Intercalation of Proflavine and a Platinum Derivative of Proflavine into Double-Helical Poly(A)

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ABSTRACT The equilibria and kinetics of the interactions of proflavine (PR) and its platinum-containing derivative $[\{PtCl(tmen)\}_2\{HNC_{13}H_7(NHCH_2CH_2)_2\}]^+$ (PRPt) with double-stranded poly(A) have been investigated by spectrophotometry and Joule temperature-jump relaxation at ionic strength 0.1 M, 25°C, and pH 5.2. Spectrophotometric measurements indicate that base-dye interactions are prevailing. T-jump experiments with polarized light showed that effects due to field-induced alignment could be neglected. Both of the investigated systems display two relaxation effects. The kinetic features of the reaction are discussed in terms of a two-step series mechanism in which a precursor complex DS_I is formed in the fast step, which is then converted to a final complex in the slow step. The rate constants of the fast step are $k_1 = (2.5 \pm 0.4) \times 10^6\ M^{-1}\ s^{-1}$, $k_{-1} = (2.4 \pm 0.1) \times 10^3\ s^{-1}$ for poly(A)-PR and $k_1 = (2.3 \pm 0.1) \times 10^6\ M^{-1}\ s^{-1}$, $k_{-1} = (1.6 \pm 0.2) \times 10^3\ s^{-1}$ for poly(A)-PRPt. The rate constants for the slow step are $k_2 = (4.5 \pm 0.5) \times 10^2\ s^{-1}$, $k_{-2} = (1.7 \pm 0.1) \times 10^2\ s^{-1}$ for poly(A)-PR and $k_2 = 9.7 \pm 1.2\ s^{-1}$, $k_{-2} = 10.6 \pm 0.2\ s^{-1}$ for poly(A)-PRPt. Spectrophotometric measurements yield for the equilibrium constants and site size the values $K = (4.5 \pm 0.1) \times 10^3\ M^{-1}$, $n = 1.3 \pm 0.5$ for poly(A)-PR and $K = (2.9 \pm 0.1) \times 10^3\ M^{-1}$, $n = 2.3 \pm 0.6$ for poly(A)-PRPt. The values of k_1 are similar and lower than expected for diffusion-limited reactions. The values of k_{-1} are similar as well. It is suggested that the formation of DS_I involves only the proflavine residues in both systems. In contrast, the values of k_2 and k_{-2} in poly(A)-PRPt are much lower than in poly(A)-PR. The results suggest that in the complex DS_{II} of poly(A)-PRPt both proflavine and platinum residues are intercalated. In addition, a very slow process was detected and ascribed to the covalent binding of Pt(II) to the adenine.

INTRODUCTION

The intercalative properties of acridines (Lerman, 1961) have been recognized as the basis of their mutagenic and antitumor activities (Ferguson and Denny, 1991). Proflavine (3,6-diaminoacridine), also denoted as PR in the following, causes frameshift mutations in viruses, in bacteriophages, and in bacteria, but shows DNA-breaking activity in mammalian cells (Ferguson and Denny, 1991). This and other chromosomal mutations seem to be related to the ability of proflavine to stabilize DNA-topoisomerase II intermediates (Ferguson and Denny, 1991; Ripley et al., 1988; Brown et al., 1993). The antineoplastic activity of some acridine derivatives and of other intercalators is the result of their interaction with topoisomerase II (Capranico et al., 1990; Corbett et al., 1993; De Isabella et al., 1993) and has been exploited in the clinical treatment of human cancers.

cis-Diamminedichloroplatinum (II) (*cis*-DDP), widely used in chemotherapy, is itself capable of producing lesions in DNA (Lippard, 1983; Fichtinger-Schepman et al., 1986; Brabec and Leng, 1993), and the combined use of intercalating drugs with *cis*-DDP has proved successful against some forms of solid tumors (Pizzocaro et al., 1985). Recently, bifunctional molecules have been synthesized that link some *cis*-DDP like residues to an intercalator (Bowler et al., 1984, 1989; Bowler and Lippard, 1986; Sundquist et al., 1990; Ceci et al., 1993), with the aim of producing compounds showing toward nucleic acids the interacting characteristics of both the functional groups. One of them is the new compound $[\{PtCl(tmen)\}_2\{HNC_{13}H_7(NHCH_2CH_2)_2\}]^+$, (tmen = *N*,*N*,*N*′,*N*′-tetramethylethylenediamine), which from now on will be denoted as PRPt, where two platinum residues are linked to a proflavine molecule (cf. Fig. 1).

It should be noted that most of the kinetic studies on the binding of intercalators to nucleic acids involve DNA, whereas binding to RNA has been relatively less studied. Despite the abundance of studies performed by different techniques, the results and the conclusions as well are rather different. For instance, the DNA-phenanthridine system has been found to display a single relaxation (Jovin and Striker, 1977; Macgregor et al., 1985), two relaxations (Wakelin and Waring, 1980; Meyer-Almes and Porschke, 1993), and even three relaxations (Breslow and Crothers, 1975). Concerning the DNA-acridine system, depending on the structure of the dye, different behaviors have been observed as well. A single relaxation with a reciprocal relaxation time that tends to level off for high polymer concentrations was measured (Marcandalli et al., 1988), but two (Wakelin and Waring, 1980) and three separate effects (Li and Crothers, 1969) were also observed. Nevertheless, all authors agree in asserting that the excluded site model should apply to all of the investigated systems, including poly(A)·poly(U)-PR (Schmechel and Crothers, 1971). In contrast, the single-stranded nucleic acids exhibit a totally different mode of binding. Hammes and Hubbard (1966) investigated the in-

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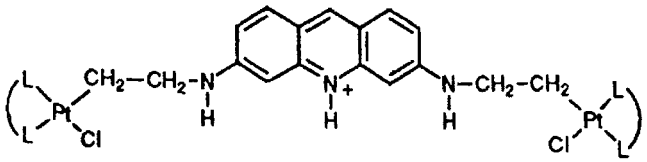

FIGURE 1 PRPt ≡ $[\{PtCl(tmen)\}_2\{HNC_{13}H_7(NHCH_2CH_2)_2\}]^+$. Tmen ≡ L-L ≡ *N,N,N′,N′*-tetramethylethylenediamine.

teraction of acridines with single-helical poly(A) by the T-jump method and concluded that the stacking between the dye molecules bound externally to the polymer chain plays an important role in the dynamics of the interaction.

To gain information on the mode of binding of bifunctional drugs to ribonucleic acids, we have performed a temperature-jump study of the interaction of PR and PRPt with double-helical poly(A). The structure of double-helical poly(A) has been elucidated by crystallographic (Rich et al., 1961), NMR (Lerner and Kearns, 1981), and Raman (Scovell, 1978) studies and proved to be quite different from those of standard RNA double helices. Actually, the adenines pair through two C_6-NH_2···N_7 and two C_6-NH_2···^-O_2P═ hydrogen bonds (Scovell, 1978), and the phosphate interacts electrostatically with the proton bound to the N_1 of adenine in the opposite chain (Rich et al., 1961). The resulting structure exhibits two parallel strands and a single groove and requires each base pair to be at least partially protonated to prevent strand dissociation. The presence of only one type of base, as well as only one type of groove, allows an easier interpretation of the experimental data (Wakelin and Waring, 1980) and makes poly(A) an especially interesting model for the study of interactions between ligands and ribonucleic acids. A further aim of this investigation is to confirm the recent assertion of previous authors (Meyer-Almes and Porschke, 1993) that the use of the T-jump technique with Joule heating yields correct results, provided that the artifacts due to field-induced alignment are properly controlled.

MATERIALS AND METHODS

Materials

Poly(A) was purchased from Pharmacia Biotech (Uppsala, Sweden) in the form of lyophilized sodium salt (mean length ~600 units). The polynucleotide solutions were standardized spectrophotometrically, using an extinction coefficient of 1.01×10^4 M^{-1} cm^{-1} at 257 nm (Janik, 1971). Proflavine hydrochloride was purchased from Sigma (St. Louis, MO), and the concentration of the stock solutions was determined by measuring the absorbance at 444 nm and using $\epsilon = 4.2 \times 10^4$ M^{-1} cm^{-1} (Schwarz et al., 1970). PRPt was synthesized and purified as described elsewhere (Ceci et al., 1993). Stock solutions of PRPt were prepared by dissolving weighed amounts of the solid in doubly distilled water. The absorption spectrum and the extinction coefficient of PRPt in the visible region appear to be practically the same as those of proflavine. All of the solutions were kept in the dark at 4°C and were used within 1 week. Sodium perchlorate was used to adjust the ionic strength, *I*, in the poly(A)-PR system, whereas sodium nitrate was employed with the poly(A)-PRPt system, because PRPt is scarcely soluble in perchlorate medium. Sodium cacodylate (1×10^{-2} M) was used to keep the pH of the solutions at the desired value.

Methods

The double-stranded conformation of poly(A) was obtained by gradually lowering the pH of the solution from an initial value of 7 to the value of 5.2 and waiting for at least 2 h so that the transition could come to completion (Maggini et al., 1994) Measurements of pH were made with a Radiometer Copenhagen (Copenhagen, Denmark) PHM-84 pH meter equipped with a combined glass electrode in which the liquid junction was a 3 M NaCl solution.

The binding equilibria were studied in the visible region, where only the dyes absorb. Spectrophotometric measurements were made using a Perkin-Elmer and Co GmbH (Überlingen, Germany) Lambda 17 UV/VIS spectrophotometer. The absorption spectra of both dyes change upon binding to poly(A), displaying hypochromism and bathochromic shifts. Spectrophotometric titrations were performed by adding increasing amounts of poly(A) to a dye solution. The data at $I = 0.1$ M were evaluated according to the Benesi and Hildebrand (1949) method, whereas the titrations at $I = 0.01$ M were analyzed by fits to the McGhee and von Hippel (1974) equation. Unless specified otherwise, concentrations of poly(A) are given in units of moles of base pairs per dm^3.

The kinetic measurements were carried out by the temperature-jump technique with Joule heating and absorbance detection. To check the influence of the electric field on the kinetics, the Messanlagen T-jump instrument (Göttingen, Germany) was modified by inserting a polarizer in front of the entrance cell window. A test was made to check the quality of the light at the exit of the T-jump monochromator (Bausch and Lomb no. 338602). With the cell filled with water, the light intensity was measured for different directions of the polarizer with respect to the transient electric field. A sinusoidal dependence of the light intensity on the rotation angle indicated that the light from the monochromator is polarized, and the value of the minima yielded ~35% polarization. Furthermore, the quality of the cell windows with respect to their ability to depolarize the incident light was tested as follows: a beam of vertically polarized light from a 633-nm He-Neon laser was allowed to cross a Nicol's prism initially set parallel to the incident light. The measured photocurrent at the exit of the prism was 1.9×10^{-3} A. The Nicol's prism was then rotated by 90°, and the measured current intensity was reduced to 1×10^{-6} A. The cell was then filled with water and inserted in front of the prism. No increase in the photocurrent was observed, meaning that light depolarization by the cell windows was negligible.

Each of the two investigated systems displays two relaxation effects. The kinetic curves were collected by a Tektronix (Beaverton, OR) 2212 storage oscilloscope, transferred to a PC and evaluated according to the method of Provencher (1976). Because our data storage device does not have a dual sampling scale, all of the experiments with the poly(A)-PRPt system, where the two relaxation effects differ by about two orders of magnitude, have been recorded first at a high and then at a low sampling rate. In such a way the two effects could be analyzed separately.

RESULTS

Equilibria

The transition of poly(A) from the single-strand to the double-strand conformation depends on pH and on the ionic strength. At $I = 0.1$ M and 25°C the melting pH is 5.9, and at the working pH of 5.2 the transition is complete (Maggini et al., 1994). Furthermore, we note that under the conditions of our experiments the duplex of poly(A) is half-protonated (Maggini et al., 1998).

Poly(A)-PR

Fig. 2 shows the spectral changes occurring in the poly(A)-PR system when increasing polymer amounts are added to a fixed dye amount. The clearly visible red shift indicates dye-base interactions, whereas the single, well-defined isosbestic point suggests the occurrence of a relatively simple binding process. This can be represented by the apparent reaction

$$D + S \leftrightarrow DS \quad (1)$$

where DS denotes the total bound dye, D the free dye, and S the binding sites on the polymer that are potentially free. Preliminary titrations, performed at 444 nm, revealed that the binding of proflavine to double-stranded poly(A) is rather weak. To enhance the extent of reaction one should increase the reactant concentrations. The total dye concentration, C_D, could not be conveniently augmented, owing to possible dimerization of proflavine for $C_D > 5 \times 10^{-5}$ M (Hammes and Hubbard, 1966), and therefore the measurements were performed in an excess of polymer. Under these circumstances the variable *r*, defining the ratio of bound dye to total polymer concentration, D_S, never exceeded the value of 0.06, thus making the binding at an isolated site largely favored. The data, plotted according to the Benesi and Hildebrand (1949) procedure, yielded good straight lines, as shown by plot *a* of Fig. 3. The ratio of intercept to slope gives *K*, the apparent equilibrium constant of reaction 1 (see Table 1). The data were then analyzed according to the Scatchard (1949) method. The resulting plots are almost parallel to the *x* axis, and the intercept on the *y* axis gave for *K* the same value as that obtained by the Benesi-Hildebrand procedure. Similar behavior has been observed with other systems, where the binding constants are small and the equilibria could be measured only at low values of *r* (Diebler et al., 1987). Obviously, for such systems the evaluation of the site size, *n*, could be very difficult. To estimate

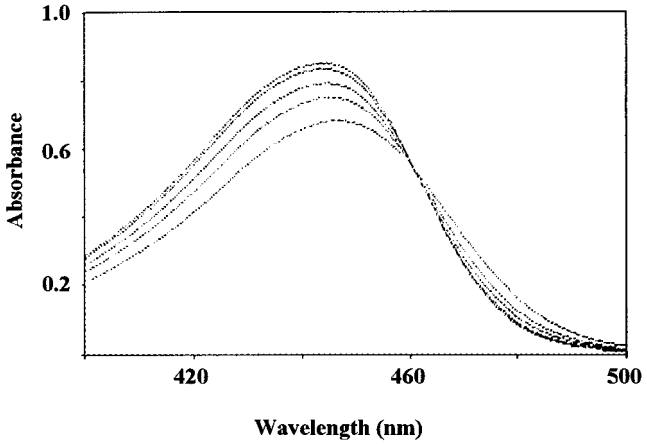

FIGURE 2 Spectra of the poly(A)-PR system. pH = 5.2, I = 0.1 M, T = 25°C. Total proflavine concentration $C_D = 2.0 \times 10^{-5}$ M. From top at 444 nm: C_S/C_D = 0, 2.5, 10, 20, 40. The total poly(A) concentration, C_S, is given in units of base pairs.

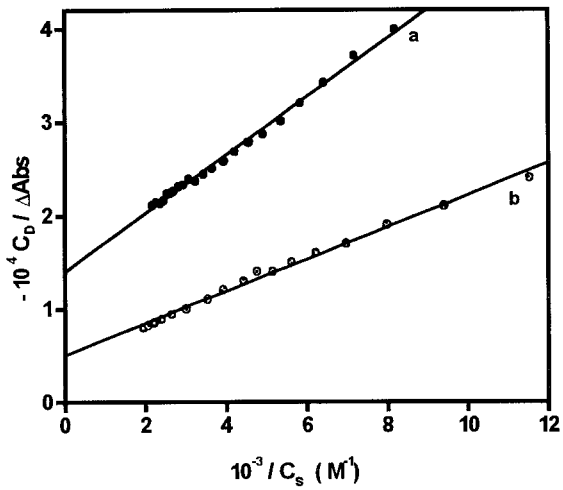

FIGURE 3 Spectrophotometric titrations evaluated according to the Benesi-Hildebrand method. (*a*) The poly(A)-PR system. (*b*) The poly(A)-PRPt system. pH = 5.2, T = 25°C, I = 0.1 M, λ = 444 nm. The concentration of poly(A), C_S, is given in units of base pairs.

n, we have performed a titration at an ionic strength of 0.01 M, where complete saturation is achieved quite easily, as indicated by the shape of the titration curve, where initially the absorbance decreases linearly and, as more titrant is added, suddenly becomes constant. The intersection of the two straight lines drawn through the linear portions of the titration curve corresponds to $C_S/C_D = n$. The site size determined in this way is 1.3 ± 0.5.

Poly(A)-PRPt

The acid dissociation constant of PRPt was determined spectrophotometrically. Its value (pK_A = 9.5 ± 0.05) enabled us to establish that at the working pH of 5.2 the dye is fully protonated.

The spectral changes occurring in the poly(A)-PRPt system, when increasing polymer amounts are added to a fixed dye amount, are qualitatively similar to those observed for the poly(A)-PR system. Again, an isosbestic point and a red shift are observed. The spectrophotometric titrations were analyzed according to the procedure used for the poly(A)-PR system. Plot *b* of Fig. 3 provides the value of *K* reported in Table 1. From a titration at I = 0.01 M it has been found that n = 2.3 ± 0.6. This result is substantiated by the analysis of the data according to the McGhee and von Hippel (1974) equation.

Temperature-jump experiments

Electric field effects

The discharge of the capacitor in temperature-jump experiments with polymers causes field-induced alignment. Because the polymer molecules, when oriented, give rise to electric dichroism, the solution behaves like a dichroic light polarizer (Dourlent et al., 1974; Meyer-Almes and Por-

TABLE 1 Parameters for the binding of acridine dyes to double-stranded Poly(A)

Dyes	n	$K/10^3$ (M^{-1})	$K_{kin}/10^3$ (M^{-1})	$k_1/10^6$ ($M^{-1}s^{-1}$)	$k_{-1}/10^3$ (s^{-1})	k_2 (s^{-1})	k_{-2} (s^{-1})
PR	1.3 ± 0.5	4.5 ± 0.1	3.8 ± 0.9	2.5 ± 0.4	2.4 ± 0.1	447 ± 55	173 ± 13
PRPt	2.3 ± 0.6	2.9 ± 0.1	2.8 ± 0.4	2.3 ± 0.1	1.6 ± 0.2	9.7 ± 1.2	10.6 ± 0.2

All measurements refer to 25°C and ionic strength 0.1 M. The following parameters have been evaluated from spectrophotometric titrations with absorbance detection: n, number of base pairs occupied by the dye; K, apparent equilibrium constant. The other parameters have been derived from T-jump experiments: $K_{kin} = (k_1/k_{-1})(1 + k_2/k_{-2})$ is the apparent equilibrium constant derived from kinetics; k_1 and k_{-1} have been derived according to Eq. 5; k_2 and k_{-2} have been derived according to Eq. 6, where $A = k_2$ and $B = k_{-2}$.

schke, 1993). As a consequence, orientation and deorientation effects could be monitored by directing toward the cell a beam of linearly polarized light.

The effects of the applied electric field on the poly(A)-Pr system have been tested at 460 nm (isosbestic point). Under these conditions the signals associated with chemical reactions are suppressed, and any observed relaxation should be thus ascribed to electric field alignment. Fig. 4 *A* shows some orientational effects of the polymer-dye complex at $I = 0.01$ M recorded for different values of the polarization angle ψ (*curves a–c*). The amplitude is reversed on going from $\psi = 0°$ to $\psi = 90°$ and is reduced to a negligible value for $\psi \cong 55°$, the magic angle. The T-jump curves, when recorded under magic angle conditions, display only chemical effects, i.e., effects free from orientational contributions (Dourlent et al., 1974; Meyer-Almes and Porschke, 1993). It is important to note that the magnitude of the orientational effect is remarkably reduced by the addition of salt and by decreasing the length of the polymer (Meyer-Almes and Porschke, 1993). The length of our poly(A) sample is ~600 bp, and the results of Fig. 4 *B* (*curves d* and *e*) show that, at $I = 0.1$ M, the orientation effects could be neglected. In light of these results we have concluded that our kinetic experiments could be safely carried out without a polarizer, and we have avoided its use to get a higher signal-to-noise ratio.

Poly(A)-PR

The T-jump experiments were carried out at 430 nm because this wavelength provides the best signals. The dye concentration ranged between 1×10^{-5} M and 3×10^{-5} M, whereas the poly(A) concentration was changed by up to 5.15×10^{-4} M. Two relaxation effects, differing by about one order of magnitude in the time scale and of opposite amplitude, were observed (cf. Fig. 4 *B*). This behavior indicates that at last two complexes are formed in nonnegligible amounts. The results indicate that the apparent reaction 1 could be appropriately described by the individual steps

$$D + S \underset{k_{-1}}{\overset{k_1}{\longleftrightarrow}} DS_I \tag{2}$$

$$DS_I \underset{k_{-2}}{\overset{k_2}{\longleftrightarrow}} DS_{II} \tag{3}$$

$$D + S \underset{k_{-3}}{\overset{k_3}{\longleftrightarrow}} DS_{II} \tag{4}$$

Assuming that the fast effect is associated with step 2, a linear dependence of the reciprocal fast relaxation time, ${\tau_1}^{-1}$, on the reactant concentration is expected according to Eq. 5, where C_s replaces the sum of the equilibrium concentrations of the reactants, because the polymer is present in excess:

$$\tau_1^{-1} = k_1 C_S + k_{-1} \tag{5}$$

A plot of ${\tau_1}^{-1}$ versus C_S is shown in Fig. 5 *A*. The points are rather scattered because, as shown in Fig. 4 *B*, the amplitude of the fast effect is small and the noise is large. The reciprocal slow relaxation time, ${\tau_2}^{-1}$, is expressed by the relationship

$$\tau_2^{-1} = (K_1 A C_S)/(1 + K_1 C_S) + B \tag{6}$$

where K_1 is the equilibrium constant of step 2. Assuming that only steps 2 and 3 are operative (series mechanism), then $A = k_2$ and $B = k_{-2}$. In the other limiting case, the parallel mechanism, step 3 is excluded and $A = k_3/K_1$, whereas $B = k_{-3}$. A plot according to Eq. 6 is shown in Fig. 5 *B*. The solid line is calculated by using for K_1 the ratio k_1/k_{-1}. Note that in Eqs. 5 and 6 the total site concentration C_S should be replaced, in principle, by $C_S f(r) - [D]f'(r)$, where the variables $f(r)$ and $f'(r)$ account for possible site overlapping (McGhee and von Hippel, 1974; Jovin and Striker, 1977; Macgregor et al., 1985). The approximation is justified by the fact that, under our experimental conditions, the values of $f(r)$ approach unity and $C_S \gg [D]f'(r)$. The values of the rate constants are collected in Table 1. It should be noted that the relaxation amplitudes of the two observed effects in the poly(A)-PR system are of opposite sign. Assuming that the difference in extinction coefficients between products and reactants has the same sign for both normal modes of reaction, one could conclude that the reaction enthalpies of the two steps are of opposite sign. Similar behavior has been found in the DNA-proflavine system (Li and Crothers, 1969).

Poly(A)-PRPt

The T-jump measurements have been performed at 445 nm in a range of poly(A) concentrations C_S up to 4×10^{-4} M and PRPt concentrations C_D up to 2×10^{-5} M. The

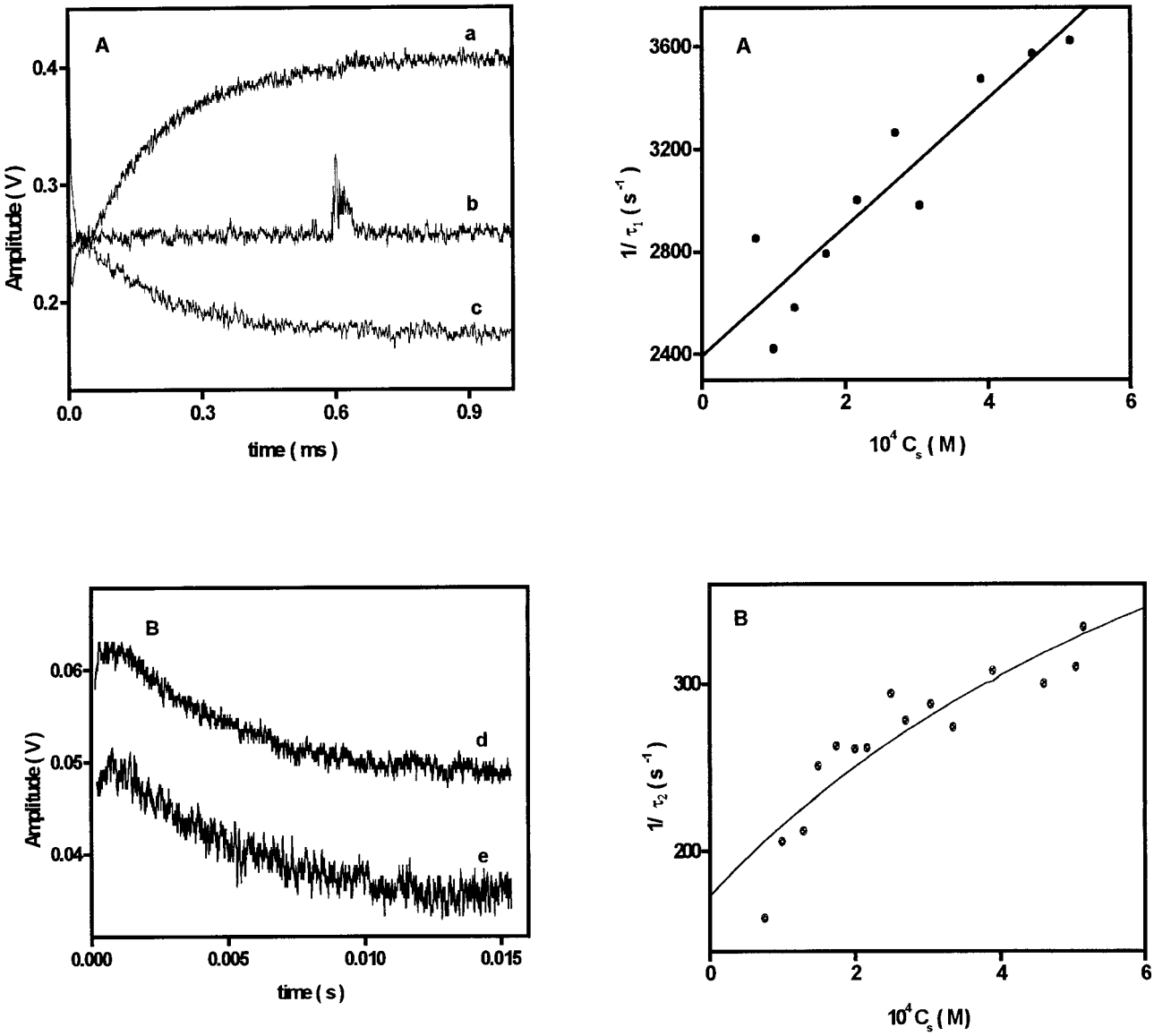

FIGURE 4 Effects of the transient electric field on the poly(A)-PR system (investigated with joule heating and absorbance detection). pH = 5.2, $C_D = 3.0 \times 10^{-5}$ M, $C_S/C_D = 3.33$, $T = 25$°C. (*A*) $I = 0.01$ M, rise time = 1 μs, heating time = 49 μs. The traces have been recorded with different directions of the polarizer with respect to the transient electric field: (*a*) parallel, (*b*) magic angle (55°), (*c*) perpendicular. The wavelength is that of the isosbestic point (460 nm), where the effects of the chemical reaction are suppressed. (*B*) $I = 0.1$ M, rise time = 50 μs, heating time = 7.4 μs, $\lambda = 430$ nm. (*d*) No polarizer, $\tau_1 = 0.41$ ms and $\tau_2 = 4.2$ ms. (*e*) Magic angle (55°), $\tau_1 = 0.38$ ms and $\tau_2 = 4.6$ ms.

FIGURE 5 Reciprocal relaxation times as a function of the reactant concentrations for the poly(A)-PR system. pH = 5.2, $I = 0.1$ M, $T = 25$°C. (*A*) Fast effect. (*B*) Slow effect.

relaxation curves of the poly(A)-PRPt system are also biexponential, but, in contrast to poly(A)-PR, the relaxation times differ by about two orders of magnitude (Fig. 6) and the amplitudes display the same sign. The two relaxation times were analyzed according to Eqs. 5 and 6; the results are shown in Fig. 7. A plot of $1/\tau_2$ versus the reactant concentration, shown in Fig. 7 *B*, yields the values of *A* and *B*. The solid line is calculated by using Eq. 6 and $K_1 = k_1/k_{-1}$.

In addition to the effects investigated by T-jump, a very slow reaction was detected by mixing poly(A) and PRPt and measuring the absorbance at long time intervals. The half-life of this chemical change, measured for $C_D = 1 \times 10^{-5}$ M and $C_S = 4 \times 10^{-4}$ M, was found to be ~50 h at 25°C. This process is too slow to be ascribed to polynucleotide-dye noncovalent binding. On the other hand, the half-life of this slow reaction is on the same order of magnitude as that of similar processes involving covalent interaction of platinum(II) drugs with polynucleotides (Bruhn et al., 1990). Within the time interval from sample preparation to the end of each T-jump experiment (~1 h), the extent of this reaction was too small to affect the relaxation measurements.

DISCUSSION

The experiments with polarized light show that the problems due to field-induced orientation can be completely

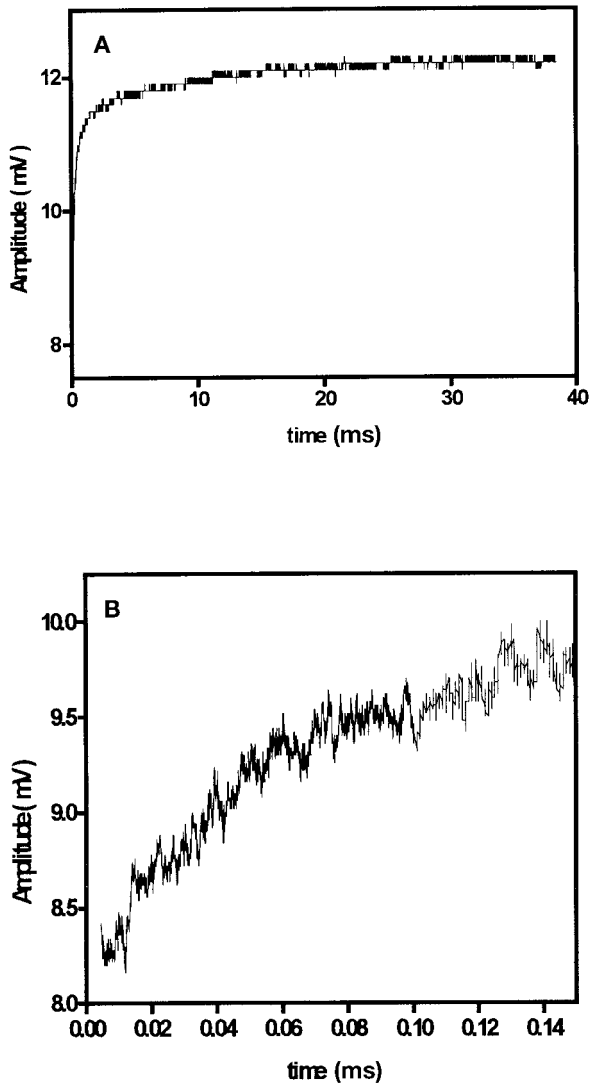

FIGURE 6 Temperature-jump relaxation detected by absorbance for the poly(A)-PRPt system. $C_D = 1.23 \times 10^{-5}$ M, $C_S/C_D = 10$, pH = 5.2, I = 0.1 M, T = 25°C, λ = 445 nm. (*A*) The curve is the result of the accumulation of three repeated experiments recorded with a rise time of 1 ms and shows two kinetic effects well separated on the time scale. The relaxation time of the slow effect is $\tau_2 = 87$ ms. (*B*) Fast effect. The curve is the result of the accumulation of four repeated experiments recorded with a rise time of 10 μs, and its analysis provides $\tau_1 = 0.53$ ms.

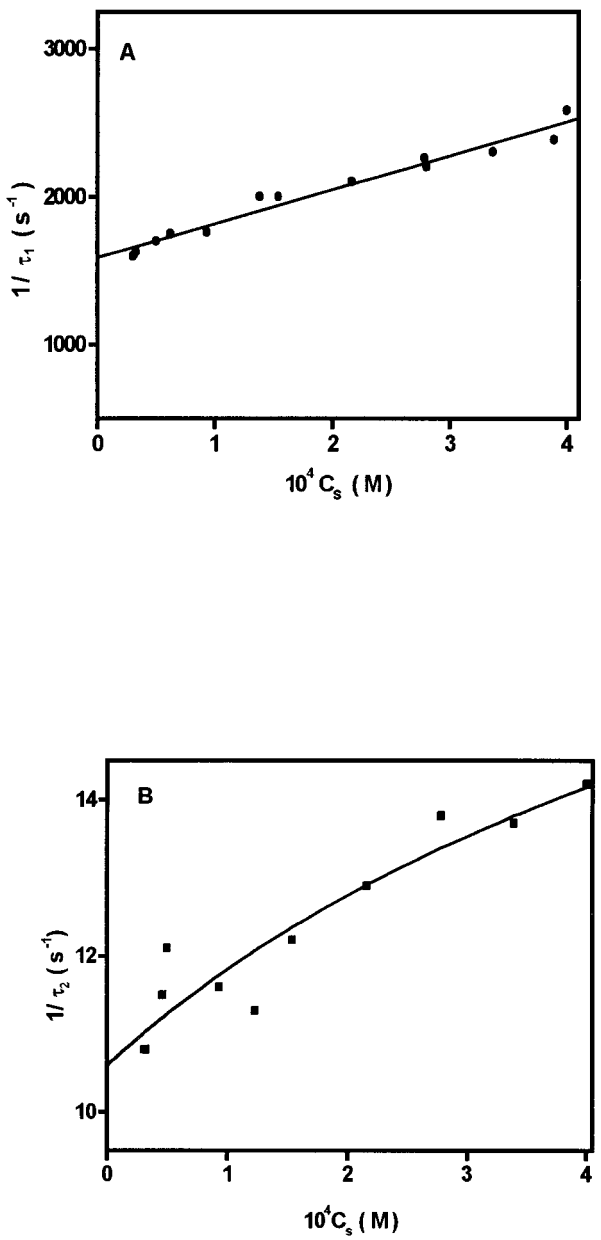

FIGURE 7 Reciprocal relaxation times as a function of the reactant concentrations for the poly(A)-PRPt system. pH = 5.2, I = 0.1 M, T = 25°C. (*A*) Fast effect. (*B*) Slow effect.

avoided by working under magic angle conditions or, if the polymers are sufficiently short (like our poly(A)), at a suitable ionic strength. The assertion by Meyer-Almes and Porschke (1993) that the temperature-jump technique with Joule heating, when used with appropriate care, does not introduce artifacts provoked by field alignment is thus confirmed.

The binding of PR to double-stranded poly(A) is different from the binding to the single-stranded polymer. In the latter case the spectra display a blue shift at moderate P/D ratios, which indicates the formation of external complexes stabilized by dye-dye stacking interactions (Dourlent and Helene, 1971; Ciatto, 1994). Moreover, the single-stranded poly(A)-PR system exhibits positive cooperativity, as indicated by down-curved Scatchard plots (Ciatto, 1994) and by a spectrum of relaxation times obtained from T-jump experiments (Hammes and Hubbard, 1966). In contrast, the red shift observed at any P/D ratio, for both systems investigated here, reveals that the binding process is dominated by dye-base interactions (intercalation), whereas equilibria and kinetics indicate the absence of positive cooperativity. Concerning the fastest of the two observed chemical effects, one must exclude the possibility that this is due to dye dimerization, because proflavine does not aggregate for concentrations less than 5×10^{-5} M (Schwarz et al., 1970).

This observation has been confirmed by our T-jump experiments performed in the absence of polymer, which did not reveal any relaxation effect. The conclusion is that the fast effect should be ascribed to dye-polymer interaction. The values of k_1 are, with both PR and PRPt, definitely lower than expected for a diffusion-controlled reaction. Two models have been proposed to interpret the reduced rate of the binding process represented here by reaction 2: the first model requires that the encounters be diffusive, but only a fraction of them lead to intercalation (Macgregor et al., 1985). The second model envisages the diffusion-controlled formation of an external complex (let us indicate it by D·S), stabilized by electrostatic interactions, followed by the rate-determining step, where D·S is converted to a more stable form (Meyer-Almes and Porschke, 1993). We do not know the stability constant of D·S, but we note that for the DNA-ethidium system the value of $\sim$100 M^{-1} has been estimated (Meyer-Almes and Porschke, 1993). Because at pH 5.2 each base pair of poly(A) bears only a negative charge (Maggini et al., 1998), we could argue that the value of the stability constant of D·S in the poly(A)-PR system should be well below 100 M^{-1}. Hence we expect that the plot of Fig. 5 *A* should not display the curvature revealing the formation of D·S. Similar considerations apply to the poly(A)-PRPt system as well. Moreover, we note that the values of k_1 for PR and PRPt are similar, and the same holds for the values of k_{-1} (cf. Table 1). This suggests that the strengths of the dye-polymer interactions in the intermediate complexes DS_I are similar, i.e., the interactions involve only the acridine, whereas the platinum residues do not contribute noticeably to the stability of DS_I in the poly(A)-PRPt system. The hypotheses about the nature of the intermediate complex are rather controversial. Most of the authors who have investigated DNA-dye systems express the idea that the bound form corresponding to DS_I would be an "outside complex" more stable than the electrostatic ion pair D·S, because of additional dye-base interactions. Wakelin and Waring (1980), by investigating the interaction of acridines and phenanthridines with DNA, observed the formation of two bound forms and proposed that these forms represent molecules associated, respectively, with the mayor and the minor groove of the helix. This argument does not hold in our case because poly(A) exhibits only a single groove. Schelhorn et al. (1992), from a thermodynamic study of the DNA-PR system, conclude that the form corresponding to DS_I is a "preintercalated" complex. Very recently Porschke (1998) has developed an electrooptical method that allows the characterization of the individual relaxation processes in terms of structures of intermediates. This author, investigating by his new technique the DNA-ethidium system, confirmed that two complexes are formed at different rates and demonstrated that in both complexes the dye is intercalated, whereas the "outside" complex is negligible.

The values of k_2 and k_{-2} are definitely lower for poly(A)-PRPt than for poly(A)-PR. We ascribe this difference to the role of the platinum residues in the process of formation of the final complex DS_{II}. Concerning this complex, we favor the idea that proflavine and platinum are both inside for the following reasons: first, the titrations at ionic strength 0.01 M show that the site size for binding of PRPt is twice that for binding of PR, suggesting that the base pair between two adjacent cavities could open, thus making room for both residues. Furthermore, we have observed that during the course of the very slow reaction, where platinum binds covalently to the adenine, the visible absorption maxima remain red-shifted relative to the free compound. This result, while indicating that the intercalation of the proflavine residue persists during the covalent binding of Pt(II) to the adenine, designates DS_{II} as an intermediate of the very slow reaction. Moreover, to give further support for our hypothesis, we notice that Bowler et al. (1984) report that the intercalator-linked platinum complexes $[Pt(AO(CH_2)_nen)Cl_2]$ (with $n = 3$ and 6) bind covalently to DNA, while the AO (acridine orange) moiety is intercalated. Finally, a comparison between Figs. 4 and 6 shows that whereas the amplitudes of the fast step bear the same sign, the amplitudes of the slow step bear opposite signs. Because the experiments have been recorded on the same side of the isosbestic point, we should conclude that the reaction enthalpy of the slow process in poly(A)-PRPt differs in sign from that of the slow process in poly(A)-PR. This result might also support the previously expressed idea in favor of a structure of DS_{II} where both proflavine and platinum residues are "inside." Actually the need to make a large cavity to allocate the Pt moiety of the dye as well could alter the energetics of the slow step to such an extent that the sign of $\Delta H°$ is reversed.

According to the above considerations, the series mechanism seems to be largely favored. Actually, concerning the poly(A)-PRPt system, it is much easier to form DS_{II} by direct insertion of the platinum residues into the polymer cavities already occupied by the acridine residues rather than by the parallel process, where DS_I should dissociate into the free reactants, which, in turn, should reassociate to give DS_{II}.

The parallel mechanism was first advanced by Bresloff and Crothers (1975) for the DNA-ethidium system. However, in such a case the interconversion between the two bound forms occurs mainly by the additional bimolecular step $S + DS_I \Leftrightarrow DS_{II} + S$, where the interstrand dye transfer is accumplished with no dissociation of the dye from from either of the strands. This mechanism, which requires a linear concentration dependence of both relaxation times, should be ruled out, because in our systems the plots of $1/\tau_2$ versus the poly(A) concentration are curved.

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