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Dye-induced aggregation of single stranded RNA: A mechanistic approach

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Abstract

The binding of proflavine (D) to single stranded poly(A) (P) was investigated at pH 7.0 and 25 °C using T-jump, stopped-flow and spectrophotometric methods. Equilibrium measurements show that an external complex PD_I and an internal complex PD_{II} form upon reaction between P and D and that their concentrations depend on the polymer/dye concentration ratio (C_P/C_D). For $C_P/C_D < 2.5$, cooperative formation of stacks external to polymer strands prevails (PD_I). Equilibria and T-jump experiments, performed at $I = 0.1$ M and analyzed according to the Schwarz theory for cooperative binding, provide the values of site size ($g = 1$), equilibrium constant for the nucleation step ($K^* = (1.4 \pm 0.6) \times 10^3\ M^{-1}$), equilibrium constant for the growth step ($K = (1.2 \pm 0.6) \times 10^5\ M^{-1}$), cooperativity parameter ($q = 85$) and rate constants for the growth step ($k_r = 1.2 \times 10^7\ M^{-1}\ s^{-1}$, $k_d = 1.1 \times 10^2\ s^{-1}$). Stopped-flow experiments, performed at low ionic strength ($I = 0.01$ M), indicate that aggregation of stacked poly(A) strands do occur provided that $C_P/C_D < 2.5$.
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Nowadays increasing interest is being turned to those polynucleotide structures, different from typical double helices, that play important roles in biology and related sciences. Among these structures, single stranded polynucleotides deserve special attention owing to the importance of natural RNA. Moreover, single stranded structures are acquiring further importance as a useful tool for specific regulation of gene expression [1] since the recent development of the antigen strategy [2], where a single oligonucleotide strand is used to recognize a double stranded structure by binding to it. The resulting triplex structures often need to be stabilized [3] and intercalators can play a positive role for this purpose.

Changes in the secondary structure of long single stranded polynucleotides are promoted by suitable variations of temperature, pH and salt concentration [4–7] whereas the combined action of pH and salt concentration can favour the formation of multiple aggregates [8]. The addition of dyes provides a tool for monitoring such structural variations of the secondary polymer structure [9]. However, the role of the dye can be more complex, as shown for the poly(A)/proflavine system here investigated, where dye stacking external to the polymer chain favours the formation of multiple strands. This phenomenon, also displayed by linear polymers other than nucleic acids [10], appears to be of some significance regarding the control of self-association and assembly of biological particles. It was found that the staining of DNA and RNA in unfixed cells give different signals, indicating that the binding to polynucleotides double strands is different from the binding to single strands [9]. The binding of acridines to double strands has been thoroughly studied and almost universally interpreted according to the excluded site intercalation model [11]. In contrast, the characteristics of the interaction of acridine dyes with single strands of nucleic acids have been much less investigated. Among the few

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equilibrium studies, the one on the poly(A)/proflavine system by Dourlent and Helene [12] and a subsequent investigation by von Tscharner and Schwarz [13] on the poly(A)/acridine orange system appear to be the closest to the present investigation. The kinetic aspect of the interaction of RNAs with acridines and phenanthridines have been even less investigated. To be cited the studies by Bittman [14] and Tritton and Mohr [9] on the RNA binding of ethidium, and an early temperature-jump study by Hammes and Hubbard [15] on poly(A)/acridine systems, where concerning proflavine, only qualitative observations were made. Note that, presently, the tendency towards the study of the nucleic acid–small molecule interaction is that of making use of oligonucleotides with specifically designed reactions sites. This strategy presents the advantage of yielding information about the reaction of interest, while possible interfering phenomena are suppressed grace to the reduced chain length. By contrast, the use of polynucleotides allows to put into evidence phenomena that manifest themselves only when the chains are sufficiently long, as, for instance, dye-stack formation driven by the polymer secondary structure.

On the basis of the above mentioned arguments, we felt that it could be interesting to contribute filling the gap by carrying out an investigation on the kinetics and equilibria of the interaction of proflavine with single stranded poly(A).

Materials and methods

Materials

Polyadenylic acid (poly(A)) and oligoriboadenylic acid hexamer ($(Ap)_5$-A) were purchased from Pharmacia Biotech (Piscataway, NJ) as the lyophilised sodium salts and were dissolved in water. The purity of the polynucleotide was checked from the absorbance ratios at three different wavelengths and found in agreement with the values given in the analysis certificate. Stock solutions of poly(A) were standardized spectrophotometrically, using $\varepsilon = 10100\ M^{-1}\ cm^{-1}$ at 257 nm [16] and stock solutions of $(Ap)_5$-A were standardized using $\varepsilon = 9230\ M^{-1}\ cm^{-1}$ at 260 nm according to sample certificate. The concentrations are expressed in molarity of phosphate units. Stock solutions of proflavine (PR) from Sigma (St. Louis, MO, USA) were standardized spectrophotometrically, using $\varepsilon = 42000\ M^{-1}\ cm^{-1}$at 444 nm [17], and kept in the dark at 4 °C. All chemicals were analytical grade and used without further purification. The ionic strength of the solutions was adjusted by suitable additions of sodium chloride, although sodium perchlorate or nitrate where found to give substantially the same results. All experiments have been done at pH 7.0, where poly(A) adopts a single strand structure [7]. The pH was maintained at the value of 7.0 with 0.002 M sodium cacodylate ($(CH_3)_2AsO_2Na$) buffer. All solutions were prepared with doubly distilled water, which was also used as the reaction medium.

Methods

Measurements of pH were made by a Metrohm 713 (Herisau, Switzerland) pH-meter equipped with a combined glass electrode.

A Perkin–Elmer Lambda 35 spectrophotometer (Wellesley, MA, USA) was used to record absorption spectra and to perform spectrophotometric titrations. The apparatus is equipped with jacketed cell holders, with temperature control to within ±0.1 °C. All measurements were made at 25 °C. Spectrophotometric titrations on poly(A)/PR interaction were carried out at $I = 0.1$ M, pH 7.0 and $\lambda = 444$ nm, by adding increasing amounts of a poly(A) solution to the spectrophotometric cell containing the dye. Polystyrene cuvettes from Kartell (Noviglio (MI), Italy) were used in place of glass cuvettes in order to minimize dye absorption on the cell walls. The optical path length of the cuvettes (l) was $l = 1$ cm.

The T-jump apparatus, used to investigate the binding kinetics at $I = 0.1$ M, is made in our laboratory and based on the Riegler et al. prototype [18] except that photomultipliers are replaced by suitable silica photodiodes (Hamamatsu, S1336, Japan). A tungsten lamp—monochromator system is used as a monochromatic light source. Although both absorbance and fluorescence changes can be simultaneously measured, in the present study the absorbance detection mode was prevalently employed. The relaxation curves were collected by an Agilent 54,622A (Palo Alto, CA, USA) storage oscilloscope, transferred to a PC and evaluated using the fitting package by Jandel (AISN software, Mapleton, OR, USA). The stopped-flow apparatus, used to investigate polymer aggregation at $I = 0.01$ M, was constructed in our laboratory [19]. It is made by a Hi-Tech (Bradford on Avon, UK) SF-61 mixing unit connected to the spectrophotometric line by two optical guides and can measure both fluorescence and absorbance changes. The acquired signal is transferred via a GPIB interface to a personal computer, where it is analyzed by a non-linear least-square procedure expressly devised to fit multi-exponential functions [20]. The signal change, expressed in volts, is proportional to the absorbance variation. Each experiment was repeated at least 10 times, and average values of the obtained time constants were used.

Results

Species distribution

Absorbance spectra were recorded for solutions with different values of the C_P/C_D ratio, where C_P and C_D denote, respectively, the analytical concentrations of poly(A) and proflavine. A single, well defined, isosbestic point (472 nm) is observed for $C_P/C_D < 1.5$ (Fig. 1A) revealing that at low polymer-to-dye ratio a single bound species is present. Moreover, the hypochromic effect and the blue shift shown in the figure suggest that the dye is

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