Base specificity in the interaction of ethidium with synthetic polyribonucleotides

---

Yu. Babayan⁺, G.Manzini, L.E.Xodo and F.Quadrifoglio^{1*}

Department of Biochemistry, Biophysics and Macromolecular Chemistry, University of Trieste, I-34127 Trieste and ¹Institute of Biology, University of Udine, I-33100 Udine, Italy

---

Received April 15, 1987; Revised and Accepted June 12, 1987

Abstract

Base specificity in the interaction of ethidium with double stranded synthetic RNA homopolymers has been studied by means of spectroscopic (UV-visible absorption and fluorescence), microcalorimetric and dilatometric techniques. The results show a strong base specificity in this interaction, the association constant with polyA:polyU being more than three order of magnitude higher than with polyG:polyC. The interaction is mainly enthalpy driven, the differences in affinity being essentially entropic in origin. These evidences along with the dilatometric data suggest that the observed base specificity may arise from the different extent of water release upon intercalation.

Introduction

Ethidium is one of the most widely used and studied aromatic dyes in biochemical and biophysical investigation on nucleic acids. The widespread interest in this molecule is due to several reasons connected to its interaction with double stranded nucleic acids, among which : the fluorescence quantum yield enhancement, that has made this molecule one of the most commonly used nucleic acids staining agents; the fact that it is one of the first molecules for which the intercalative mode of interaction with DNA was established (1,2) and hence has become a prototype for the physico-chemical investigations on intercalators; and not least its frameshift mutagenicity (3,4).

Whereas its interaction with deoxyribonucleic acids has been thoroughly investigated in many aspects (5-12) and with many different techniques (1,13,14), that with double stranded ribonucleic acids, even if certainly not ignored (1,15,16), has received less attention, and some aspects are worth of further investigation. The aim of this paper is to present some results about the interaction of ethidium with three synthetic double stranded polyribonucleotides.

Experimental

Ethidium bromide was a sample from Sigma. PolyA:polyU, polyI:polyC, polyG and polyC were samples from Pharmacia. Double stranded polyG:polyC (as purchased from Sigma) was used for the absorption and fluorescence titrations and for the dilatometric measurements, whereas for the calorimetric measurements polyG:polyC was prepared by heating an aqueous solution of polyG

and polyC in equimolar amounts at 100°C for two hours. The stoichiometry of the complex obtained was found to be 1:1 according to the method of "continuous variation" (17). The absorption and CD spectra of the equimolar product were almost coincident with that of the Sigma product and with that of an analogous sample reported in the literature (18). The molar absorbance values used for the different compounds were as follows: ϵ^{480} for ethidium bromide ($5850 \text{ M}^{-1}\text{cm}^{-1}$) and ϵ^{260} (per phosphate group) for the polymers polyA:polyU (7140), polyI:polyC (5000), polyG (9900), polyC (5100) and polyG:polyC (7900).

The usual solvent condition was: 0.1 M NaCl, 0.01 M Tris, pH= 7.5.

Binding isotherms were determined by means of spectrophotometric and spectrofluorometric titrations using a CARY 219 (Varian) Spectrophotometer and a Perkin Elmer MPF-3 Spectrofluorometer, respectively, both equipped with stoppered quartz cuvettes and thermostatted cell holder. In the case of spectrofluorometry λ_{exc} was 510 nm and no inner filter correction was necessary owing to the very low concentration (and absorbance) of the ethidium solutions. Typically an ethidium solution ($c=10^{-4}\text{M}$ for absorption and $c<10^{-5}\text{M}$ for fluorescence) was titrated at constant concentration with increasing amounts of each polymer containing the same concentration of ligand. The dimerization equilibrium of ethidium ($K^0=70 \text{ M}^{-1}$ in 0.1 M NaCl (19)) was taken into account but the dimer concentration resulted negligible in most of the cases.

Thermal denaturation profiles for polyA:polyU-ethidium complexes at different binding ratios were followed through the variation with temperature of the absorbance at 260 nm, using an Haake F3 thermocryostat equipped with an Haake PG10 temperature programmer. Heating rates were 0.1 °C/min.

The calorimetric measurements were performed by means of both batch and flow type LKB isothermal microcalorimeters. Corrections for the heats of dilutions of both ethidium and polymer resulted less than 10% of the heat effect and almost within the limits of experimental error.

Dilatometric measurements were carried out with Carlsberg dilatometers following a procedure previously described (20). The polymer concentration was about 0.01 M (phosphate) and the amount of ethidium sufficient to give a binding ratio $r=0.2$. The solvent was aqueous 0.01M NaCl, 0.01 M Tris, to keep the ionic strength lower than in the optical experiments in order to guarantee a nearly complete binding of the dye to polyG:polyC. The corrections made for the volume variation on dilution of both polymer and dye resulted to be one order of magnitude lower than the binding effects.

Results

The reversible interaction of ethidium with polyA:polyU has been investigated by means of absorption and fluorescence spectrophotometry, following the well known batho- and hypochromic effect on the visible

absorption band and the fluorescence quantum yield enhancement of this ligand in consequence of its interaction with double stranded nucleic acids. Fig.1 shows, in the form of a Scatchard plot, the results of both spectrophotometric and spectrofluorometric titrations of ethidium with increasing amounts of polymer (0.1M NaCl, 0.1M Tris, pH 7.5, room temperature). A quite high affinity as well as anticooperativity are evident in this interaction. A saturation stoichiometry of one ethidium molecule every two base pairs is clearly suggested by the plot. These data have been fitted with the equation describing the multiple site exclusion model of McGhee and von Hippel (21), taking into account the cooperativity parameter ω :

$$\frac{r}{Cr} = K_b (1-nr) \left[\frac{(2\omega-1)(1-nr)+r-R}{2(\omega-1)(1-nr)} \right]^{n-1} \left[\frac{1-(n+1)r+R}{2(1-nr)} \right]^2 \quad 1)$$

$$R = [1-(n+1)r]^2 + 4\omega r(1-nr)]^{1/2}$$

Figure 1. Scatchard plot of the equilibrium binding for ethidium-polyA:polyU, in 0.1 M NaCl, 0.01 M Tris, pH=7.5, 25°C. Data have been obtained from fluorescence (●) and from absorbance (▲) titrations. The curve is the best fit using equation 1, with parameters $n=2$, $\omega=0.4$, $K_b=2.5 \cdot 10^6$. r is expressed on bp basis.

Figure 2. Enthalpy change in the interaction of ethidium with polyA:polyU (O) and with polyG:polyC (●) in 0.1 M NaCl, 0.01 M Tris, pH=7.5, 25°C, as a function of r (concentration of polymer expressed in bp).

An intrinsic binding constant $K_b = (2.5 \pm 0.5) 10^6 \text{ M}^{-1}$ ($\Delta G_b = -8.8 \pm 0.2 \text{ Kcal/mol}$), an anticooperative parameter $\omega = 0.4 \pm 0.1$ ($\Delta G_{ac} = +0.55 \pm 0.15 \text{ kcal/mol}$) and a stoichiometry of 2 base pairs per ethidium have been found.

The enthalpy of interaction has been measured by means of isothermal flow microcalorimetry and the results are shown in Fig. 2. The value of ΔH_b appears nearly independent of the binding ratio r and is equal to $-7.2 \pm 0.3 \text{ kcal}$ per mole of ethidium bound. An independent determination of ΔH_b has been achieved through fluorescence titrations at different temperatures (see Fig.3) and the van't Hoff analysis of the temperature dependence of K_b (evaluated at each temperature through the use of the McGhee-von Hippel equation) has provided a value of $\Delta H_b = -8 \pm 2 \text{ kcal/mol}$, in fairly good accord with the microcalorimetric results.

The thermal denaturation of polyA:polyU in the presence of varying amounts of ethidium has been followed through the absorbance variation at 260 nm. The results are shown in Fig. 4. Biphasic melting profiles have been obtained in the presence of substoichiometric amounts of ligand, in agreement with Waring(22). This behaviour is expected, on the basis of the theoretical treatment of McGhee (23), in consequence of a sufficiently high value of the binding constant. Incidentally it is worth of mention the fact that an analogous result (also quantitatively: about 10°C of difference between the two subsequent denaturation steps) has been obtained for polyA:polyU in the presence of a non-intercalator, DAPI, which is, however, characterized by a very similar value of K_b (24).

A series of analogous measurements has been performed in order to study the interaction of ethidium with polyG:polyC. Spectrophotometric titrations of 10^{-4} M ethidium with polyG:polyC (Sigma sample) have evidenced the fact that even a polymer concentration of 10^{-3} M is far from promoting a nearly quantitative binding and that this condition is approximately fulfilled with an excess of 10^{-2} M . A quantitative analysis of these data, parallel to that

Figure 3. Scatchard plots of the equilibrium binding for ethidium-polyA:polyU at different temperatures: (●) 17°C, (▲) 30°C, (○) 37°C. Data have been obtained from fluorescence titrations, using as buffer 0.1M NaCl, 0.01 M Tris, pH=7.5. The lines drawn represent the best fit using equation 1. r is expressed on bp basis.

Figure 4. Thermal denaturation of polyA:polyU in the presence of various amounts of ethidium, in 0.1M NaCl, 0.01 M Tris, pH=7.5. The curves correspond to the following concentrations of polymer (C_p) and of ethidium (C_{et}):

(a) $C_p = 1.7 \cdot 10^{-5}$ M (bp)	$C_{et} = 0$
(b) $C_p = 1.6 \cdot 10^{-4}$ M	$C_{et} = 1.1 \cdot 10^{-5}$ M
(c) $C_p = 1.2 \cdot 10^{-4}$ M	$C_{et} = 1.1 \cdot 10^{-5}$ M
(d) $C_p = 0.8 \cdot 10^{-4}$ M	$C_{et} = 1.1 \cdot 10^{-5}$ M
(e) $C_p = 3.9 \cdot 10^{-5}$ M	$C_{et} = 1.1 \cdot 10^{-5}$ M

Figure 5. Relative fluorescence intensity (fluorescence of the dye in the presence of polymer normalized by the fluorescence in the absence of polymer) as a function of increasing amounts of polymer. Buffer used is 0.1 M NaCl, 0.01 M Tris, pH=7.5. $C_{et} = 8 \cdot 10^{-6} M$, $25^{\circ}C$.

performed in the case of polyA:polyU, could not be done: however it was clear that the saturation is not lower than 0.2 moles of ethidium per base pair and that the association constant is in the order of $10^3 M^{-1}$. A better estimate of the affinity of ethidium for this polymer has been reached through a spectrofluorometric titration, following the fluorescence enhancement of $8 \mu M$ ethidium upon addition of increasing (excess) amounts of polyG:polyC (see Fig.5).

In the presence of an excess of polymer (i.e. of binding sites), and hence at sufficiently low values of r , one can see by inspection of the McGhee-von Hippel equation (21) that, if the product nr is not higher than about 0.05, then its simplification to

$$\frac{r}{C_f} = \frac{C_b}{C_f C_p} \approx K_b \quad 2)$$

does not affect the K_b value by more than 10% ($C_b + C_r = C_r$ are the concentrations of bound, free and total ethidium. C_b is the polymer concentration in base pairs). The relative fluorescence intensity, F_{rel} , (i.e. the fluorescence measured in the presence of increasing amounts of polymer normalized by the fluorescence of

the dye in the absence of polymer) can be written as :

$$F_{rel} = \frac{C_f}{C_T} + R_f \frac{C_b}{C_T} = 1 + (R_f - 1) \frac{C_b}{C_T} \quad 3)$$

where R_f is the ratio of the fluorescence quantum yields of the bound and free ethidium molecules. Substitution of eq. 2 in eq. 3 and rearrangement yields :

$$\frac{1}{F_{rel} - 1} \approx \frac{1}{R_f - 1} + \frac{1}{K_b (R_f - 1)} \cdot \frac{1}{C_p} \quad 4)$$

in order to show that a plot of $1/(F_{rel} - 1)$ versus $1/C_p$ is nearly linear and provides estimates for R_f and K_b . This has been done and the result is shown in the insert of Fig 5. The values of $R_f = 20 \pm 5$ and $K_b = (0.8 \pm 0.2) 10^3 M^{-1}$ have been obtained, the last one confirming the previous guess from the spectrophotometric evidences. The same experiment and data treatment have been done with polyI:polyC, another synthetic polymer which is known to bind rather poorly ethidium (16), and the results are shown in Fig. 5, too. In this case the values obtained are $R_f = 35 \pm 2$ and $K_b = (1.5 \pm 0.3) 10^4 M^{-1}$: K_b is higher than in the case of polyG:polyC, but well below the value found for polyA:polyU; on the contrary R_f is markedly higher than the value for polyG:polyC, but not too lower than the value of about 40 found for polyA:polyU.

The enthalpy change on binding has been measured also in the case of polyG:polyC by means of isothermal batch type microcalorimetry. The results are reported in Fig 2. After normalization of the heats evolved by the amounts of ethidium bound, as estimated from the knowledge of K_b , the enthalpy of binding resulted independent of r within the experimental error and a value of $\Delta H_b = -7.5 \pm 0.5$ kcal/mol has been obtained. The summary of the thermodynamic data is displayed in Table 1.

The volume change on binding has been measured by dilatometry for the interaction of ethidium with polyA:polyU and with polyG:polyC, and it resulted to be negative in both cases (-26 ± 1 ml/mol and -21 ± 2 ml/mol ethidium, respectively) with a small but significant difference.

Discussion

The main features that emerge from the data are the great difference in affinity for ethidium between polyA:polyU and the other two polymers considered. It is known that polyA:polyU binds ethidium much more than polyI:polyC does, both at 0.04 M Tris (2) and 1.0 M $NaNO_3$ (16). This difference of about two orders of magnitude in the binding constant is found also in the more

Table I

Summary of thermodynamic data for the interaction of ethidium with synthetic double stranded polyribonucleotides in 0.1 M NaCl, 0.01 M Tris, pH= 7.5, 25°C.

Polymer	K_b (M^{-1})	$-\Delta G_b$ (Kcal/mol)	$-\Delta H_b$ (Kcal/mol)	ΔS_b (cal/mol.*K)	$-\Delta V_b$ (ml/mol)	n^a
PolyA:polyU	$(2.5 \pm 0.5)10^6$	(8.8 ± 0.2)	(7.2 ± 0.3)	$+5 \pm 2$	26 ± 1	2
PolyG:polyC	$(0.8 \pm 0.2)10^3$	(4.0 ± 0.3)	(7.5 ± 0.5)	-12 ± 3	21 ± 2	<5
PolyI:polyC	$(1.5 \pm 0.3)10^4$	(5.8 ± 0.2)	nd	nd	nd	<5

^aexclusion parameter as defined by McOhee and von Hippel (21) in base pairs per ligand

"physiological" ionic strength used in this work. The difference is even more pronounced between polyA:polyU and polyG:polyC, whose K_b values differ by more than three orders of magnitude, thus giving an answer to a rather old question raised about specificity of ethidium binding to polynucleotides (2). A consequent consideration is that the largest difference in affinity is between polyA:polyU and the other two polymers, and not between these two: this should indicate that the role of the $-NH_2$ group of guanine in position 2 does not play a critical role in the specificity of ethidium interaction.

Regarding the saturation stoichiometry, we have been able to determine this parameter only in the case of polyA:polyU, for which it resulted to be one ethidium molecule every two base pairs, not differently from what it has been found with natural and synthetic DNAs. This result is at variance with that reported by Bresloff and Crothers (16) of one ethidium every three base pairs. We believe that this difference is due to two reasons: the first is that from the present data the interaction of two ligands separated by two base pairs appears anticooperative, which makes the approach to saturation even more difficult than the simple site exclusion effect, the second is that the higher ionic strength (and the type of anion NO_3^- instead of Cl^-) used by Bresloff and Crothers (16) is sufficient to depress the binding in the range of $r > 0.33$ below the normal limit of detection. In other words the stoichiometry of one ethidium every two base pairs in double stranded RNAs is not forbidden by severe steric hindrances or other energetic barriers but only slightly less easy than in the case of DNA owing to anticooperativity. Another observation reported by Bresloff and Crothers (16) is a slight positive cooperativity (of about -0.6 kcal/mol) in the interaction of ethidium with polyA:polyU. We were not able to observe this phenomenon with fluorescence measurements both by adding polyA:polyU to $1.5 \times 10^{-7} M$ ethidium or adding dye to $4 \times 10^{-6} M$ polyA:polyU. In the first case the scattering of the experimental data at $r < 0.05$ did not allow to

confirm or exclude the very small effect seen by Bresloff and Crothers. In the second type of experiment no sigmoidal increase of fluorescence on addition of ethidium to the polymer was noticed, as it should be in case of cooperativity. It could seem conflicting the presence of both negative and positive cooperativity in the same system: but, even if unlikely, cooperativity in the interaction of two ethidium molecules three or more base pairs apart does not necessarily exclude anticooperativity in the interaction of two ethidium molecules separated by only two base pairs.

Nelson and Tinoco (15) have studied the interaction of ethidium with a double stranded oligoribonucleotide, (CAAAAAG:CUUUUUG) and found a saturation stoichiometry of 3 ethidium molecules per duplex, in agreement with a stoichiometry of one ethidium per two base pairs. From the analysis of the denaturation profiles they were not able to distinguish between the possibility of stronger binding to the sites adjacent to the terminal CGs without anticooperativity and anticooperativity ($\alpha=0.1$) without site specificity. Our data suggest that both effects (sequence specificity and anticooperativity) are likely to be present. Their K_b value in the anticooperative hypothesis is $2.5 \times 10^5 \text{ M}^{-1}$, lower by one order of magnitude than our value for polyA:polyU, but clearly not conflicting because of their higher ionic strength and of the absence of polyelectrolyte effect in their case.

The main evidences from the microcalorimetric experiments are:

a) the ΔH_b values for polyA:polyU and polyG:polyC are very similar to each other and to those measured with DNA ; b) they are lower in absolute value than that deduced by Nelson and Tinoco (15) from the van't Hoff analysis of the melting curves of their oligomeric duplex ($-14 \pm 4 \text{ kcal/mol}$). The difference between the two sets of data appears significant. However the intrinsic experimental errors and the number of assumptions associated to a van't Hoff analysis, the lower values obtained by Nelson and Tinoco when the fitting of the data were limited to the high r melting curves ($\Delta H=11 \text{ kcal/mol}$) make that difference not so significant. Were the difference real, it should not be easy to find a physical reason for such a behaviour; c) the main driving force for the interaction of ethidium with these synthetic RNA duplexes is essentially enthalpic, whereas the strong selectivity between polyA:polyU and polyG:polyC is due to the entropy of binding, whose contribution to ΔG_b is comparatively weak but sufficient, being of opposite sign, to make the difference. A further evidence in this sense is provided by the dilatometric data. The values, reported in Table I, are in good accord with the results of Torgerson et al(25), who found a $\Delta V_b = -25 \text{ ml/mol}$ for the binding of ethidium to tRNA using static pressure techniques. Somewhat smaller data were obtained for the interaction of ethidium with two synthetic DNAs (26), namely poly(dAdT) (-15 ml/mol) and poly(dGdC) (-9 ml/mol). Since the negative volume change on ethidium binding to double stranded nucleic acids can be attributed to the release of "structured" water from both the drug and the polymer binding sites into the

bulk, it is tempting to suggest that the marked difference in affinity of ethidium for the two RNAs considered, which is mainly entropic in origin, may correlate with a higher water release from the binding site in the case of polyA:polyU with respect to polyG:polyC. On speculative grounds one would suggest that, if the water release in this interaction can be roughly compared with the process of ice melting, for which $\Delta V = -2$ ml/mol ca. and $\Delta S = +5$ cal/K mol ca., our data for $\Delta V_{\text{AU}} - \Delta V_{\text{GC}} = -5$ ml/mol and $\Delta S_{\text{AU}} - \Delta S_{\text{GC}} = +17$ cal/K mol could mean that on interacting with an ethidium molecule polyA:polyU releases about three water molecules more than polyG:polyC does.

Acknowledgement

This work has been financially supported by M.P.I. Dr. Yuri Babayan acknowledges the Italian Ministero degli Affari Esteri for the financial support during his stage at the BBCM Department in Trieste.

*On leave of absence from Molecular Physics and Biophysics Department, Yerevan State University, Mravyan 1, Yerevan 375049, USSR

*To whom correspondence should be addressed

References

- 1) Waring, M.J., (1965) *J. Mol. Biol.* **13**, 269-282.
- 2) Waring, M.J., (1966) *Biochim. Biophys. Acta* **114**, 234-244.
- 3) Streisinger, G., Okada, Y., Emrich, J., Newton, J., Tsugita, A., Terzaghi, E., Inouye, M., (1966) *Cole Spring Harbor Symp. Quant. Biol.* **31**, 77-84.
- 4) McCann, J., Choi, E., Yamasaki, E., Ames, B.N., (1975) *Proc. Natl. Acad. Sci. USA* **72**, 5135-5139.
- 5) Muller, W., Crothers, D.M., (1975) *Eur. J. Biochem.* **54**, 267-277.
- 6) Krugh, T.R., Reinhardt, C.G., (1975) *J. Mol. Biol.* **97**, 133-162.
- 7) Krugh, T.R., Wittling, F.N., Cramer, S.P., (1975) *Biopolymers* **14**, 197-210.
- 8) Kastrop, R.V., Young, M.A., Krug, T.R., (1978) *Biochemistry* **17**, 4855-4865.
- 9) Patel, D.J., Canuel, J.L., (1976) *Proc. Natl. Acad. Sci. USA* **73**, 3343-3347.
- 10) Bauer, W., Vinograd, J., (1970) *J. Mol. Biol.* **47**, 419-435.
- 11) Arnott, S., Chandrasekaran, R., Birdsall, D.L., Leslie, A.G.W., Ratliff, R.L., (1980) *Nature (London)* **283**, 743-745.
- 12) Helgott, D.C., Kallenbach, N.R., (1979) *Nucleic Acids Res.* **7**, 1011-1017.
- 13) LePecq, J.B., Paoletti, C., (1967) *J. Mol. Biol.* **27**, 87-106.
- 14) Tsai, C.-C., Jain, S.C., Sobell, H.M., (1975) *Proc. Natl. Acad. Sci. USA* **72**, 626-632.
- 15) Nelson, J.W., Tinoco, I.Jr., (1984) *Biopolymers* **23**, 213-233.
- 16) Bresloff, J.L., Crothers, D.M. (1981) *Biochemistry* **20**, 3547-3553.
- 17) Job, P., (1928) *Ann. Chim.* **9**, 113.
- 18) Gray, D.M., Tinoco, I.Jr., Chamberlin, M.J., (1972) *Biopolymers* **11**, 1235-1258.
- 19) Reinhardt, C.G., Krugh, T.R., (1978) *Biochemistry* **17**, 4845-4854.
- 20) Delben, F., Quadrioglio, F., Giancotti, V., Crescenzi, V., (1982) *Biopolymers* **21**, 331-341.
- 21) McGhee, J.D., von Hippel, P.H., (1974) *J. Mol. Biol.* **86**, 469-489.
- 22) Waring, M.J., (1974) *Biochem. J.* **143**, 483-486.
- 23) McGhee, J.D., (1976) *Biopolymers* **15**, 1345-1375.
- 24) Manzini, G., Xodo, L.E., Barcellona, M., Quadrioglio, F., (1985) *Nucleic Acids Res.* **13**, 8955-8967.
- 25) Torgerson, P.M., Drickamer, H.G., Weber, G., (1980) *Biochemistry* **19**, 3957-3960.
- 26) Macgregor, R. B. Jr., Clegg, R.M., Jovin, T.M., (1985) *Biochemistry* **24**, 5503-5510.