent the actual meter reading and are uncorrected for the deuterium isotope effect.

Proton NMR spectra were obtained at 100 MHz by using a JEOL FX 100S spectrometer outfitted with a 10-mm sample probe. A 180°–τ–90° pulse sequence was employed and the delay time (τ) was adjusted for optimum nulling of the residual water resonance. Spectra were collected using the "block averaging" technique; 8K word Fourier transforms were obtained with a spectral width of 1000 Hz. Typically 100 free induction decays were transformed and averaged. Hexamethyldisiloxane (HMDS) in CCl_4 contained in a coaxial tube served as the external standard. The probe temperature was 32 °C. Electron spin resonance (ESR) spectra were recorded by using a Varian E-3 spectrometer.

RNase activity assays (in the presence and absence of ATA) were performed at 24 °C essentially according to Kalnitsky et al. (1959) using yeast RNA (Calbiochemical Co.) as a substrate. Percent activity was calculated as 100 times the ratio of the inhibited RNase assay to the control RNase assay. Each assay was done in triplicate, and the resulting error was ca. ±5%.

Results

The inhibitory activities of the high molecular weight fractions (fraction I) of commercially available and the deuterated ATA prepared by us were compared using the assay procedure described above; the activity of deuterated ATA fraction II was also determined. As shown in Figure 1, the most active preparation was fraction I of the commercially available material, with fractions I and II of the deuterated ATA displaying high and marginal inhibitory RNase activity, respectively. The ultraviolet–visible spectra of both types of fraction I were very similar to those published previously (Schleich et al., 1978; González et al., 1979).

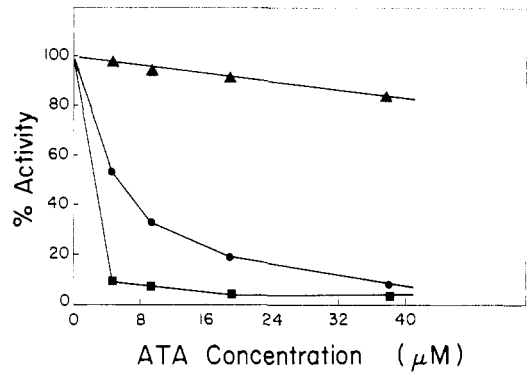

FIGURE 1: Aurintricarboxylic acid inhibition of RNase activity. Aldrich fraction I ATA (■); deuterio-ATA fraction I (●); deuterio-ATA fraction II (▲). See Materials and Methods for details.

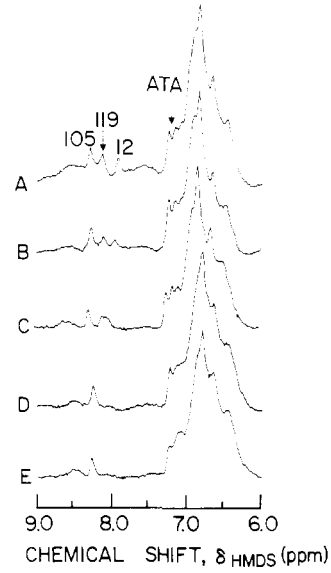

FIGURE 2: Low-field portion of the 100-MHz ^1H NMR spectrum of RNase in the presence of increasing aurintricarboxylic acid concentrations (monomer molar equivalents): (A) no ATA; (B) 1.2 mM ATA; (C) 2.4 mM ATA; (D) 6.0 mM ATA. Protein concentration is 1.5 mM in 0.2 M NaCl; pH 6.5 (uncorrected); temperature = 32 °C; 100 accumulations in the Fourier transform mode. See Materials and Methods for complete NMR details. The arrow denotes the position of the residual proton resonance of deuterated ATA.

^1H NMR spectra were obtained for RNase (1.5×10^{-3} M) between the pH values 6.5 and 10.0 at 0.5 pH unit intervals, both in the absence and in the presence of different fraction I deuterio-ATA concentrations. (The lower limit of pH 6.5 was chosen because precipitation was observed below this pH at the ATA concentrations of interest.) As shown in Figure 2 (spectrum A) the C(2)–H resonances of histidyl residues 105, 119, and 12 are clearly evident at pH 6.5 in the presence of 0.2 M NaCl. The assignments were made according to Patel et al. (1975). As observed previously (Roberts et al., 1969), the C(2)–H resonance of His-48 is not evident under these solvent conditions. Upon the addition of increasing

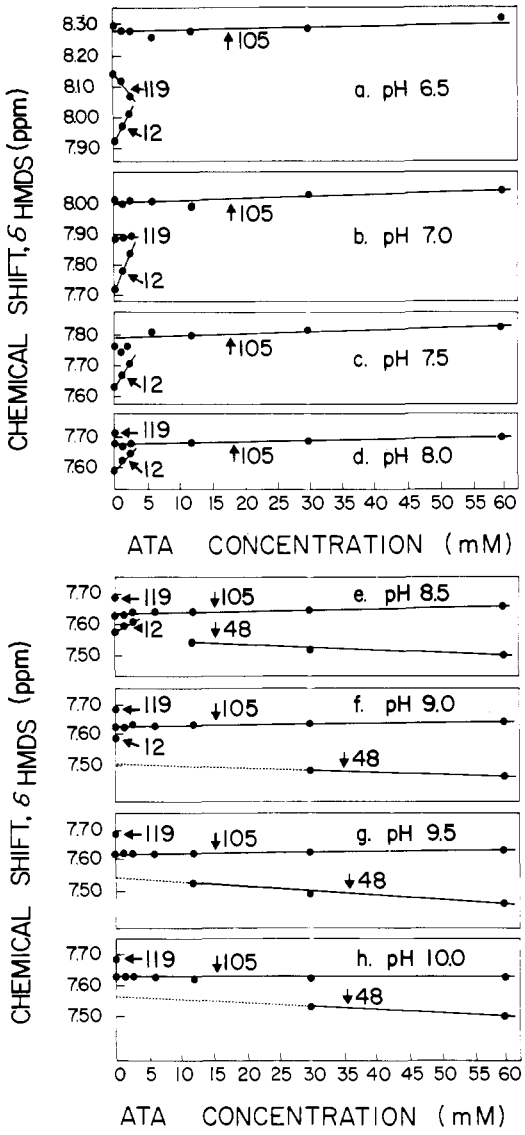

FIGURE 3: Effect of aurintricarboxylic acid on the chemical shift of the RNase C(2)–H histidyl residue resonances at different pH values (uncorrected): (a) pH 6.5; (b) pH 7.0; (c) pH 7.5; (d) pH 8.0; (e) pH 8.5; (f) pH 9.0; (g) pH 9.5; (h) pH 10.0. Protein concentration is 1.5 mM. ATA concentration is expressed in monomer molar equivalents. See Figure 2 for NMR details. All evident resonances are included.

concentrations of deuterio-ATA at pH 6.5, the C(2)–H resonances of His-119 and -12 are observed to move upfield and downfield, respectively, while the chemical shift of the C(2)–H resonance of His-105 sustains a very slight downfield shift; a slight broadening of these resonances (except His-105) also occurs (spectra B and C, Figure 2). Coalescence occurs at an ATA concentration (monomer molar equivalents) of 2.4×10^{-3} M (spectrum C, Figure 2). At higher ATA concentrations ($>6.0 \times 10^{-3}$ M), the broadening is so severe that both of these resonances are no longer apparent (spectra D and E, Figure 2). These spectral data are also shown in Figure 3a where the chemical shift of each C(2)–H resonance is plotted against ATA concentration. Similar behavior was also observed at higher pH values (Figure 3b–h). At all pH values examined, the C(2)–H chemical shift of His-105 sustained a very small downfield shift with increasing dye concentrations. In each case where the C(2)–H resonance of His-12 was apparent (pH 6.5–8.5), increasing ATA concentrations resulted in a pronounced downfield shift (Figure 3a–e); above pH 8.5 this resonance was not apparent even at the lowest

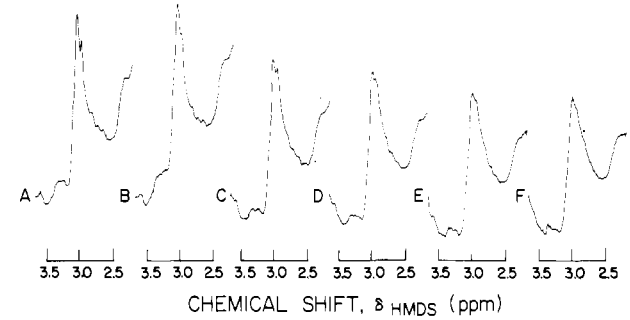

FIGURE 4: High-field portion of the 100-MHz ^{1}H NMR spectrum of RNase in the presence of increasing aurintricarboxylic acid concentrations (monomer molar equivalents): (A) no ATA; (B) 1 mM ATA; (C) 2 mM ATA; (D) 3 mM ATA; (E) 4 mM ATA; (F) 6 mM ATA. Protein concentration is 1.5 mM in 0.2 M NaCl at pH 6.5 (uncorrected). See Figure 2 for NMR details.

ATA concentration employed (1.2×10^{-3} M), presumably because of merger with the His-105 resonance (Figure 3f–h). In contrast to the behavior observed for the C(2)–H resonance of His-119 at pH 6.5, increasing ATA concentrations at pH 7.0 produced little change in the chemical shift of this resonance (Figure 3b). At concentrations of ATA greater than 2.4×10^{-3} M, this resonance broadens beyond detection (Figure 3b). Above pH 7.5 the His-119 resonance is not evident at the lowest concentration of ATA used (Figure 3c–h) due to merger with the nearby His-105 resonance. At pH values greater than 8.0 a new resonance appears in the chemical shift range of 7.45–7.55 ppm, but only at ATA concentrations of 12×10^{-3} M or greater (Figure 3e–h). The chemical shift of this resonance moves slightly upfield in a linear fashion with increasing ATA concentration (Figure 3e–h); extrapolation to zero inhibitor dye concentrations yields a chemical shift value of 7.53 ± 0.02 ppm.

The aliphatic region of the ^{1}H NMR spectrum of RNase also exhibited changes upon the addition of deuterio-ATA. The most pronounced spectral changes occurred in the relatively broad resonance located at 3.0 ppm. This resonance is dominated by the overlapping ϵ-methylene protons of the 10 lysyl residues present in the protein (Brown & Bradbury, 1975). Figure 4 shows the broadening effect of this resonance with increasing ATA concentrations at pH 6.5. This effect was observed throughout the pH range examined in our experiments.

We also investigated the effect of ATA on the interaction of 3′-CMP and 2′-CMP with RNase. The ^{1}H NMR spectrum of RNase A complexed with these mononucleotides was first reported by Meadows & Jardetzky (1968). They showed that 3′-CMP was bound at the active site of the enzyme, thereby inducing downfield shifts in the C(2)–H resonances of His-12 and -119 and in the C(6)–H and C(5)–H resonances of cytidine. We repeated this experiment and our results are in complete accord with the published work, as shown in Figure 5 (spectrum A). Increasing deuterio-ATA concentrations elicited three spectral effects (Figure 5): (1) the collapse of the merged His-12 and -119 resonances; (2) an upfield shift of the cytidine C(6)–H and C(5)–H resonance doublets; (3) an increase in the intensity of the cytidine doublet resonances (relative to the intensity of His-105). At an inhibitor concentration of 6.0×10^{-3} M, the RNase spectrum is identical (with the exception of the cytidine resonances) with that obtained using the same concentration of inhibitor without 3′-CMP. The broad low resonance that remains downfield from the C(2)–H resonance of His-105 arises from the residual peptide NH protons (Roberts et al., 1969).

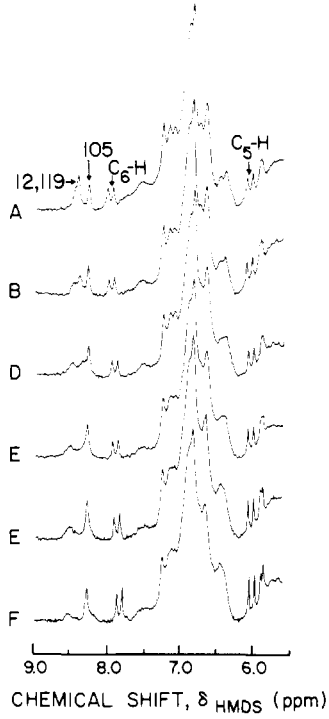

FIGURE 5: Low-field portion of the 100-MHz ^{1}H NMR spectrum of RNase (1.5 mM) and 3′-CMP (1.9 mM) in the presence of increasing aurintricarboxylic acid concentrations (monomer molar equivalents): (A) no ATA; (B) 1 mM ATA; (C) 2 mM ATA; (D) 3 mM ATA; (E) 4 mM ATA; (F) 6 mM ATA. The solution contained 0.2 M NaCl at pH 6.5 (uncorrected). See Figure 2 for NMR details.

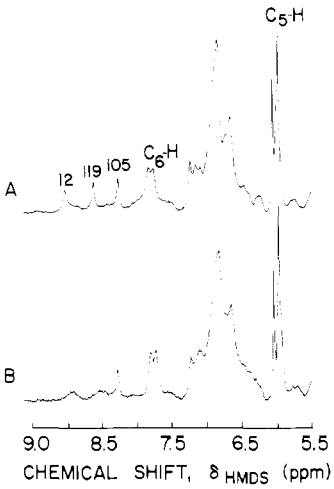

FIGURE 6: Low-field portion of the 100-MHz ^{1}H NMR spectrum of RNase (1.5 mM) and 2′-CMP (8.5 mM) in (A) the absence and (B) the presence of 8.6 mM aurintricarboxylic acid. The solution contained 0.2 M NaCl at pH 6.5 (uncorrected). See Figure 2 for NMR details.

The effect of polymeric ATA on the interaction between 2′-CMP and RNase A was also investigated. The spectrum of RNase in the presence of saturating amounts of 2′-CMP is shown in Figure 6 (spectrum A), whereas spectrum B of this figure demonstrates the effect of the addition of an equimolar concentration of deuterio-ATA polymer (relative to 2′-CMP). The His-12 and -119 C(2)–H resonances almost disappear, remaining as very broad resonances, and the C(6)–H and C(5)–H cytidine resonances sharpen and sustain an intensity increase concomitant with an upfield shift.

We were initially puzzled by the relatively selective resonance broadening induced by the binding of the ATA polymer

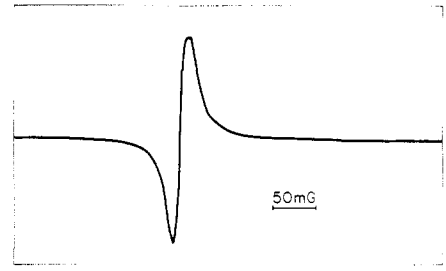

FIGURE 7: ESR spectrum of deuterioaurintricarboxylic acid fraction I. The ATA concentration in water is 1.2 mM.

to RNase. We therefore explored the possibility that the ATA polymer was a paramagnetic species. The ESR spectrum of the deuterio-ATA polymer (fraction I) is shown in Figure 7 and establishes the paramagnetic character of the polymer. Fraction I prepared from commercially available material gave a very similar ESR spectrum.

Discussion

The synthesis of ATA was first described by Caro (1892), and over the years several modifications of this procedure have been described, culminating with a supposedly pure preparation by Smith et al. (1949). More recently, interest in ATA has been evinced by molecular biologists who employ this triphenylmethane dye for the inhibition of a variety of cellular processes that ultimately depend on the formation of a protein–nucleic acid complex. However, it has been reported that pure ATA has one-tenth the biological activity of the commercial preparations which contain several components (Huang & Grollman, 1972). In previous work, we have shown that the active components of ATA are polymeric and that inhibitory activity varies directly with increasing molecular weight (Schleich et al., 1978; González et al., 1979).

The preparation of ATA according to the procedure of Welcher (1947) yields a product containing a considerable amount of polymeric material whose inhibitory activity is comparable to that of the polymeric components obtained from commercially available preparations. We synthesized a totally deuterated ATA sample according to this procedure, followed by fractionation on the basis of size. The higher molecular weight fraction, designated fraction I (M_r >3500), was similar in inhibitory activity to fraction I prepared from the commercially available product in the RNase activity assay (Figure 1). Furthermore, fraction II obtained from the deuterated preparation possessed marginal inhibitory activity, reaffirming our previous reports that ATA activity is dependent upon polymeric molecular weight. Also, the ultraviolet–visible spectral properties of both fraction I preparations were nearly identical. We therefore conclude that the deuterated ATA preparation closely approximates the highly active commercially available material that is so widely utilized.

Histidyl residues 12 and 119 are located in the active site of RNase, and the chemical shifts of their C(2)–H resonances are a sensitive monitor of inhibitor binding (Meadows et al., 1969). The chemical shift and line broadening data shown in Figures 2 and 3 imply that at all dye concentrations employed, the ATA polymer binds in the active site of the enzyme. Since His-105 is located outside of the active site and is freely exposed to the solvent milieu (Patel et al., 1975), the observation that its chemical shift is unaffected by the binding of ATA would further imply that no extensive binding occurs on the exterior of the protein in the vicinity of this residue. The new resonance which emerges at pH values greater than 8.0 in the presence of ATA we suggest arises from the C(2)–H of His-48. This assignment is supported by the observations

that this resonance sharpens considerably in the presence of NaOAc (Roberts et al., 1969) and at pH values greater than 6.5 (Patel et al., 1975) and the fact that it is the most upfield of the histidyl C(2)–H resonances (Roberts et al., 1969; Patel et al., 1975). Furthermore, consideration of the Harker X-ray structure of RNase A visualized by a molecular modeling computer graphics system reveals that His-48 is buried and located as far away from the active-site region as His-105, thereby rendering it nonsusceptible to the broadening effects described above.

Examination of the aliphatic region of the RNase NMR spectrum reveals that the ATA polymer interacts strongly with the lysyl residues as evidenced by the increased broadening of the resonance located at 3.0 ppm. This effect is apparent over the entire pH range and at the lowest ATA concentrations examined. This observation is plausible with the interpretations made above since 3 of the 10 lysyl residues and 3 of the 4 arginyl residues of RNase A lie at or near the active site and because of the pK values of all the lysyl residues (except lysine-41 which is in the active site) is $\sim$10.9 (Brown & Bradbury, 1975)).

The interaction between RNase and the nucleoside monophosphates, 3′-CMP and 2′-CMP, has been well characterized by proton NMR spectroscopy (Meadows & Jardetzky, 1968; Meadows et al., 1969). We therefore felt it useful to investigate the interaction of ATA with the enzyme complexed to these nucleoside monophosphates. The merger and eventual collapse of the His-12 and -119 resonances, concomitant with the chemical shift and line width changes in the cytidine C(5)–H and C(6)–H resonances with increasing ATA concentration, imply that the dye occupies the enzyme active site with the displacement of the nucleoside monophosphate.

The above observations suggest that the mechanism of action of ATA in the inhibition of protein–nucleic acid complex formation is competition between the nucleic acid and the polymeric ATA for binding in the active site. However, the fact that polylysine–DNA complex formation is also inhibited by ATA (Schleich et al., 1978; Gonzáles et al., 1979) indicates that this polyanionic dye is also capable of interfering with electrostatic interactions, which are a contributory driving force in protein–nucleic acid complex formation.

The major line broadening effect exerted by the ATA polymer arises from its paramagnetic character, the consequence of dipolar interaction between the nuclear and electron magnetic spin moments. (Such interaction has an r^{-6} distance dependence.) Other potential sources of line broadening include increases in the correlation time for protein tumbling induced by polymeric dye binding and lifetime broadening arising from chemical exchange. A significant increase in the rotational correlation time of the protein is unlikely because the C(2)–H resonance for His-105 is not detectably broadened by the binding of polymeric ATA (Figures 2, 5, and 6). Broadening due to chemical exchange of a diamagnetic ATA species resulting in a mixture of free and bound protein is likewise expected to be a minor contribution because at high ATA to RNase ratios the affected resonances would sharpen in response to the increasing fraction of protein–ATA complex. In fact, the opposite effect is observed; i.e., extremely broad His-12 and -119 resonances occur at high ATA to RNase ratios. Our observation that the ATA polymer is a stable polyradical is not entirely unexpected, since stable poly(triphenylmethane) radicals have been reported (Braun, 1968). The ESR spectrum of the ATA polymer is similar to that reported for such compounds. This unanticipated finding is significant in that it may provide a powerful means of probing the nucleic acid binding site of proteins by NMR spectroscopy, since ATA is the only well-documented compound capable of interacting with a broad variety of nucleic acid binding proteins.

Acknowledgments

We thank Professor Robert Langridge for time and instruction in the use of the Molecular Modelling System located at the University of California, San Francisco. This facility is supported by Grant RR 1081 from the National Institutes of Health.

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